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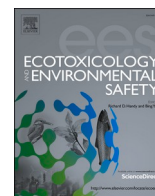
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Effects of florfenicol exposure during early life on toxicity, gut microbiota, and fecal metabolome in SD rats

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ABSTRACT

Florfenicol (FLO) is a third-generation veterinary antibiotic with a high residue detection rate in food, which cause the toxicity of FLO even at low doses, receiving notable attention. The impact of FLO exposure during early life on health and gut microbiota is still unclear. Here, the effects of FLO exposure on toxicity, gut microbiota, drug resistance genes, and the fecal metabolome during early life were investigated in suckling Sprague-Dawley (SD) rats. The results showed that FLO exposure during early life significantly increased the body weight, and WBC and LY levels in the blood, induced inflammation in the liver and intestines. FLO had a dose-dependent effect on the alpha and beta diversity of the gut microbiota, increasing the ratio of Firmicutes to Bacteroides and the abundance of some pathogenic bacteria, and changing the abundance of bacteria related to energy metabolism and inflammation, also promoted the enrichment of drug resistance genes. The fecal metabolome also demonstrated the effect of FLO exposure on metabolic pathways related to energy metabolism and inflammation. In conclusion, this research shows that FLO exposure during early life can lead to excessive weight gain, an inflammatory response, gut microbiota imbalance, the enrichment of drug resistance genes, and effects on related metabolic pathways.

1. Introduction

Florfenicol (FLO) is a synthetic derivative of chloramphenicol, which is primarily used to treat most gram-positive and gram-negative bacteria infections in animals(Lis et al., 2011), especially chloramphenicol-ethylsulfonamycin broad-spectrum resistant gram-negative bacteria infections, as FLO has a better curative effect and an improved ability to inactivate bacteria relative to chloramphenicol (Varma et al., 2010). As a third-generation animal-specific amido alcohol antibiotic, FLO uses sulfomethyl (-SO_2CH_3) to replace the nitro group (-NO_2) in chloramphenicol. Therefore, FLO does not cause aplastic anemia in the body or cause mutagenic, carcinogenic or teratogenic effects, which makes FLO one of the most widely used antibiotics in the aquaculture industry(M et al., 1983). However, studies have shown that FLO still has certain acute and chronic toxicity and immunotoxicity(Bretzlaff et al., 2010). Most countries have stipulated the

maximum residue limit (MRL) for FLO in foods of animal origin (MRL 200–3000 mg/kg). Due to the higher MRL, FLO has become one of the veterinary drugs with the highest residue detection rate in foods of animal origin (Wang et al., 2016).

The gut microbiota is closely related to fat metabolism and energy homeostasis, and is also influenced by other factors such as dietary habits, cesarean section and breastfeeding (Mayorga et al., 2017; Pihl et al., 2016). Among these, antibiotic exposure is a potential threat. Early life is a critical period that gut microbiota colonize and establish. Exposure to antibiotics during this period may not only cause toxic damage but may also interfere with the normal gut microbiota, leading to a series of metabolic diseases(Altobelli et al., 2003). Previous studies have shown that antibiotics, such as vancomycin, penicillin and chlortetracycline, can alter the composition of gut microbiota, affecting the metabolism of bile acids, thereby increasing fat accumulation(Ilseung et al., 2012). Epidemiological studies have also reported a positive

association between antibiotic use in children and childhood obesity (Antti et al., 2015; Yallapragada et al., 2015). Monitoring of antibiotic levels in urine revealed that the rate of obesity in school-aged children was associated with levels of antibiotics such as florfenicol and methotrexate(Wang et al., 2016). Since FLO is widely present in foods of animal origin although its residual amount is often lower than the MRL, the impacts of FLO exposure during early life on the gut microbiota and metabolism are still unclear and worthy of attention considering the sensitivity experienced during early life,

In this study, different exposure doses of FLO (2, 20, 200, 2000 µg/kg BW) were administered by gavage for 15 days in suckling SD rats. Toxicological changes in hematology, serum biochemistry and histopathology were evaluated. High-throughput sequencing of 16S rRNA was used to clarify the changes in the gut microbiota after FLO exposure. Changes in resistance genes in the gut microbiota were detected using qPCR. The changes in fecal metabolomes after FLO exposure were characterized by UPLC-Q/TOF. This research provides knowledge respecting the potential risks of low-level FLO exposure during early life. These information may be helpful for further studies on the dietary intake of FLO residue in infants and young children.

2. Material and methods

2.1. Animals experiment

Suckling SD rats (birth 48 h) from 6 mother rats were purchased from Chengdu Dashuo Experimental Animal Co., Ltd. (Chengdu, China). The pup rats were kept in a barrier-class animal room of 22 ± 2 ℃ and 55 ~ 75% relative humidity in a 12/12 h light/dark cycle with mother rats. Mother rats were fed standard rat food (AIN-93 G) and sterile water. The pup rats from the same mother rat were randomly divided into 5 groups (each group included 8–12 rat pups from 6 different mother rats, and randomly included males and females). On the 7th day after birth, the pup rats were given respectively different doses of FLO by oral gavage for 15 days (0, 2, 20, 200 or 2000 µg/kg BW FLO in 0.9% saline). The dosage of FLO was determined according to the residual amount of FLO in food and the ADI value. After weaning, the pup rats were fed with standard rat food and sterile water. Fecal samples of pup rats were collected every 5 days from the day after the end of FLO exposure and stored at − 80 ℃ until analysis. On the 20th day after the end of FLO exposure (aged 42 days after birth), the pup rats were sacrificed by CO_2 anesthesia. The liver, spleen, kidney, duodenums, and blood from the heart were collected for experiments.

All experimental animal procedures were in accordance with the relevant guidelines and principles and approved by the Experimental Animal Ethics Committee of Chengdu Medical College.

2.2. Analyses of blood and serum biochemical

Rat heart whole-blood was collected respectively in an anticoagulant blood collection tube with EDTA for hematology analysis and in an EDTA-free blood collection tube and centrifuged at 3000 rpm for 10 min for serum biochemical analysis.

The white blood leucocyte count (WBC), red blood cell count (RBC), hemoglobin (HGB), platelet count (PLT), neutrophil count (NE), average red blood cell volume (MCV), lymphocyte count (LY), mean hemoglobin (MCH), red blood cells deposited (HCT), and mean hemoglobin concentration (MCHC) were measured using a blood analyzer (ADVIA 2120, Siemens, Germany). Glutamate transpeptidase (GGT), alkaline phosphatase (ALP),serum alanine aminotransferase (ALT), albumin (ALB), globulin (GLB), total protein (TP), white ball ratio (A/G), aspartate aminotransferase (AST), urea, total cholesterol (CHOL), creatinine, triglycerides (TG) were analyzed by a biochemical analyzer (AU2700, Olympus, Japan).

2.3. Histological analysis

The liver, spleen, kidney, and duodenal tissue of rat pups were fixed with 10% neutral buffered formalin and processed in accordance with standard protocols (dehydration with gradient alcohol, transparent tissue with xylene, impregnation with wax, paraffin embedding, block sections, patches, and baked slices) after 48 h, were stained with hematoxylin-eosin (HE). Histopathology was examined using a microscope (E200, Nikon, Japan).

2.4. Analysis of gut microbiota

Stool samples of suckling SD rats were snap-frozen in liquid nitrogen. Metagenomic DNA was isolated using the TIANamp Stool DNA Kit (Tiangen Biotech, Beijing, China). PCR was performed for amplifying the V3-V4 region of the 16S rRNA gene by the forward primer 5′-ACTCCTACGGGAGGCAGCAG-3′ and the reverse primer 5′- GGAC-TACHVGGGTWTCTAAT-3′. The PCR products were high-throughput sequenced on an Illumina MiSeq platform (Illumina, CA, USA) by Majorbio Co. Ltd. (Shanghai, China).

The alpha and beta diversities were analyzed to identify the species diversity on the online Majorbio I-Sanger Cloud Platform (www. i-sanger.com). The Ace, Sobs, Simpson, Shannon, and Chao1 indexes are used to characterize the biodiversity richness. The distance matrix was visualized by utilizing principal coordinate analyses (PCoA) based on beta diversity. The important difference species are screened out using linear discriminant analysis effect size (LEfSe) analysis tool by performing cladogram construction and linear discriminant analysis (LDA).

2.5. Real-time quantitative PCR analysis

Total DNA of the intestinal flora was extracted from the fecal samples by a TIANamp Stool DNA Kit (Tiagen Biochemical Technology Co., Ltd., Beijing, China). According to the requirements of the International Standard for Real-time Fluorescent Quantitative PCR (MIQE), specific primers were designed (Supplementary Table 1). The different resistance genes of FLO in the intestinal flora (such as floR, fexA, and Cfr) were detected using real-time quantitative PCR detection system (CFX96™, Bio-Rad, Hercules, USA) with Talent Fluorescence Quantitative Assay Kit (Tiagen Biochemical Technology Co., Ltd., Beijing, China). The PCR procedure was a three-step method: 94 ℃ denaturation for 3 min, followed by 40 cycles of 94 ℃ denaturation for 5 s, annealing at a given temperature for 10 s, extension at 72 ℃ for 15 s, and plate reading at 72 ℃ for 5 s. After the amplification cycle, the solution curve of the RCR product was detected to eliminate the interference of nonspecific amplification. A negative control was set during the process of PCR amplification to eliminate contamination. The 16S rRNA gene was used as an internal control. The changes in the abundance of resistance genes were analyzed by the $2^{-\Delta\Delta Ct}$ method.

2.6. UPLC-Q-TOF analysis of fecal metabolites

The metabolic compounds in fecal samples were extracted using ultrapure water, methanol and acetonitrile, and were detected using UPLC-Q-TOF (The LC30 UPLC system (Shimazu, Japan)) connected with an high-resolution mass spectrometry system in the positive ion mode (X500R, AB Sciex, CA, USA) as previously described(Dai et al., 2020). Mass spectral data were processed for peak identification, peak alignment, characteristic peak extraction using Mark-View softwar (ABSCIEX, CA, USA). The baseline calibration, noise reduction and singularity elimination were performed according to QC samples. Then, the data were analyzed on an online MetaboAnalyst platform (version 5.0, www. metaboanalyst.ca)(Pang et al., 2021). The potential biomarkers related to FLO exposure were screened in accordance with the loading plot, the S-plot, and the variable importance in the project (VIP) value (VIP > 1.0). The key metabolites screened out were identified by

searching available mass spectrometry results, such as the MassBank of North America (MoNA, massbank.us), Human Metabolome Database (HMDB, www.hmdb.ca), and Metabolite and Tandem MS Database (METLIN, Metlin.scripps.edu). The pathways of the metabolites were annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG, www.kegg.com).

2.7. Statistical analysis

All data were expressed as the mean±S.E.M. The statistical analysis was carried out by Student's *t*-test for the comparison between two groups, or ANOVA followed by Duncan's shortest significant range test for the comparison between multiple groups using SPSS 22 software (IBM, chicago).

3. Result

3.1. Clinical observations

Repeated oral exposure to suckling SD rats with different doses of FLO for 15 days did not cause death. None of the clinical signs such as mental state, behavior, daily activities, or appearance of the exposure groups showed treatment-related changes, compared with the control group. In addition, the body weight growth rate of the 200 and 2000 μg/kg BW FLO exposure groups was significantly higher than that of the control group and showed a dose-dependent effect (Fig. 1). This suggests that FLO exposure during early life may affect the body weight of suckling SD rats.

3.2. Hematology

The effects of FLO exposure on hematological indexes are shown in Supplementary Table 2. The WBC and LY were significantly increased in the 20, 200 and 2000 μg/kg BW FLO exposure groups, indicating that FLO exposure may be the cause of an inflammatory response. Although there were significant changes in HGB and HCT there was no strict dose-dependent trend, which implies that these changes may not be related to exposure to FLO during early life.

3.3. Blood chemistry

The blood biochemical indexes of the suckling SD rats after FLO exposure during early life are showed in Supplementary Table 3. The results demonstrated that most of the blood biochemical indexes were not significantly different. Although the creatinine after FLO exposure during early life was significantly increased, while the CHOL was significantly decreased, there was no strict dose-dependent trend. However, the AST was significantly increased in the 2–2000 μg/kg BW FLO exposure groups, suggesting that FLO exposure may cause liver damage or inflammation.

3.4. Histopathology

No abnormalities were observed on the histopathology of the suckling SD rats. The organ index for all groups did not show a dose-dependent relationship (data not shown). However a series of treatment-related pathological changes in the liver tissue and intestinal tissue appeared in the FLO exposure groups (Fig. 2), including non-obvious liver lobes, swelling and degeneration of liver cells around veins, inflammatory lesions, slight fat degradation, and spot necrosis in liver tissue, as well as degeneration and necrosis of intestinal mucosal epithelial cells and inflammatory cell infiltration in the intestinal mucosal layer, compared with the control group.
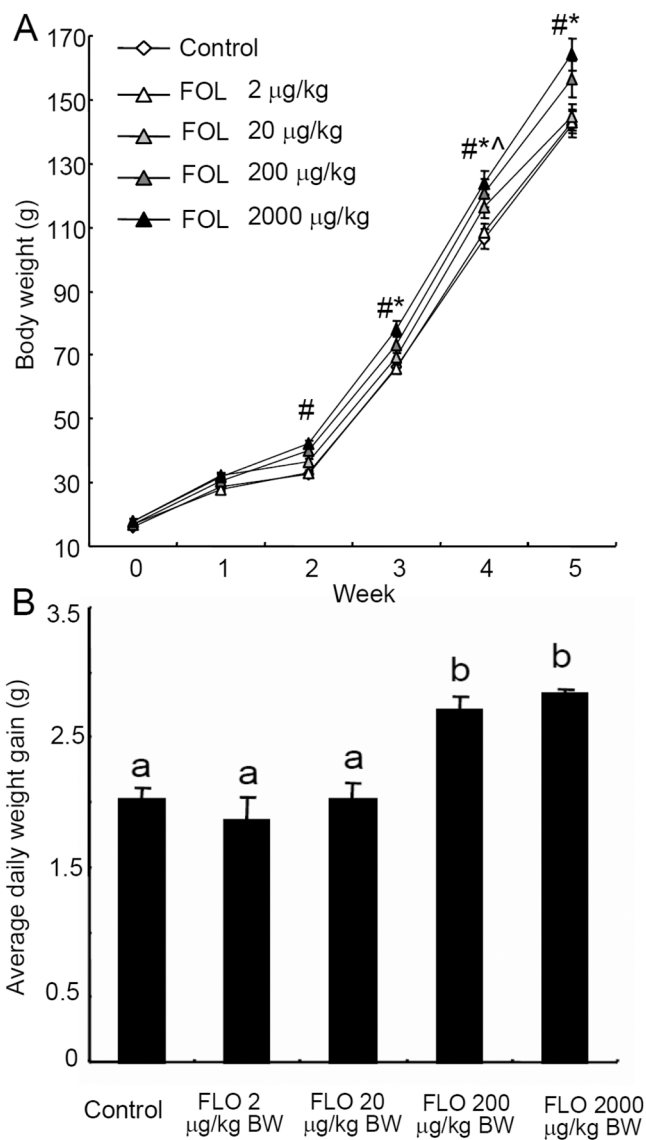

Fig. 1. Changes of body weight (A) and average daily weight gain (B) in suckling SD rats after FLO exposure during early life. Data were presented as the mean ± SEM (n = 5). # *P* < 0.05 (FLO 2000 μg/kg BW vs. Control), * *P* < 0.05 (FLO 200 μg/kg BW vs. Control), ^ *P* < 0.05 (FLO 20 μg/kg BW vs. Control), $ *P* < 0.05 (FLO 2 μg/kg BW vs. Control). Bars with the different letters represent a significant difference between groups (*P* < 0.05).

3.5. Effects of FLO exposure during early life on the gut microbiota

A total of 1772,859 usable pyrophosphate sequences were obtained from 45 samples with an average sequence length of 443.86 bp. After the above obtained sequences were optimized by discarding the chimeric sequences and the unqualified sequences using Usearch software, 1010,745 sequences were obtained. The 22,461 valid sequences were obtained for each sample by random extraction to a uniform sequences amount according to the minimum sequences number in samples. The above valid sequences were clustered into 349 operational taxonomic units (OTU) by QIIME software with a 97% similarity level. Rarefaction curves based on the Shannon, Sob, and Simpson indexes indicated that the amount of sequencing data was large enough to reflect the biological diversity (Supplementary Fig. 1).

The results of alpha diversity analysis based on the number of OTU sequences of each sample are shown in Table 1. The ANOVA of alpha diversity indexes showed that different FLO exposure concentrations
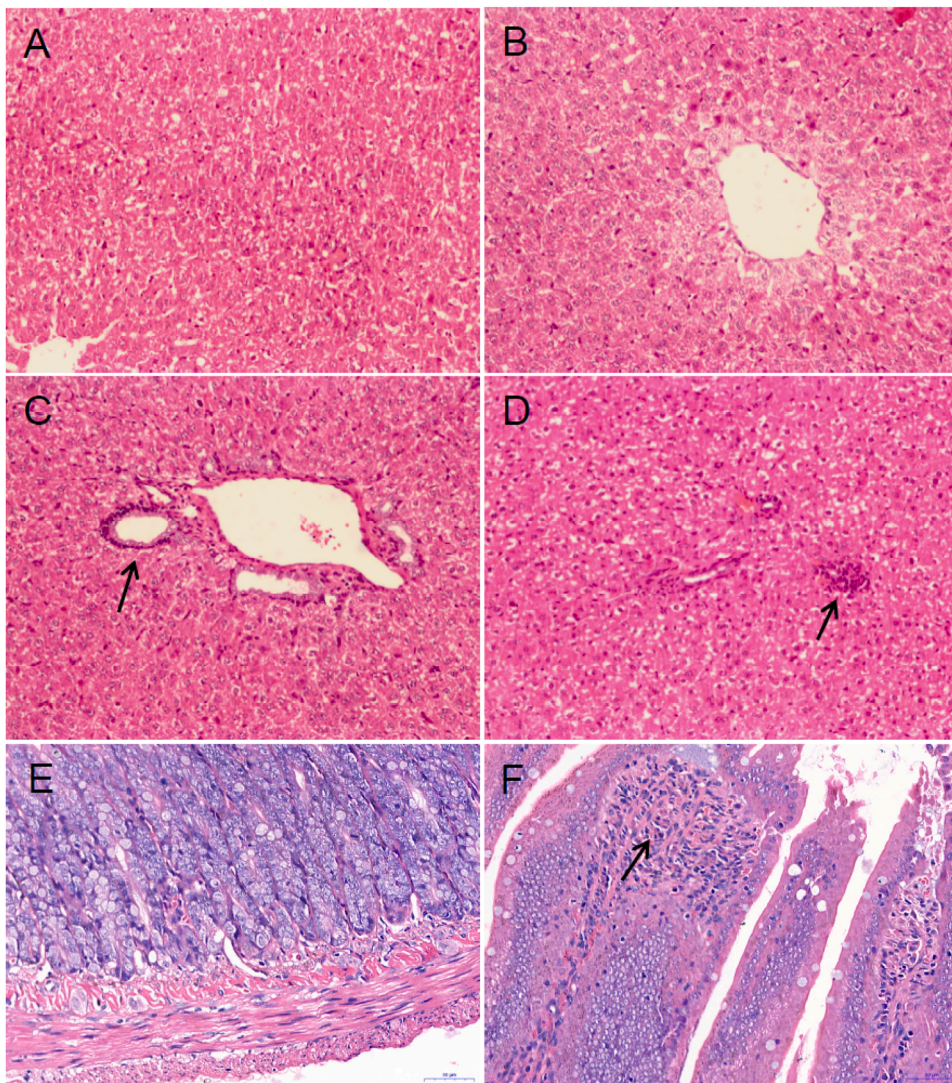
Fig. 2. Changes of FLO exposure on of histopathology in liver tissue and intestinal tissue. (A) Normal liver in the control group (×200, HE); (B) Vacuolar degeneration of hepatic cells in the 2000 µg/kg FLO exposure group (×200, HE); (C) Hepatocyte swelling and inflammatory lesions in the 2000 µg/kg FLO exposure group; (D) Point necrosis and mild fat degradation in the 200 µg/kg FLO exposure group; (E) Normal intestinal tissue in the control group (×400, HE); (F) Mucosal epithelial cell infiltration in the 2000 µg/kg FLO exposure group (×400, HE).

Table 1
Effects of FLO exposure on diversity index of gut microbiota in suckling SD rats.

FLO exposure concentration (µg/kg BW)	Withdrawal period (day)	Sobs	Shannon	Simpson	ACE	Chao 1
Control	0	225 ± 18.58	3.21 ± 0.12	0.09 ± 0.01	268.5 ± 21.49	275.08 ± 19.9
2		206.33 ± 6.67	3.15 ± 0.18	0.1 ± 0.02	248.34 ± 10.94	252.16 ± 12.93
20		222 ± 10.02	3.19 ± 0.14	0.11 ± 0.02	257.13 ± 9.47	255.86 ± 9.96
200		244 ± 4.04	3.66 ± 0.06	0.06 ± 0.01	281.93 ± 9.13	286.97 ± 11.15
2000		212 ± 5.13	3.16 ± 0.15	0.11 ± 0.02	256.1 ± 2.82	260.34 ± 0.99
Control	10	277 ± 13.89	3.17 ± 0.12	0.12 ± 0.01	329.6 ± 9.71	340.67 ± 9.22
2		259 ± 47.27	3.64 ± 0.29	0.06 ± 0.02	288.93 ± 49.7	289.77 ± 51.19
20		288.67 ± 24.66	3.61 ± 0.44	0.09 ± 0.05	321.49 ± 21.12	318.99 ± 21.6
200		269 ± 19.66	3.46 ± 0.22	0.08 ± 0.02	324.38 ± 15.27	327.47 ± 23.9
2000		233.33 ± 13.45	2.78 ± 0.34	0.21 ± 0.07	261.96 ± 17.96	265.95 ± 17.86
Control	20	238.33 ± 8.69	2.83 ± 0.31	0.16 ± 0.04	284.71 ± 18.83	292.98 ± 15.61
2		267.67 ± 14.77	3.34 ± 0.35	0.1 ± 0.04	315.77 ± 16.89	324.47 ± 12.23
20		267.67 ± 22.81	3.36 ± 0.26	0.09 ± 0.02	317.6 ± 29.17	330.04 ± 35.52
200		335 ± 18.58	3.57 ± 0.14	0.08 ± 0.01	378.82 ± 12.66	382.42 ± 15.48
2000		321.67 ± 19.91	3.25 ± 0.35	0.18 ± 0.06	356.33 ± 18.09	369.38 ± 21.78

Data were presented as mean ± SEM (n = 3).

significantly affected the community diversity indexes (such as Simpson and Shannon), while the withdrawal period after FLO exposure to community richness indexes (such as Chao1, Sobs and Ace) had a significant impact (Supplementary Table 4). Multiple comparisons of FLO exposure concentration using Duncan's method revealed that only

20 µg/kg BW FLO exposure can significantly change the Shannon and Simpson indexes (Supplementary Fig. 2A, B). This indicates that low-level FLO exposure during early life has a significant impact on the diversity and homogeneity of the gut microbiota. Multiple comparisons of the withdrawal period using Duncan's method showed that the Sobs,

Ace, and Chao1 indexes of the withdrawal period at 10 and 20 days significantly increased compared with that of withdrawal period at 0 days, and there was no significant difference in the Sobs, Ace, and Chao1 indexes of withdrawal period at 10 and 20 days (Supplementary Fig. 2C–E). This implies that the number of species in the gut microbiota of suckling SD rats increased rapidly and stabilized after FLO exposure and withdrawal for 10 days. Therefore, the sample data from the withdrawal period 10 days after FLO exposure were used for beta diversity analysis ($P < 0.001$, ANOSIM test on binary Euclidean distances) to reflect the differences in microbial communities between sample groups. The two-dimensional PCoA score plot displayed a distinct separation pattern and indicated that the gut microbiota has a structural difference between FLO exposure groups and the control groups (Fig. 3C).

The species composition of the gut microbiota was determined by taxonomic assignment using the Ribosomal Database Project (RDP) classifier. The 668 OTUs were classified into 14 phyla, 24 classes, 33 orders, 58 families, 154 genera, and 274 species. At the phylum level, *Firmicutes* and *Bacteroides* were the dominant phyla, accounting for more than 90% of the gut microbiota (Fig. 3A), and the ratio of *Firmicutes* and *Bacteroide* increased with increasing FLO exposure concentration (Supplementary Fig. 3F). At the family level, *Lactobacillaceae* and *Bacteroidales_S24_7_group* were the two most abundant *Bacteroidetes* families

(Fig. 3B). However, the abundances of only 4 families were significantly impacted with a dose-dependent effect under FLO exposure (ANOVA, $P < 0.05$), such as *Bifidobacteriaceae*, *Prevotellaceae*, *Bacteroidales_S24–7_group*, and *Erysipelotrichaceae*. The taxonomy cladograms generated by LEfSe analysis showed that the predominant different species of FLO exposure similar to the above analysis (Fig. 3D). The relative abundance of biomarkers at the genus level and FLO exposure concentration showed a dose-dependent effect (Supplementary Fig. 3). The genera whose abundance decreased with increasing FLO exposure concentration included *Staphylococcus*, *Chlamydia*, *Jeotgalicoccus*, *Prevotellac_9*, *Prevotellaceae_UCG_001*, *Christensenellaceae_R_7_group*, and *Corynebacterium_1*. The genera whose abundance increased with increasing FLO exposure concentration included *Intestinimona*, *Ruminococcaceae_UCG_013*, *norank_f_Lachnospiraceae*, and *Ruminiclostridium*. However, 2000 μg/kg BW FLO exposure still significantly reduced the abundance of these genera. In addition, the abundances of *Adlercreutzia*, *Parasutterella*, *norank_f_Ruminococcaceae*, *DesuLfovibrio*, *norank_f_Bacteroidales_S24_7_group*, *AllobacµLum*, *Bifidobacterium*, *Tyzzerella*, and *norank_f_Erysipelotrichaceae* increased significantly after only 2 μg/kg BW FLO exposure, but with the increase of FLO exposure concentration, their abundances showed a gradual decreasing trend, compared with the control group.
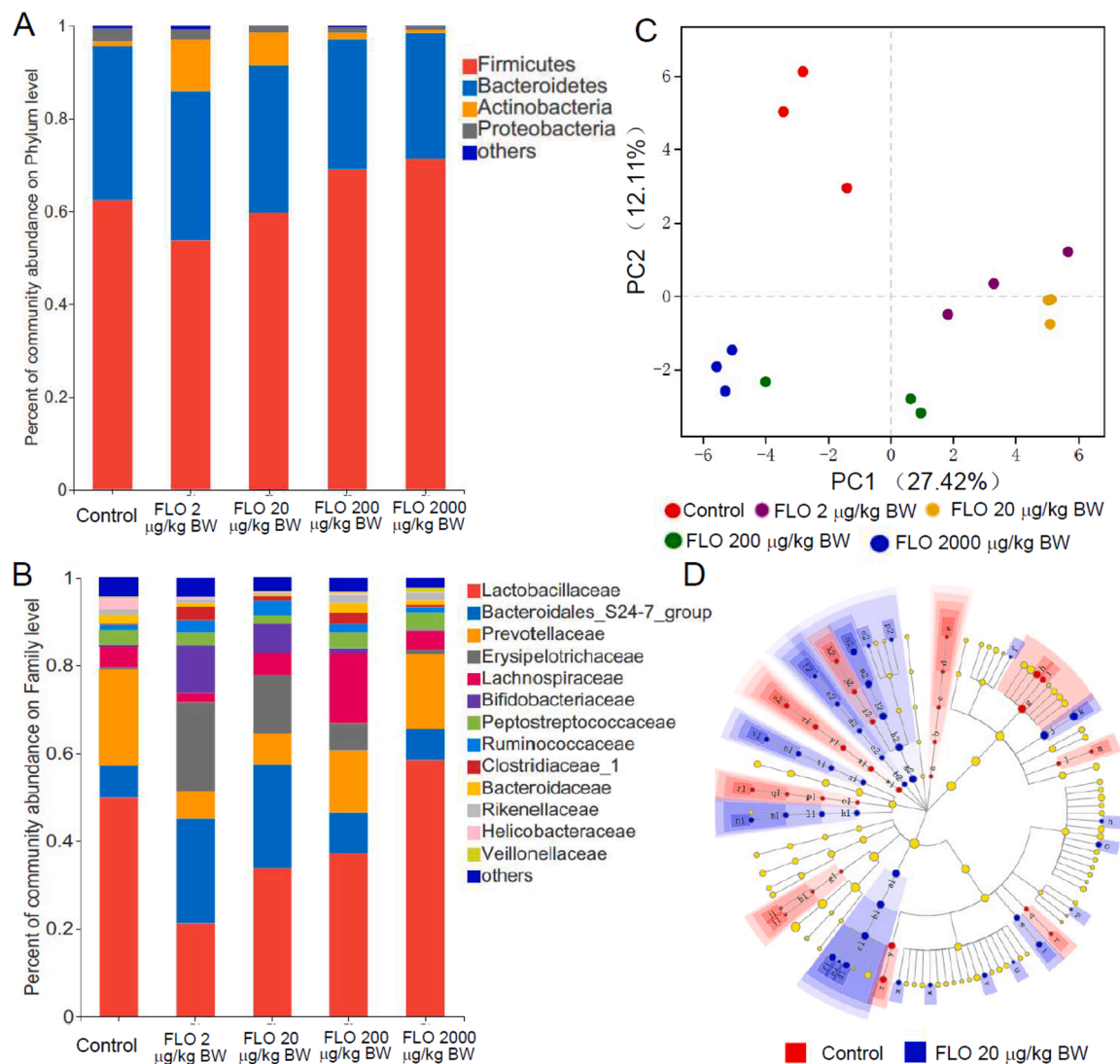

Fig. 3. Effects of FLO exposure on the gut microbiota in suckling SD rats. Changes of relative abundance on phylum (A) and family (B) level. PCoA two-dimensional score plots based on gut microbiota OTU data (C). Taxonomic cladogram from phylum to genus based on the LEfSe analysis (D).

3.6. Effect of FLO exposure during early life on drug resistance genes

Real-time fluorescent quantitative PCR was preformed to understand the effect of FLO exposure during early life on drug resistance genes in the gut microbiota of suckling SD rats. The changes in the abundance of the typical florfenicol resistance genes floR, fexA and cfr in the gut microbiota are shown in Fig. 4. The results showed that the abundance of the resistance genes floR and fexA increased significantly in samples of withdrawn 0 days after FLO exposure, and fexA was more sensitive to FLO exposure, while the abundance of cfr did not significantly change. However, in the sample of withdrawal period at 10 days, FLO exposure caused a significant increase in the abundance of floR, fexA, and cfr, with a dose-dependent effect. This indicates that the proportion of resistant bacteria, and the relative abundance of resistance genes increased significantly after FLO exposure during early life. In addition, the difference in the relative abundance of drug resistance genes in samples with different withdrawal periods after FLO exposure also implied that the change in the proportion of bacteria containing drug resistance genes in the gut microbiota reflects a time course of enrichment.
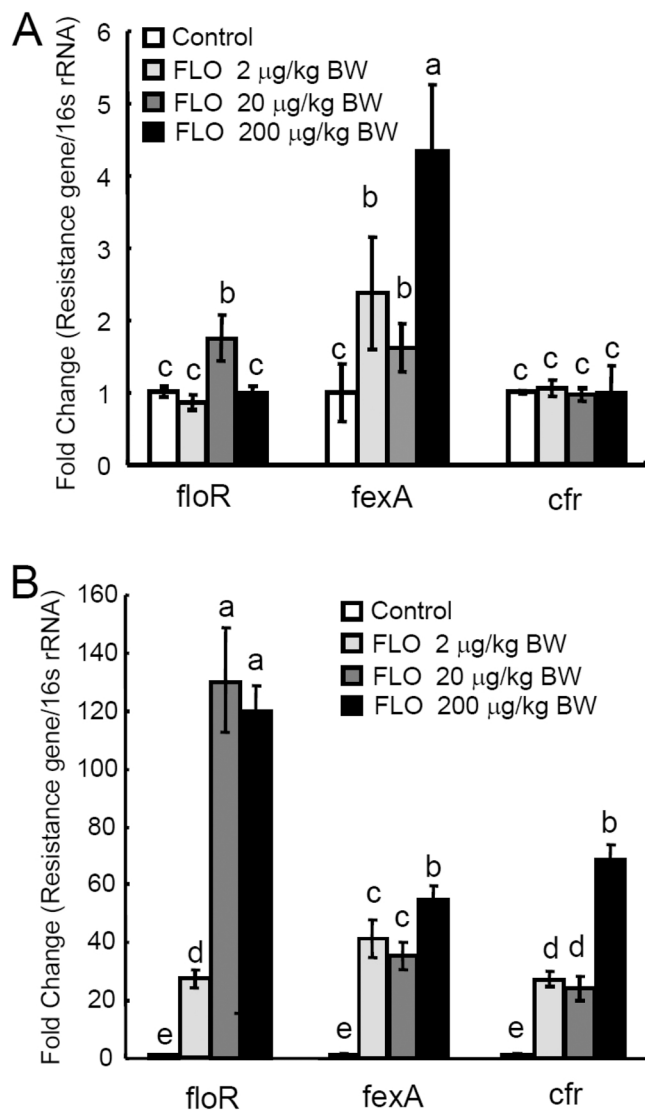
3.7. Effect of FLO exposure during early life on fecal metabonomic

To understand the effects of FLO exposure during early life on fecal metabolites, fecal samples from the withdrawal period at 10 days after FLO exposure were measured by UPLC-QTOF. The 253 detectable peaks were observed. To distinguish the differences in fecal metabolites, the PLS-DA model, as a supervised multivariate statistical method, was used to analyze the multidimensional metabolite abundance scale. The cross-validation results suggested that the model had good predictive ability and was not overfitted ($Q^2 = 0.69$ and $R^2Y = 0.99$). The PLS-DA score plots showed that the control group was clearly distinguished from the 20 µg/kg BW FLO exposure group, closed to the 2 µg/kg BW FLO exposure group, and all groups were mainly distributed in different quadrants (Fig. 5). These results indicate that a lower dose of FLO exposure can also cause significant differences in the fecal metabolome of SD rats.

To further explore the impact of FLO exposure on metabolism, the hierarchical cluster heatmap was drawn in accordance with the abundance of the metabolites (Supplementary Fig. 4). The samples from the FLO 20 µg/kg BW exposure groups clustered into different trees, however, the 2 µg/kg BW FLO exposure group and control group were mixed with each other at a closer distance. This indicates that 20 µg/kg BW FLO exposure during early life has a significantly higher impact on fecal metabolites than the effect of 2 µg/kg BW FLO exposure. A total of 52 metabolites that played important roles in distinguishing whether FLO exposure were screened in accordance with the VIP value (VIP > 1). Seventeen metabolites as potential biomarkers of FLO exposure were identified by comparing the exact mass and MS/MS spectrum from the metabolomics databases (METLIN HMDB, and MoNA). The information of 17 metabolites including *m/z* value, molecular formula, exact mass, KEGG ID, compound name, VIP value and change trend are shown in Table 2, and most of them were significantly changed after FLO exposure ($P < 0.05$). The metabolic pathways of the potential biomarkers were clarified by metabolite enrichment analysis using the KEGG database. The results showed that FLO exposure during the early life had a significant effect on phenylalanine and tyrosine metabolism (phenylalanine, L-tyrosine), the urea cycle, and aspartic acid metabolism (L-glutamate amino acid, citrulline), niacin and niacinamide metabolism (L-glutamic acid, niacin) in suckling SD rats.

4. Discussion

As a derivative of chloramphenicol, FLO has been widely used in veterinary medicine because of its safer and broad-spectrum properties (Wang et al., 2016). The current study found that the LD50 values of FLO

Fig. 4. Relative abundances of the resistance genes floR, fexA and cfr by qPCR at 0 days (A) and 10 days (B) after the oral administration of florfenicol. All data are presented as the mean ± SEM (n = 3). Bars with the different letters represent a significant difference between groups ($P < 0.05$).
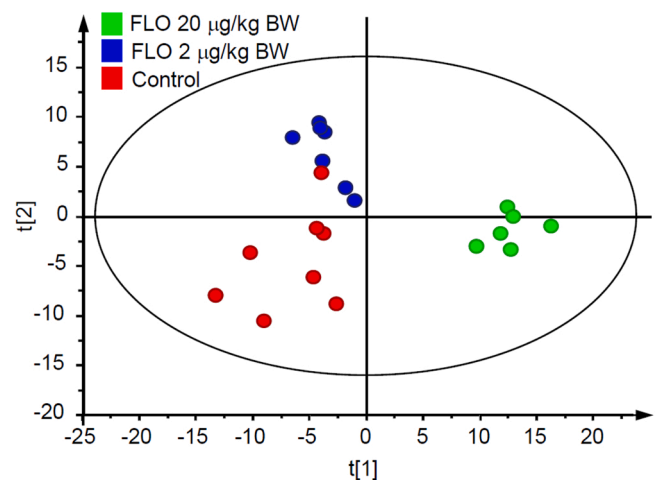

Fig. 5. PLS-DA two-dimensional score plot based on fecal metabolites (n = 6–8).

Table 2
Effects of FLO exposure on the biomarkers in fecal samples.

M/Z	Excat mass (Da)	Elemental composition	Postulated identity	KEGG ID	VIP values	FLO 20 μg/kg BW vs. Control
176.1041	175.0957	C6H13N3O3	Citrulline	C00327	1.8879	↑
124.0383	123.0320	C6H5NO2	Nicotinic acid	C00253	2.8903	↑
162.0536	161.0476	C9H7NO2	2-Indolecarboxylic acid	–	3.4631	↑
159.0757	158.0691	C6H10N2O3	4-Methylene-L-glutamine	C01109	1.9892	↑
359.2861	324.0777	C17H13ClN4O	Alpha-hydroxyalprazolam		1.4438	↓
136.0743	135.1047	C9H13N	Amphetamine	–	2.4287	↑
182.0808	181.0739	C9H11NO3	L-Tyrosine	C00082	1.528	↑
112.0059	111.0796	C5H9N3	Histamine	C00388	1.2023	↓
137.0452	136.0385	C5H4N4O	Hypoxanthine	C00262	2.7475	↑
162.0553	161.0477	C9H7NO2	Indole-3-carboxylic acid	C19837	3.0481	↑
148.0600	147.0532	C5H9NO4	L-Glutaminic acid	C00025	1.1103	↓
222.0968	221.0899	C8H15NO6	N-Acetylgalactosamine	C01074	1.8343	↓
391.2838	390.277	C24H38O4	Nutriacholic acid	–	1.983	↓
220.1175	219.1107	C9H17NO5	Pantothenic acid	C00864	1.1684	↑
166.0855	165.079	C9H11NO2	Phenylalanine	C00079	1.1385	—
130.0857	129.079	C6H11NO2	Pipecolic acid	C00408	1.1736	—
138.0537	137.0477	C7H7NO2	Trigonelline	C01004	1.2529	↓

(Selected ion [M + H] +).↑indicates significantly increased at P < 0.05; ↓indicates significantly decreased at P < 0.05; —indicates no statistical significance.

are as high as 2000–5000 mg/kg with acute exposure, and almost no acute toxicity(Nahler, 2009). Mice showed no significant effect on body weight or various tissues and organs after 500 mg/kg BW FLO exposure by gavage(Hu et al., 2016). In this study, doses of FLO exposure by gavage were determined based on the residue range of FLO in foods of animal origin. Even after 20 days of FLO exposure during early life, suckling SD rats did not show changes related to FLO exposure in terms of clinical symptoms such as behavior, mental state, food and water consumption, daily activities or appearance. At the same time, there were no obvious abnormalities in the main organs, such as the heart, liver, spleen, lung, and kidney, which is consistent with previous research(Hu et al., 2016). However, some studies reported that FLO may affect RBC and PLT production, and the underlying mechanism may involve impairment of bone marrow hematopoiesis during FLO treatment, resulting in moderate atrophy of the hematopoietic lineage, which is partially replaced by adipocytes(Hassanin et al., 2014). Since no significant inflammation or necrosis was observed, moderate atrophy of the hematopoietic lineage was mainly attributable to drug-induced apoptosis(Hu et al., 2014). Abnormal reductions in RBCs and PLTs were not observed in our study, which may be due to the low dose of FLO exposure in this study.

It is widely believed that FLO has a strong immunosuppressive effect, decreasing leukocyte and lymphocyte concentrations and inhibiting lymphocyte function. In addition, the phagocytic capacity of neutrophils and macrophages may be inhibited by FLO. However, this study found that the WBCs and LYs were significantly increased in the FLO exposure groups (P < 0.05). Combined with the inflammatory lesions and lymphocyte infiltration in the liver and intestinal tissues, we found that low-dose FLO exposure during early life may cause low-level inflammatory reactions. Some studies have shown that exposure to antibiotics during early life such as penicillin and erythromycin may disrupt intestinal microbes and inhibit the innate immune response of the ileum, cause intestinal disorders and damage the integrity of the intestine. This process often leads to the release of intestinal gram-negative bacterial endotoxins into the blood, which in turn stimulates the inflammatory response mediated by Toll-like receptor 4 (TLR4), ultimately leading to the immune response and low-level inflammation(Jin et al., 2016), and childhood anaerobic antibiotic exposure is also closely related to inflammatory bowel disease(Örtqvist et al., 2019).

There is no doubt that antibiotics play a critical role in improving human health, but their widespread use has had some unintended consequences, such as the potential to promote growth(Högberg et al., 2014). In the 1950s, subtherapeutic doses of antibiotics were found to accelerate weight gain in livestock(Lassiter, 1955), and a similar result was also observed in this study. The mechanism may involve the direct action of antibiotics on the intestine and the link between alterations in gut microbiota and changes in human metabolism (Tremaroli and Bä ¤Ckhed, 2012; Turnbaugh et al., 2009). A recent study found that antibiotics increased the amount of body fat in mice and were associated with changes in the composition of the gut microbiota(Ilseung et al., 2012). Antibiotics not only increase energy intake and regulate satiety through hormonal changes, but also affect the expression of genes related to the conversion of carbohydrates to short-chain fatty acids (SCFAs) in microorganisms (Turnbaugh et al., 2006). The gut microbiota in infants is more susceptible to interference (Vael et al., 2011). Several recent studies on children have shown that antibiotic exposure during early life can lead to weight gain and aggravate the risk of obesity (Murphy et al., 2014). Changes in the gut microbiota are related to obesity and metabolic disorders (Vanessa, 2013), in which the F/B ratio is a biomarker for assessing lipid metabolism(Cani et al., 2009). The increase in the F/B ratio and changes in several bacterial species can promote obesity development in obese mouse models of diet-induced or genetic phenotypes(Cani et al., 2008), which is consistent with the observations in this study.

In addition, the abundance and composition of the gut microbiota also produced significant changes after FLO exposure during early life. Among the bacterial genera whose abundance was significantly reduced after FLO exposure, *Christensenellaceae_R_7_group* is believed to be related to effective obesity intervention (Liu et al., 2019b) and regulation of abnormal glucose tolerance (Gong et al., 2020), and can improve the ability of the host to metabolize energy and substances(Sun et al., 2019). *Prevotellaceae_UCG_001 and Prevotellac_9*, members of *Prevotellaceae*, are rich in enzymes that degrade cellulose and xylan (De Filippo et al., 2010). They can influence the body's energy expenditure (Carvalho and Saad, 2013) by regulating the protein kinase pathway (AMPK) associated with energy metabolic homeostasis and can produce SCFAs and degrade dietary fiber by regulating the protein kinase pathway (AMPK) related to the balance of energy metabolism(Song et al., 2019; Xiao et al., 2020), which affects energy consumption and reduces intestinal inflammation(Scheppach and Weiler, 2004).

However, the abundance changes of other bacterial genera showed that the proliferation of these bacterial genera was stimulated in the low-dose FLO exposure groups, and the growth of these bacterial genera was inhibited in the high-dose FLO exposure group. This suggests that low-dose FLO exposure on these bacteria may produce hormesis effects (Rial et al., 2011) and has a greater impact on the gut microbiota. Among these bacteria, *Ruminococcaceae_UCG_013*, *Intestinimonas*, *Ruminiclostridium*, and *norank_f_Ruminococcaceae* are members of *Ruminococcaceae*. Some research proves that the metabolic process of *Ruminococcaceae* can produce an inflammatory polysaccharide and cause inflammatory bowel disease (Henke et al., 2019; Ma et al., 2020). The abundance of *Ruminococcaceae* is proportional to gastrointestinal

symptoms such as chronic constipation and diarrhea (Dan et al., 2020), and also is positively associated with immune A protein nephropathy (IgAN) (De Angelis et al., 2014). *Intestinimonas* has also been found that can produce butyrate using lysine, which is closely related to obesity and intestinal inflammation(Song et al., 2020). *Norank_f_Lachnospiraceae* and *Tyzzerella* come from *Lachnospiraceae*. *Lachnospiraceae* is associated with metabolic diseases such as obesity and diabetes(Li et al., 2019) and can affect blood glucose regulation by causing pancreatic β-cell dysfunction in sterile mice(Kameyama and Itoh, 2014). Some studies have also shown that a high abundance of *norank_f_Lachnospiraceae* has an inhibitory effect on fatty acid metabolism(Yu et al., 2020). *Tyzzerella* is also a potentially pathogenic bacterium(Fomenky et al., 2018) whose abundance is susceptible to dietary patterns and is high in populations with poorly structured diets(Liu et al., 2019a). *Norank_f_Erysipelotrichaceae, AllobacuLum* belongs to the *Erysipelotrichaceae* family, which is believed to be related to colon inflammation. For example *Erysipelotrichaceae* has a higher abundance in the gut microbiota of colitis mouse model(Chen et al., 2017). *Desulfovibrio* is a sulfate-reducing bacterium that can produce toxins and destroy the mucosal barrier and is closely related to inflammation(Zhang et al., 2010). In short, significant changes in the abundance of the abovementioned bacterial genera may be among the reasons for the higher weight gain and inflammation in the suckling SD rats after FLO exposure.

In the gut microbiota, the survival and impact of pathogens and conditional pathogens depend on resistance to antibiotics (Agarwal and Nair, 2013). Antibiotic resistance of bacteria can be obtained through gene transfer of resistance gene genetic elements, and antibiotic resistance genes are closely related to transposons, plasmids and integrons (Stokes and Gillings, 2011). For example, the tet gene encoding the tetracycline efflux protein is normally found in plasmids in an aqueous environment(Chopra and Roberts, 2001). This study also found that florfenicol resistance genes can be enriched in the gut microbiota and released into the environment with feces. The release of the resistance genes poses a potential threat to public health and deserves further attention.

Phenylalanine, as one of the metabolites affected by FLO exposure, can be converted to tyrosine in the liver. Tyrosine is the raw material for the synthesis of catecholamines and thyroid hormones. (Zhang et al., 2014). This process is also related to energy metabolism in the liver and brain (Ramos et al., 2013). Excessive levels of tyrosine in the FLO exposure groups not only led to a decline in the body's energy metabolism (de Andrade et al., 2011) and increased body weight but also caused neurological dysfunction in pup SD rats. In the urea cycle, citrulline and arginine are recycled with each other and together maintain protein homeostasis (Ginguay and De Bandt, 2019). Some studies showed that citrulline can significantly increase the production of IL-6(Breuillard et al., 2015) and improve insulin sensitivity and inflammation in mice (Starkie et al., 2003). The increase in citrulline in the FLO exposure groups may be involved in autoimmune regulation of inflammation. Niacin is partially converted into trigonelline and its metabolites in the body. Some studies have found that trigonelline can inhibit the conversion of sugars into fat and exerts anti-inflammatory and antioxidant effects in liver and kidney cells(Afifi et al., 2016; Costa et al., 2020). In this study, the inhibition of niacin metabolism and the decrease in trigonelline content in the FLO exposure groups may be among the reasons for the faster weight gain and inflammation. However, changes in the metabolic pathways in feces are not only affected by the gut microbiota (Lin et al., 2015) but may also be related to the interaction between the liver and the intestine(Delzenne et al., 2019). Therefore, the influence of the gut microbiota that changed with by FLO exposure during early life on host metabolism and inflammation through the gut-liver axis is worthy of further study.

This study clarified the effects of FLO exposure during early life on the toxicity, gut microbiota, and fecal metabolome in SD rats. However, the effects of FLO exposure during early life on SD rats in adulthood and the related mechanisms of the gut microbiota affecting the metabolic

pathways were still not very clear and need to be further studied.

5. Conclusion

In summary the suckling SD rats were exposed to FLO by gavage administration for 15 consecutive days during early life, which induced faster weight gain and obvious inflammation in the liver and intestine. Gut microbiota analysis demonstrated that FLO exposure during early life caused changes in the composition of the intestinal bacteria, a significant decrease in diversity, and a high abundance expression of antibiotic resistance genes in the suckling SD rats. Analysis of the fecal metabolome also further confirmed the influence of FLO exposure during early life on metabolic pathways related to energy intake. Thus, FLO exposure during early life may have the potential to affect energy metabolism, induce inflammation, and increase the risk of obesity development by altering the establishment of the host gut microbiota.

CRediT authorship contribution statement

Zheng Ma: Conceptualization, Data curation, Formal analysis, Software, Writing – original draft. **Lin Lin:** Methodology, Investigation. **Xiao Yang:** Writing – review & editing. **Ya Yuan:** Conceptualization, Resources. **Xiaoyan Fu:** Writing – review & editing. **Shijia Chen:** Software. **Wenbo Hu:** Methodology. **Jinyao Chen:** Data curation. **Hang Xiao:** Software, Data curation. **Xiangyi Wei:** Writing – review & editing. **Juan Dai:** Project administration, Funding acquisition, Methodology.

Declaration of Competing Interest

The authors have declared no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.113038.

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