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Flaxseed regulated TGR5 signaling pathway mediated by gut microbiota-6α-hydroxylated bile acid metabolism to improve NAFLD in mice

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ABSTRACT

The pathophysiology of nonalcoholic fatty liver disease (NAFLD) was characterized by alterations in the intestinal microbiota and bile acids (BAs). Flaxseed powder (FLA) was rich in active components such as α-linolenic acid, dietary fiber, and lignans, which had lipid-lowering and anti-inflammatory effects. Here, we investigated whether FLA reduced liver fat and improved inflammation by modulating the gut microbiota, enriching bacteria involved in the production of 6α-hydroxylated BAs, and activating the gut-liver-BA metabolic pathway-specific receptor Takeda G protein-coupled receptor 5 (TGR5). Wild-type (WT) and *TGR5* knockout mice were set up in a low-fat control group, a high-fat model group and a flaxseed powder intervention group for 12 weeks. At the end of the experiment, we examined the levels of lipids (TC, LDL-C, HDL-C, and TG), the levels of inflammatory factors (TNF-α and IL-6), the pathological changes in the liver, and the differences in the expression of key proteins (CYP7A1, TLR4, and NF-κB) in the liver of *TGR5*$^{-/-}$ and WT mice. In the current study, we found that 12 weeks of FLA intervention significantly attenuated the progression of NAFLD in WT mice, whereas *TGR5* knockout exacerbated the extent of disease in mice with NAFLD. *TGR5* gene knockout blocked the anti-inflammatory effect of FLA, but did not block its lipid-lowering effect. The *TGR5* gene may be a key protein in the anti-inflammatory pathway of FLA, rather than a key protein in the lipid-lowering pathway of FLA. FLA intervention altered the relative abundance of gut microbiota with BA metabolizing enzymes, which in turn regulated the composition of intestinal BA, particularly the proportion of the key functional BAs 6α-hydroxylated BAs, thereby activating the intestinal BA-specific receptor TGR5, which might play a role in ameliorating inflammation. FLA might be a promising functional food for the prevention of NAFLD by modulating the microbiota and BAs.

1. Introduction

In recent years, the prevalence of nonalcoholic fatty liver disease (NAFLD) had been on the rise globally, with about one in four adults worldwide was affected by NAFLD, which was a potentially serious liver disease[1]. In Asia, NAFLD had become a major public health problem with an updated population prevalence of 34%. Meanwhile, NAFLD-associated hepatocellular carcinoma was also on the rise[2]. NAFLD could progress to liver fibrosis and liver cancer, which was distinctively characterized by the lipid droplet accumulation in hepatocytes[3] and chronic inflammatory state[4]. Therefore, it was important to understand the mechanisms resulting in NAFLD and its complications for finding preventive treatment and new strategies.

There was growing evidence proving that the enterohepatic axis played a role in NAFLD, it was worth noting that the enterohepatic axis played a key role in the regulation of bile acid (BA) metabolism,

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BA pool size, and enterohepatic circulation of BA[5]. BAs were molecules synthesized from cholesterol in the liver and then secreted to the gut where they could be further metabolized by the microbiota[5]. Intestinal microbiomes transformed BA compounds into various forms, thus greatly increasing their diversity and biological functions[6]. BA could regulate the activity of several receptors as signal molecules, especially farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5), and contributed to regulating energy consumption, lipid metabolism, and NAFLD improvement[7-9]. BA in enterohepatic circulation activated FXR, which inhibited the expression of cholesterol 7*α*-hydroxylase (*Cyp7a1*) and sterol 12*α*-hydroxylase (*Cyp8b1*) to represses BA synthesis. In the intestine, FXR induced fibroblast growth factor 15 (FGF15), which further inhibited the transcription of *Cyp7a1*[10]. In the study by Gillard et al.[11] changes in the gut-liver BA profile in nonalcoholic steatohepatitis (NASH) mice resulted in low TGR5 and FXR signal, while supplementation with secondary BA DCA restored TGR5 and FXR signals and prevented the development of NASH. TGR5 was widely expressed in epithelial cells of the gastrointestinal system, including the intestine and gallbladder, activation of TGR5 by BA in the gastrointestinal tract protected intestinal barrier function and reduced inflammation[11-13]. In addition, changes in the gut microbiota had been shown to increase endogenous production of BA with high affinity for TGR5[14], this was another mechanism to increase TGR5 activation.

The view of "Food is Medicine" had been proposed repeatedly[15]. Flaxseed, also known as linseed (*Linum usitatissimum* L.), was one of the oldest cultivated crops in the world, which was wildly used as a supplement for the nutritional benefits of its bioactive compound, such as *α*-linolenic acid, fiber, lignans, and proteins[16-17]. Flaxseed contained more than 10% soluble dietary fiber, and adequate dietary fiber intake could reduce plasma cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C)[18-20]. The latest research showed that soluble flaxseed fiber could reduce lipid reabsorption and increase excretion[21]. In addition, peptides (linusorbs, LOs) were isolated from linseed oil, and their biological activities were studied. The anti-inflammatory activity of the LO mixture was detected in mice hepatitis models induced by lipopolysaccharides (LPS)/*D*-galactosamine injection[17]. Flaxseed contained excellent bioactive compounds, which had the potential to improve lipid metabolism and inflammation, while also postponing the disease's progression[22]. However, people were unaware of the various uses and potential mechanisms of flaxseed related to lipid metabolism and inflammation in food and medicine.

The current study aimed to examine whether flaxseed powder (FLA) regulated lipid metabolism and liver inflammation in NAFLD mice by changing intestinal microbiota and BA. Our results showed that the supplementation of FLA affected the transformation of bacterial BA by changing the composition of intestinal microflora, and preventing NAFLD damage by increasing the intestinal TGR5 signal transduction in mice. Different BA distribution, namely the high abundance of hyocholic acid (HCA) species, including HCA, hyodeoxycholic acid (HDCA), and its derivatives combined with glycine and taurine, might play a key role in regulating lipid metabolism and liver inflammation, leading to its unique prevention of NAFLD.

2. Materials and methods

2.1 Animal experiments and diets

The animal studies were complied with all relevant ethical regulations and approved by the Institutional Animal Care and Use Committee of Southeast University. The animal ethics approval number was 2020-0330-011. The mice were kept in a controlled environment (22–25 °C, 12 h of light/dark cycle, humidity (40 ± 5)%), and were exposed to ultra-pure water freely in a cage ventilated separately under specific pathogen-free (SPF) conditions. The mice were allowed one week of acclimatization fed with a control chow diet.

Male wild-type (WT) C57BL/6J mice (six weeks old) and *TGR5* gene knockout (*TGR5*$^{-/-}$) mice (six weeks old) were obtained from Shanghai Nanfang Model Biotechnology Co., Ltd. Following a week of acclimatization feeding, 24 WT mice were randomly divided into three groups based on their body weight, each containing eight mice: 1) normal control group (CO) consumed a diet low in fat (D12450J), comprising 10% fat, 70% carbohydrates and 20% protein; 2) NAFLD model group (HFD) consumed a diet high in fat (D12492), comprising 60% fat, 20% carbohydrate and 20% protein; 3) FLA intervention (FLA) group were adjusted for their feed formulations based on high-fat formula feed D12492 with FLA additions of 25% (*m*/*m*), and their three energy nutrients were adjusted to achieve energy balance with the corresponding NAFLD model group. The 24 *TGR5*$^{-/-}$ mice were grouped in the same manner as the WT mice. FLA was purchased from CanMar foods Ltd., Regina, Canada. The ingredients of the FLA used were shown in Table 1, the details of the feed formulations for each group were shown in Table 2. The body weight and food intake were recorded once a week during the experiment. After 12 weeks of intervention, fecal samples were collected within 24 h at the end of the experiments.

Table 1
The ingredients of the FLA.

Item	Value
Energy (kJ/100 g)	2 208
Protein (%)	20.0
Fat (%)	43.0
Saturated fatty acids (%)	4.0
Trans fatty acids (%)	0.0
Monounsaturated fatty acids (%)	9.0
Polyunsaturated fatty acids (%)	30.0
Carbohydrate (%)	29.0
Dietary fiber (%)	24.0
Sodium (mg/100 g)	70

2.2 Serum sample collection and measurement

At the end of the experiment, mice were anesthetized using isoflurane after 12 h of fasting, but consuming water as usual. Following this, blood was extracted from the eyeballs using 1.5 mL centrifuge tubes equipped with EDTA-K_2 anticoagulants. The serum was prepared by centrifugation at 3 700 r/min for 25 min after standing at 4 °C for 2 h. Subsequently, the serum was isolated and preserved individually in apparatus at −80 °C. The levels of serum TC, TG, LDL-C, and high-density lipoprotein cholesterol (HDL-C) were determined through enzymatic techniques using commercial assay kits (Nanjing Jiancheng Co., Ltd., Nanjing, China). The levels

Table 2
Compositions of experimental diets.

Ingredient	WT_CO/KO_CO		WT_HFD/KO_HFD		WT_FLA/KO_FLA	
	Proportion (%)	Energy composition (%)	Proportion (%)	Energy composition (%)	Proportion (%)	Energy composition (%)
Protein	19.3	20	26.2	20	26.2	20
Carbohydrate	67.6	70	26.3	20	26.3	20
Fat	2.4	10	34.9	60	34.9	60
	Mass (g)	Energy (kcal)	Mass (g)	Energy (kcal)	Mass (g)	Energy (kcal)
Casein, 80 mesh	200	800	200	800	161.3	645.2
L-Cystine	3	12	3	12	3	12
Flaxseed (protein)	0	0	0	0	38.7	154.8
Flaxseed (fat)	0	0	0	0	83.2	748.8
Flaxseed (carbohydrate)	0	0	0	0	9.7	38.7
Flaxseed (dietary fiber)	0	0	0	0	46.4	0.0
Corn starch	452.2	1 809	0	0	0	0
Maltodextrin 10	75	300	125	500	117.3	469.2
Sucrose	172.8	691	68.8	275.2	68.8	275.2
Cellulose, BW200	50	0	50	0	3.6	0
Soybean oil	25	225	25	225	25	225
Lard	0	0	245	2 205	161.8	1 456.2
Mineral mix S10026	10	0	10	0	10	0
DiCalcium phosphate	13	0	13	0	13	0
Calcium carbonate	5.5	0	5.5	0	5.5	0
Potassium citrate, $1H_2O$	16.5	0	16.5	0	16.5	0
Vitamin mix V10001	10	40	10	40	10	40
Choline bitartrate	2	0	2	0	2	0
FD&C blue dye #1	0	0	0.05	0	0.05	0
Total	1 035	3 850	773.8	4 057.2	775.8	4 065.2

Note: WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, flaxseed powder intervention group; KO_CO, *TGR5* gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, *TGR5* gene knockout type flaxseed powder intervention group.

of serum interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) were measured using the commercial ELISA kits (Nanjing Jin Yibai Biological Technology Co., Ltd., Nanjing, China).

2.3 Histological examination

Liver and colon tissues were fixed in formalin, and then embedded in paraffin, and tissue sections were stained with hematoxylin and eosin (HE). NAFLD was defined as steatosis with or without inflammation, but without ballooning[23]. Non-alcoholic fatty liver (NAFL) was diagnosed and staged using the Kleiner NAFLD activity score (NAS)[24]. Utilizing an optical microscope (Olympus BX41), pathological images were captured at 400× magnifications. The results were analyzed by an external pathology researcher based on steatosis, focal and diffuse inflammation, and nuclear vacuolation. Based on the NAS score at liver biopsy, NAFLD was categorized NAFL (NAS < 3), borderline non-alcoholic steatohepatitis (NASH, NAS = 3–4), and NASH (NAS > 5). Furthermore, villus height and crypt depth were measured in at least three different areas of one slide and in more than four mice per group. The NDP viewer 2 software was used to measure ileum villus length and crypt depth.

2.4 Western blot analyses

Tissue samples from the liver and intestines were lysed in cold RIPA buffer with protease inhibitors (Yeasen, Shanghai, China). Protein levels were measured using the BCA protein assay kit (Thermo Fisher, 23227). Equal amounts of protein (100 μg per lane) were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Membranes underwent blocking at room temperature using 5% non-fat milk powder, followed by an overnight incubation at 4 °C with primary antibodies. Primary antibodies were FXR (1:1 000, mAb #72105, Cell Signalling Technology, Beverly, MA, USA), TGR5 (1:1 000, ab72608, Abcam, Cambridge, UK), CYP7A1 (1:1 000, ab138497, RRID: AB_2828001, Abcam, Cambridge, UK), FGFR4 (1:1 000, mAb #A9197, ABclonal, Wuhan, China), TLR4 (1:1 000, sc-293072, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and NF-κB p65 (1:1 000, mAb #8242, Cell Signalling Technology, Beverly, MA, USA). The relative intensity of bands was standardized to GAPDH through the application of GAPDH antibody (1:1 000, Cell Signalling Technology, Beverly, MA, USA). The membranes underwent three washes using TBST buffer, succeeded by a 2 h room temperature incubation with HRP-conjugated secondary antibody (1:20 000, SA00001-1/SA00001-2, Proteintech Group, Inc., USA). The gray values of gel bands were quantified using ImageJ software.

2.5 Gut microbiota assessment

Total genomic DNA was extracted from the cecal contents collected from all the mice at the end of the 12-week feeding. The V3−V4 variable regions of the bacterial 16S rRNA genes were amplified. All DNA samples were amplified and purified using a primer pair (338F and 806R). PCR products were mixed in equal density ratios. The method used to assess the gut microbial profile was available in the supplementary material.

2.6 Fecal BA analysis

For BA quantitation in ileum, cholic acid (CA)-D_4, chenodeoxycholic acid (CDCA)-D_4, deoxycholic acid (DCA)-D_4, glycochenodeoxycholic acid (GCDCA)-D_4, glycodeoxycholic acid (GDCA)-D_4, glycol-lithocholic acid (GLCA)-D_4, lithocholic acid (LCA)-D_4, taurocholic acid (TCA)-D_4 and tauro-ursodeoxycholic acid (TUDCA)-D_5 were used as internal standards. BAs were analyzed using ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) based on the previous work[25]. The method used to assess ileum BA was available in the supplementary material.

2.7 Statistical analysis

Data were expressed as mean ± standard deviation (SD). The significance of the differences between the groups was assigned using the two-way ANOVA of Bonferroni post-test (parametric data set) or Dunn multiple comparison test (Kruskal-Wallis test of non-parametric data set) (GraphPad Prism 8, USA). Non-normal distribution data were compared by Mann-Whitney *U* test (between two groups) or Kruskal-Wallis test (between multiple groups). All results were considered statistically significant when the *P* value was less than 0.05.

The intestinal microbiota was evaluated using QIME and R package 3.5.1. To compare the relative level of the rich taxa at the level of phylum and genus among the groups, the two-way analysis of variance (ANOVA) and the post-hoc least significance tests were carried out. The α-diversity was calculated by QIME2, including Chao1 index, Shannon index, and Pielou's evenness index, to evaluate the community diversity of microbes in the intestinal flora. Based on Bray-Curtis and Jaccard, principal coordinate analysis (PCoA) was used to evaluate β-diversity. The significant difference in β-diversity was analyzed by permutation multivariate analysis of variance (PERMANOVA). In addition, linear discriminant analysis (LDA) effect size (LEfSe) was conducted to identify biomarkers rich in taxa and functional pathways by calculating LDA scores (greater than 3) between groups, and R package 3.5.1 was used to draw heat maps. Apart from that, the phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) based on operational taxonomic units (OTUs) was used to predict the abundance of functional categories using the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs. Furthermore, Spearman's correlation analysis was used to evaluate the correlation between the change of microbial species and the host BAs, and the data were expressed as mean ± SEM and $P < 0.05$ as the significance threshold.

3. Results

3.1 Effects of FLA intervention on the physiological and biochemical parameters of WT and TGR5$^{-/-}$ mice with NAFLD

This experiment not only focused on the ameliorative effects of FLA on NAFLD but also verified the crucial role of *TGR5* gene in the study. As shown in Fig. 1A, the dynamic changes in body weight showed that the body weight of *TGR5*$^{-/-}$ mice was higher than that of WT mice under the same formulated feed, suggesting that *TGR5* gene knockout was more likely to cause weight gain in mice. Furthermore, FLA significantly delayed high-fat diet induced body weight gain in both WT and *TGR5*$^{-/-}$ mice, showing a slower increased trend than other groups, especially in WT mice body weight was almost maintained at the CO group level. As shown in Fig. 1B, after 12 weeks of intervention, the final body weights of the FLA group were significantly lower than those of the HFD group ($P < 0.01$ or 0.001). Moreover, as shown in Fig. 1C, there was no difference in the average daily feed intake among the groups of mice.

After the intervention, the organs of the mice in each group, including the liver, epididymal fat, and subcutaneous fat, were weighed, and the organ-body ratio was calculated (shown in Table 3). Under the same feed formula, the net weight of the liver, epididymal fat, and hypogastric fat in *TGR5*$^{-/-}$ mice was significantly higher than that in WT mice. The results further suggested that knockout of the *TGR5* gene resulted in a more susceptible increase in body fat content in mice. After FLA intervention, regardless of whether it was *TGR5*$^{-/-}$ mice or WT mice, the net weight of epididymal fat and hypogastric fat in the body, as well as their visceral body ratio, were significantly reduced compared to the HFD group ($P < 0.05$). The results of the study indicated that FLA could reduce fat accumulation in the body.

To compare the effects of FLA intervention on blood lipids and inflammation in WT and *TGR5*$^{-/-}$ mice and to identify the differences between them, the present study examined the serum levels of TC, TG, HDL-C, and LDL-C, as well as the serum levels of the inflammatory factors TNF-α and IL-6 in each group of mice. As shown in Figs. 2A–D, TC, TG, and LDL-C levels were significantly higher in the WT_HFD group compared to the WT_FLA group ($P < 0.05$). Compared to the KO_FLA group, the levels of TC, TG, and LDL-C were also significantly higher in the KO_HFD group ($P < 0.05$ or 0.01). These results indicated that FLA intervention could significantly reduce the levels of serum TC, TG, and LDL-C in mice, *TGR5* gene knockout did not cause a decrease in FLA's ability to improve blood lipid levels.

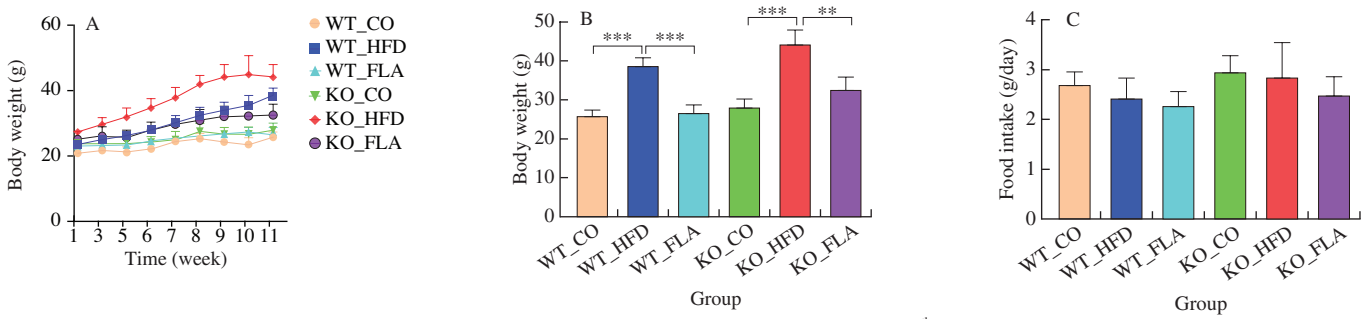

Fig. 1 (A) Effects of FLA intervention on body weight at different time points, (B) body weight at the 12th week, and (C) daily food intake of each group. WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, wild-type flaxseed powder intervention group; KO_CO, *TGR5* gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, *TGR5* gene knockout type flaxseed powder intervention group. Data were expressed as mean ± SD ($n = 8$). $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 3
Effects of FLA on body weight and organ weight of NAFLD mice.

General index	WT_CO	WT_HFD	WT_FLA	KO_CO	KO_HFD	KO_FLA
Weight (g)	25.88 ± 1.46^{d}	38.63 ± 2.13^{b}	26.50 ± 2.20^{d}	28.00 ± 2.14^{d}	44.13 ± 3.83^{a}	32.63 ± 3.25^{c}
Glucose (mmol/L)	9.59 ± 1.18^{c}	11.83 ± 1.19^{b}	10.40 ± 2.29^{bc}	8.89 ± 1.66^{c}	14.04 ± 3.93^{a}	10.44 ± 1.05^{bc}
Liver (g)	1.25 ± 0.14^{bc}	1.46 ± 0.17^{b}	1.02 ± 0.13^{c}	1.32 ± 0.43^{b}	1.71 ± 0.38^{a}	1.34 ± 0.31^{b}
Epididymal fat (g)	0.35 ± 0.18^{c}	2.47 ± 0.40^{a}	0.49 ± 0.30^{c}	0.63 ± 0.23^{c}	2.68 ± 0.49^{a}	1.54 ± 0.55^{b}
Hypogastric fat (g)	0.08 ± 0.05^{d}	1.03 ± 0.12^{b}	0.18 ± 0.13^{d}	0.22 ± 0.13^{d}	1.40 ± 0.27^{a}	0.70 ± 0.28^{c}
Liver index (%)	4.85 ± 0.66^{a}	3.77 ± 0.31^{b}	3.82 ± 0.26^{b}	4.65 ± 1.31^{a}	3.84 ± 0.59^{b}	4.11 ± 0.89^{ab}
Testicular fat ratio (%)	1.33 ± 0.65^{d}	6.37 ± 0.81^{a}	1.81 ± 1.02^{d}	2.21 ± 0.66^{d}	6.10 ± 1.10^{a}	4.60 ± 1.42^{c}
Abdominal fat bodies (%)	0.29 ± 0.19^{c}	2.67 ± 0.24^{b}	0.57 ± 0.45^{dc}	0.76 ± 0.41^{d}	3.16 ± 0.41^{a}	2.09 ± 0.76^{c}

Note: WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, wild-type flaxseed powder intervention group; KO_CO, *TGR5* gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, *TGR5* gene knockout type flaxseed powder intervention group. Data were expressed as mean ± SD ($n = 8$). Different lowercase letters in the same line represent a statistically significant difference ($P < 0.05$).

However, as shown in Fig. 2F, among the inflammatory factors, only the level of TNF-α showed a statistically significant difference among the three groups of WT mice; compared to the WT_HFD group, the levels of TNF-α were significantly lower in the WT_FLA and WT_CO groups ($P < 0.05$). In contrast, regardless of the feeding method, the TNF-α levels were high in all three groups of *TGR5*$^{-/-}$ mice, and FLA intervention did not reduce the TNF-α level. These results suggested that *TGR5* gene knockout might block the anti-inflammatory effect of FLA, and the *TGR5* gene might be a key protein in the anti-inflammatory pathway of FLA.

3.2 Effects of FLA intervention on liver histology changes in WT and TGR5$^{-/-}$ mice with NAFLD

Liver tissues of WT-type and *TGR5*$^{-/-}$ type groups of mice were stained by HE and evaluated by histological NAS. As shown in Fig. 3, HE staining was performed to observe the pathological conditions of liver cells in mice under a 400× microscope. Pathological sections showed that hepatocytes in the WT_HFD model group were swollen, vacuolated and degenerated, with inflammatory cells, and fat vacuoles were visible under light microscopy, and the cytoplasm was filled with a large number of rounded lipid droplets of varying sizes. In the WT_CO group, hepatocyte structures were structurally intact, with clear demarcation, sizes were relatively uniform, nuclei were round and clear, centrally located, cytoplasm was rich, liver lobules were normal, and neatly arranged radial liver cell cords were visible. Compared to the WT_HFD group, the WT_FLA group showed more complete and clear hepatocyte structures and boundaries, indicating that this FLA dosage could effectively prevent the occurrence and development of NAFLD disease. Compared to the WT_HFD group, the KO_HFD model group exhibited more severe fat degeneration; the KO_CO group also showed hepatocyte swelling and condensation,

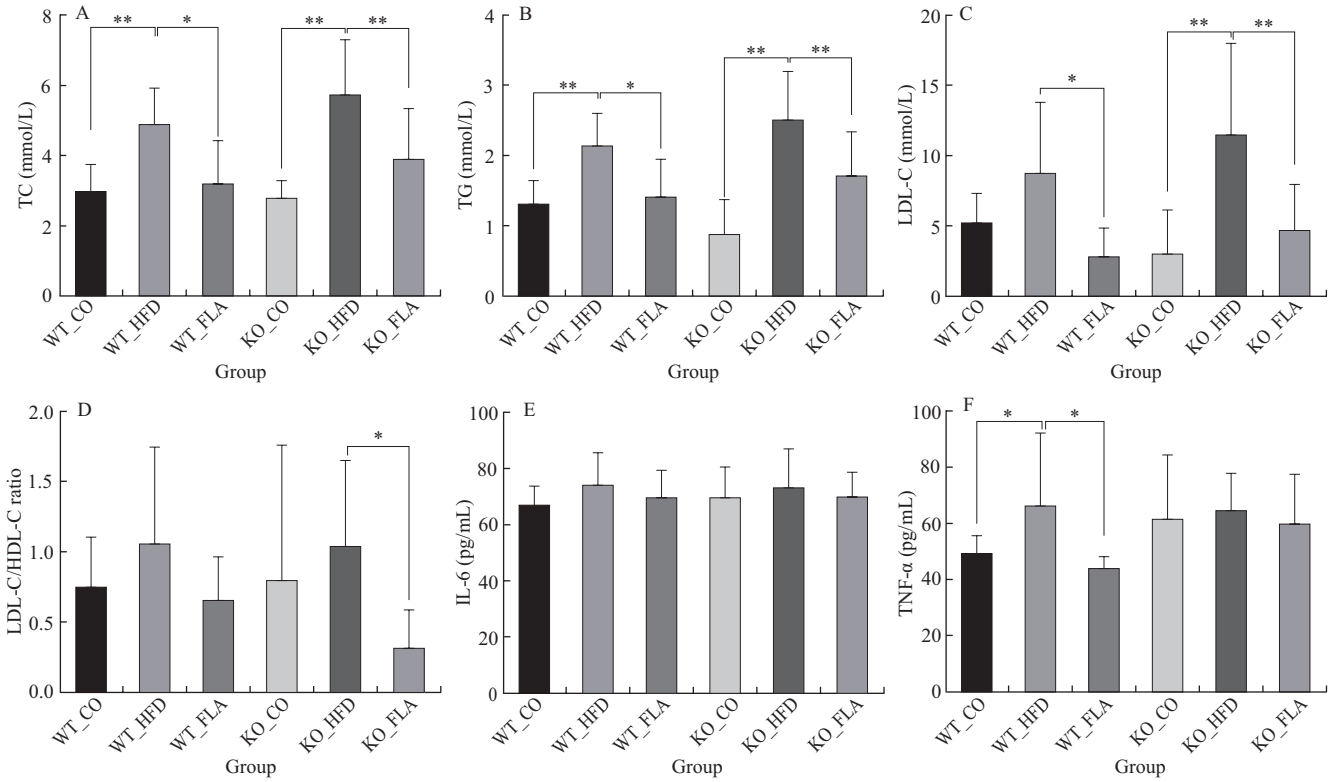

Fig. 2 Effects of FLA intervention on blood lipid and serum inflammatory factor levels in WT and *TGR5*$^{-/-}$ mice. (A) TC, (B) TG, (C) LDL-C, (D) LDL-C/HDL-C ratio, (E) IL-6, (F) TNF-α. WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, wild-type flaxseed powder intervention group; KO_CO, *TGR5* gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, *TGR5* gene knockout type flaxseed powder intervention group. Data were expressed as mean ± SD ($n = 8$). *$P < 0.05$, **$P < 0.01$.

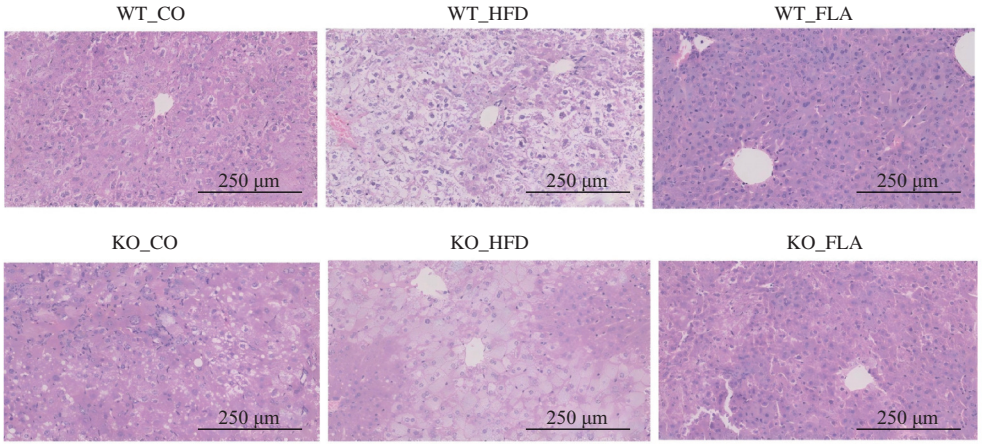

Fig. 3 Representative images of HE staining of liver sections. WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, wild-type flaxseed powder intervention group; KO_CO, *TGR5* gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, *TGR5* gene knockout type flaxseed powder intervention group.

with a few round lipid droplets of varying sizes in the cytoplasm, albeit less severe than the WT_HFD group. The swelling and concentration of lipid droplets in hepatocytes were lower in the KO_FLA group compared to the KO_CO group, but the integrity and clarity of hepatocyte structure and boundaries were poorer than in the WT_FLA group.

As shown in Table 4, FLA intervention significantly reduced the NAS of the liver in WT_FLA mice compared to WT_HFD group ($P < 0.05$); similarly, the NAS of the liver in KO_FLA mice was also lower than that of KO_HFD group. The *TGR5*$^{-/-}$ mice NAS scored higher on average compared to the WT mice.

The above results indicated that FLA could effectively prevent the occurrence and progression of NAFLD, and knocking out the *TGR5* gene exacerbated hepatocellular steatosis and the severity of NAFLD in mice.

3.3 Effects of FLA intervention on key proteins of intestinal BA signaling pathway in WT and TGR5$^{-/-}$ *mice with NAFLD*

To further verify that the lipid-lowering and anti-inflammatory efficacy of FLA was mainly mediated through FXR and TGR5, respectively, this study further examined the expression of key proteins in the hepatic BA signaling pathway in the WT and *TGR5*$^{-/-}$ groups of mice. As shown in Fig. 4, the Western blot results indicated that, compared to the WT_CO group and WT_FLA group, the key proteins TLR4 ($P < 0.05$) on the inflammation-regulating pathway and the key protein CYP7A1 ($P < 0.05$) on the lipid-regulating pathway showed higher expression in the WT_HFD group. Although there was no statistical difference, NF-κB expression tended to increase in the WT_HFD group compared to the WT_CO group and WT_FLA group. However, in the liver tissues of *TGR5*$^{-/-}$ mice, there was no significant difference in the expression of NF-κB and TLR4 proteins among KO_CO group, KO_FLA group and KO_HFD group ($P > 0.05$), suggesting that the regulation of hepatic inflammation by FLA was mainly related to the activation of TGR5. In addition, there was no significant difference in the expression of CYP7A1 protein among KO_CO group, KO_FLA group and KO_HFD group ($P > 0.05$).

3.4 Effects of FLA intervention on BA metabolism in WT and TGR5$^{-/-}$ *mice with NAFLD*

The study conducted principal component analysis (PCA) on fecal samples from each group of mice to preliminarily understand the overall metabolic differences between the groups and the degree of variation within the samples (Supplementary Fig. S1). Each point in the figure represented a sample, with different colored points indicating different samples. The closer the projection distance between two points on the axes, the more similar the BAs composition of the two samples in the corresponding dimension. As shown in Fig. S1A, a clear separation among the KO_CO, KO_HFD, and KO_FLA groups could be visualized, with the PC1 axis explaining 46.71% of the total BAs variance, and

Table 4
Effects of FLA intervention on Kleiner NAS of NAFL in WT and *TGR5*$^{-/-}$ mice.

Group	Statistics on the number of mice in each NAS score per group				NAS
	1 point	2 point	3 point	4 point	
WT_CO	5	3	0	0	1.38 ± 0.48^{b}
WT_HFD	2	2	4	0	2.25 ± 0.83^{a}
WT_FLA	6	1	1	0	1.38 ± 0.70^{b}
KO_CO	3	4	1	0	1.75 ± 0.66^{ab}
KO_HFD	2	2	3	1	2.38 ± 0.99^{a}
KO_FLA	3	4	1	0	1.75 ± 0.66^{ab}

Note: WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, wild-type flaxseed powder intervention group; KO_CO, *TGR5* gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, *TGR5* gene knockout type flaxseed powder intervention group. Data were expressed as mean ± SD ($n = 8$). Different lowercase letters in the same line represent a statistically significant difference ($P < 0.05$).

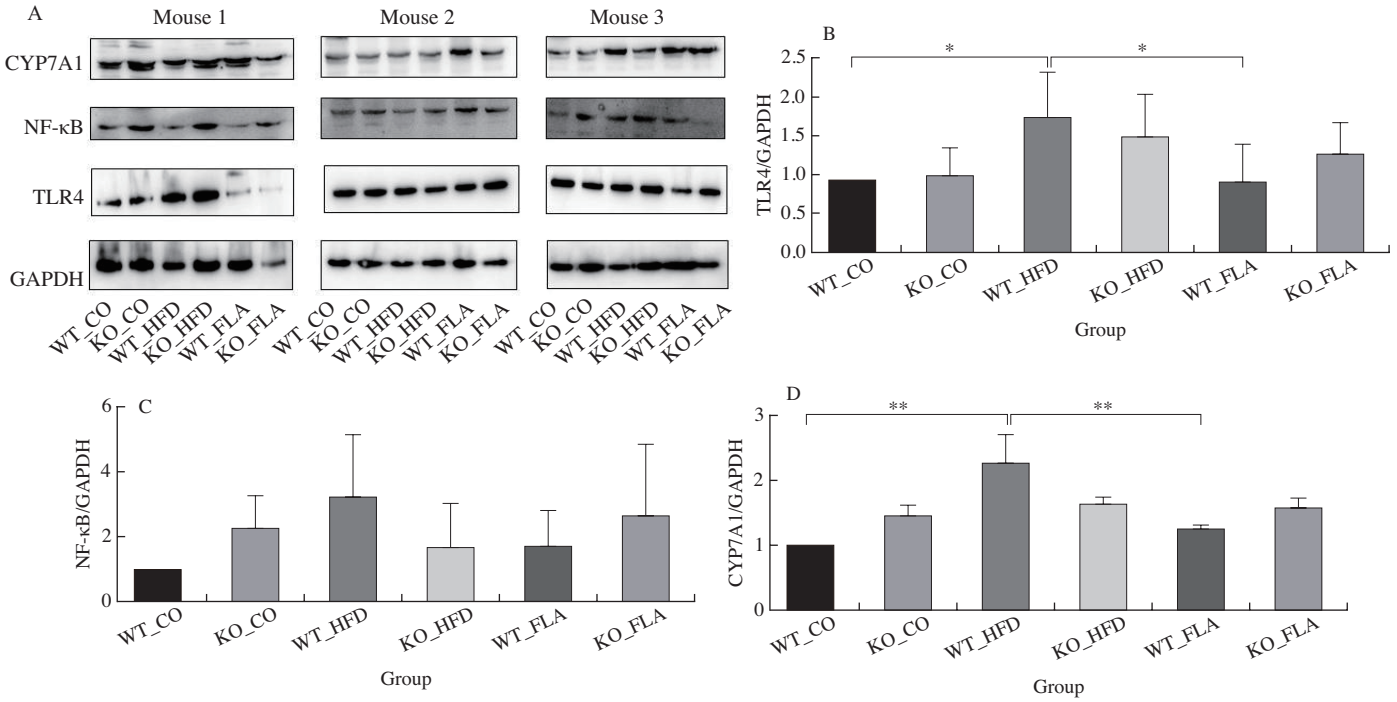

Fig. 4 Expression level of key proteins in BA metabolism pathway in liver of WT and *TGR5*$^{-/-}$ mice. (A) Representative Western blot images and quantitative analysis of (B) TLR4, (C) NF-κB p65, and (D) CYP7A1. WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, wild-type flaxseed powder intervention group; KO_CO, *TGR5* gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, *TGR5* gene knockout type flaxseed powder intervention group. Data were expressed as mean ± SD (n = 8). *P < 0.05, **P < 0.01.

the PC2 axis accounting for 14.31% of the variance. As shown in Fig. S1B, the separation trend among the WT_CO, WT_HFD, and WT_FLA groups was similar to that among the *TGR5*$^{-/-}$ mouse groups, but the separation trend was not as pronounced. The study further conducted PCA on fecal samples from WT mice and *TGR5*$^{-/-}$ mice under the same formulated feed intervention (Figs. S1C−E). The results indicated that the variability of BAs in WT mice and *TGR5*$^{-/-}$ mice under the same diet was relatively small. Among them, the variability of mouse samples fed with CO formulated feed was greater compared to those fed with HFD and FLA feeds, with the FLA feed group showing the least variability in BAs. Additionally, statistical analysis of BAs metabolites between the two groups showed that the WT-FLA group and the KO-FLA group had the fewest differential metabolites, followed by the WT_HFD group and the KO_HFD group (Table 5). These results coincided with the results of PCA, which suggested that knocking out the *TGR5* gene might not significantly affect the BAs fractions in mice feces.

Table 5
Statistics of differential metabolites.

Group	Total	Up regulated	Down regulated
KO_CO *vs*. KO_FLA	26	25	1
KO_CO *vs*. KO_HFD	20	20	0
KO_FLA *vs*. KO_HFD	21	5	16
WT_CO *vs*. KO_CO	16	8	8
WT_CO *vs*. WT_FLA	26	24	2
WT_CO *vs*. WT_HFD	19	16	3
WT_FLA *vs*. KO_FLA	5	4	1
WT_FLA *vs*. WT_HFD	18	1	17
WT_HFD *vs*. KO_HFD	11	11	0

Note: WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, wild-type flaxseed powder intervention group; KO_CO, *TGR5* gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, *TGR5* gene knockout type flaxseed powder intervention group.

The study conducted a differential analysis of fecal BAs, as shown in Fig. 5, there were statistically significant differences in the total BAs (TBAs) and primary BA concentrations among the KO_CO, KO_HFD, and KO_FLA groups (P < 0.05). TBAs and primary BAs concentrations in the intestinal feces of KO_CO and KO_FLA mice were significantly lower than those in the KO_HFD group (P < 0.05 or 0.01). The KO_HFD group had significantly higher secondary BAs than the KO_CO and KO_FLA groups (P < 0.01). Additionally, compared to the KO_HFD group, the KO_FLA and KO_CO groups showed a significant decrease in unconjugated BA concentration in the intestinal feces (P < 0.01). The conjugated BA concentrations in the KO_FLA and KO_CO groups were lower than in the KO_HFD (P < 0.01). All in all, the trend of fecal BAs in the WT_CO, WT_HFD, and WT_FLA groups was similar to that in the three *TGR5*$^{-/-}$ groups, and no statistically significant differences were found in the TBAs, secondary BAs and unconjugated BAs concentrations among them (P > 0.05).

The study further analyzed the difference in BAs between *TGR5*$^{-/-}$ mice and WT mice under the same formulated feed intervention, and there were no statistically significant differences in TBAs, primary BAs, secondary BAs, conjugated BAs, and unconjugated BAs contents (P > 0.05), although total BAs, primary BAs, secondary BAs, conjugated BAs, and unconjugated BAs concentrations of the KO_HFD group were higher than those of the WT_HFD, but the difference was not statistically significant in TBAs, primary BAs, and conjugated BAs contents (P > 0.05). Furthermore, changes in BAs under different feed formulations were based on the orthogonal partial least squares discriminant analysis (OPLS-DA) model and screened for differential metabolites, which were also analyzed. As shown in Fig. 6, compared to the WT_HFD group, the levels of HDCA, DCA, GDCA, taurodeoxycholic acid (TDCA), 23-deoxycholic acid (23-DCA), 3β-HDCA, norcholic acid (NCA), isolithocholic acid

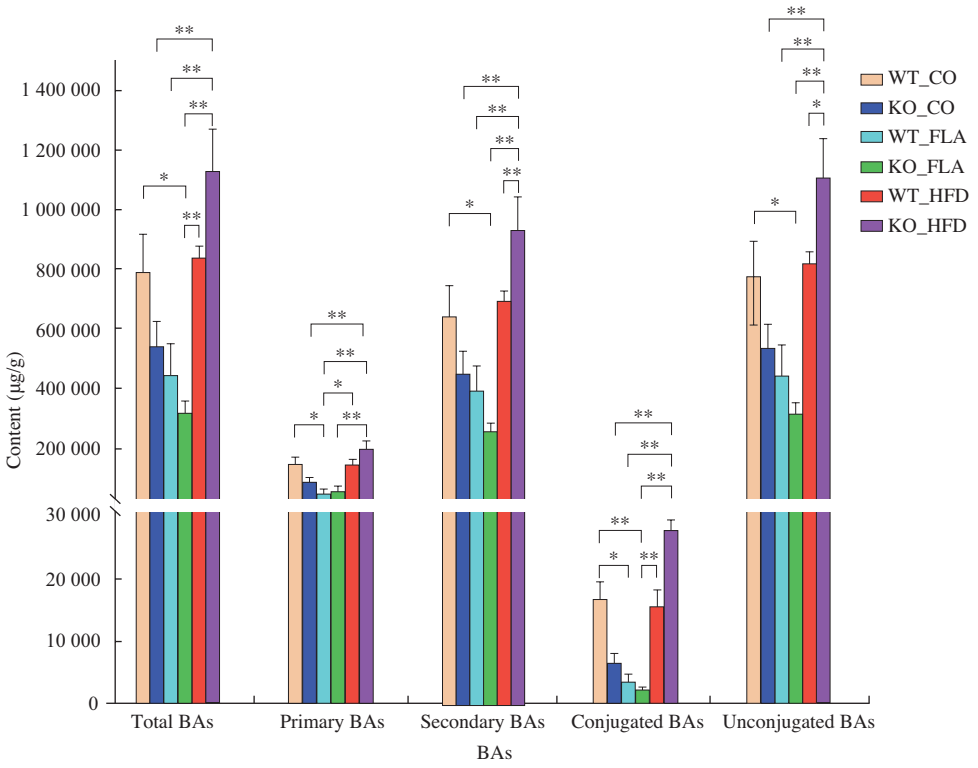

Fig. 5 Classification and analysis of fecal TBA profile in WT and *TGR5*$^{-/-}$ mice. WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, wild-type flaxseed powder intervention group; KO_CO, TGR5 gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, T*GR5* gene knockout type flaxseed powder intervention group. Data were expressed as mean ± SD (n = 8). *P < 0.05, **P < 0.01.

(ILCA), taurolithocholic acid (TLCA), ω-muricholic acid (ω-MCA), glycoursodeoxycholic acid (GUDCA), TCA, 3β-cholic acid (3β-CA), 3β-deoxycholic acid (3β-DCA), α-muricholic acid (α-MCA), and LCA in the feces of WT_CO mice were significantly reduced (P < 0.05). However, dehydrocholic acid (DHCA), glycodehydrocholic acid (GDHCA), and 7,12-diketolithocholic acid (7,12-DKLCA) significantly increased (P < 0.05). Also, compared with the WT_HFD group, the feces of mice in the WT_FLA group contained significantly higher levels of glycohyocholic acid (GHCA), murideoxycholic acid (MDCA), lithocholic acid-3-sulfate (LCA-3S), HDCA, TUDCA, 7-ketolithocholic acid (7-KLCA), HCA, hyocholic acid (THCA), ursocholic acid (UCA), 3β-HDCA, GUDCA, TLCA, isoallolithocholic acid (IALCA), taurochenodeoxycholic acid (TCDCA), ursodeoxycholic acid (UDCA), 3β-UDCA, and LCA, while isodeoxycholic acid (IDCA) significantly decreased (P < 0.05). As shown in Fig. 7, compared to the KO_HFD group, the levels of CDCA, IDCA, CA, 3-oxocholic acid (3-oxo-CA), glycocholic acid (GCA), GCDCA, NCA, TCA, 7-ketodeoxycholic acid (7-KDCA), 23-DCA, 3β-CA, 3β-DCA, UDCA, TDCA, 12-oxochenodeoxycholic acid (12-oxo-CDCA), 7,12-DKLCA, TCDCA, α-MCA, GDCA, and MDCA significantly decreased in the feces of the KO_CO group, the levels of chenodeoxycholic acid-3-β-D-glucuronide (CDCA-3Gln), GHCA, LCA-3S, HCA, HDCA, 3β-HDCA, MDCA, 7-KLCA, TUDCA, THCA, GUDCA, 3β-UDCA, UCA, TLCA, UDCA, and LCA significantly increased in the feces of the KO_FLA group, while 12-oxo-CDCA, IDCA, 3-oxodeoxycholic acid (3-oxo-DCA), 3-oxo-CA, and taurodehydrocholic acid (TDHCA) significantly decreased (P < 0.05). In *TGR5*$^{-/-}$ mice and WT mice, the differences in BAs caused by HFD and FLA intervention were mostly the same.

The above results further indicated that knocking out the *TGR5* gene did not significantly affect the BAs fractions in mice feces, but the differences in BAs changes among groups of *TGR5*$^{-/-}$ mice were obvious, indicating that *TGR5* gene knockout might lead to a more easily disrupted BAs balance in mice.

3.5 *Effects of FLA intervention on gut microbiota in WT and TGR5*$^{-/-}$ *mice with NAFLD*

The effective sequences of gut microbiota from all mice were clustered and analyzed to observe OTUs and their relative abundance. Based on the clustering results, a Venn diagram was drawn and the common and unique OTUs among the six groups were analyzed. As shown in Fig. S2, only 227 OTUs were shared by the six groups of mice; a comparison among the three groups of WT-type mice showed that the number of OTUs shared by the WT_CO, WT_HFD, and WT_FLA groups was 553, and the number of OTUs unique to them was 2 546, 2 013, and 4 053, respectively; a comparison among the three groups of *TGR5*$^{-/-}$ mice showed that the number of shared OTUs in the KO_CO, KO_HFD and KO_FLA groups was 473, and the number of unique OTUs was 2 558, 2 143 and 2 717, respectively.

The α-diversity of gut microbes was used to reflect the abundance, evenness, and diversity of microbial communities in the gut. Based on the results of the rarefaction curves, species accumulation curves, and rank-abundance curves (Fig. S3), the groups of samples were adequately sequenced, sample homogeneity was relatively good, and the sample size was sufficient to reflect the species composition of the community.

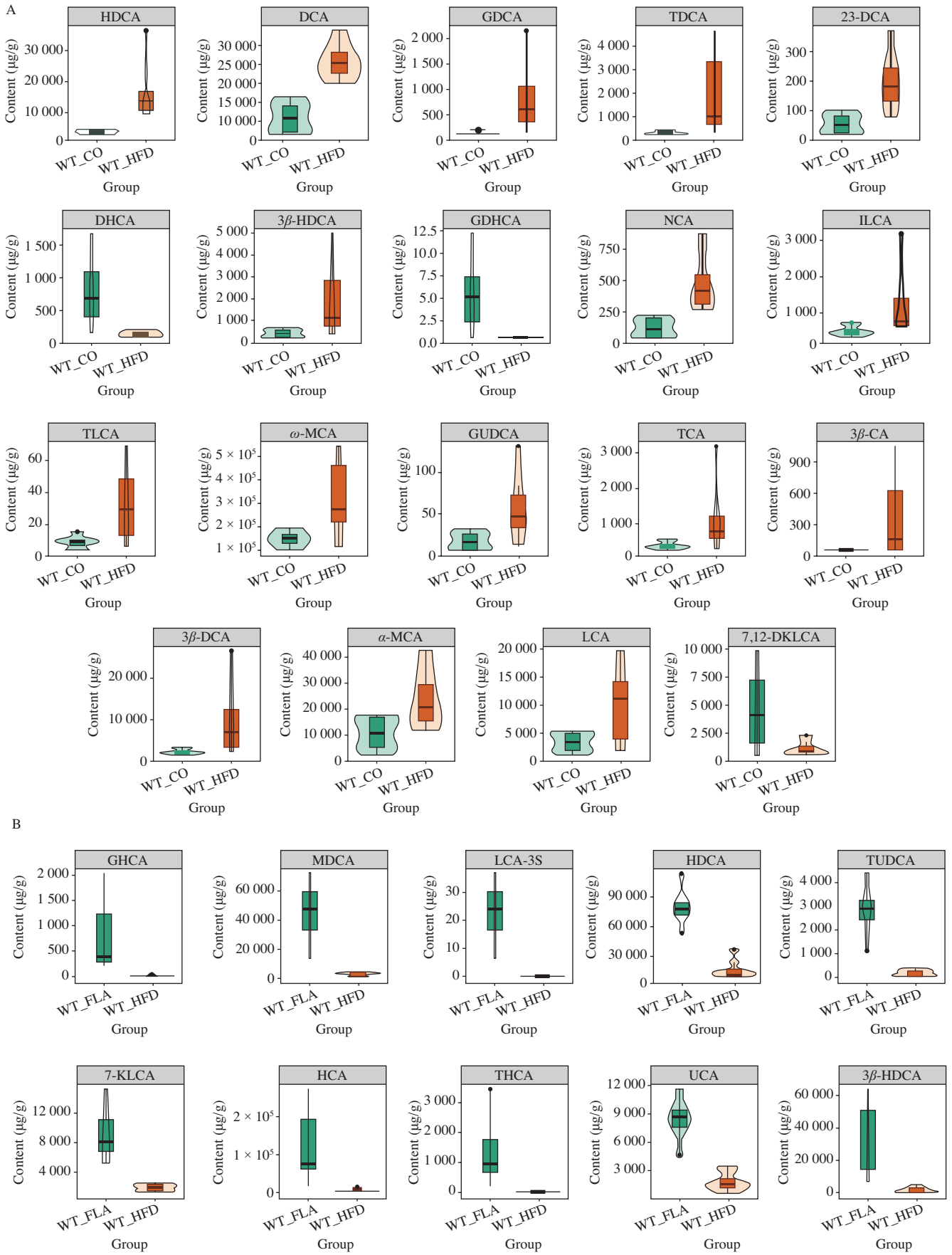

Fig. 6 Violin plots comparing BA differences among three groups of WT mice using OPLS-DA. (A) WT_CO *vs*. WT_HFD; (B) WT_FLA *vs*. WT_HFD. WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, wild-type flaxseed powder intervention group.

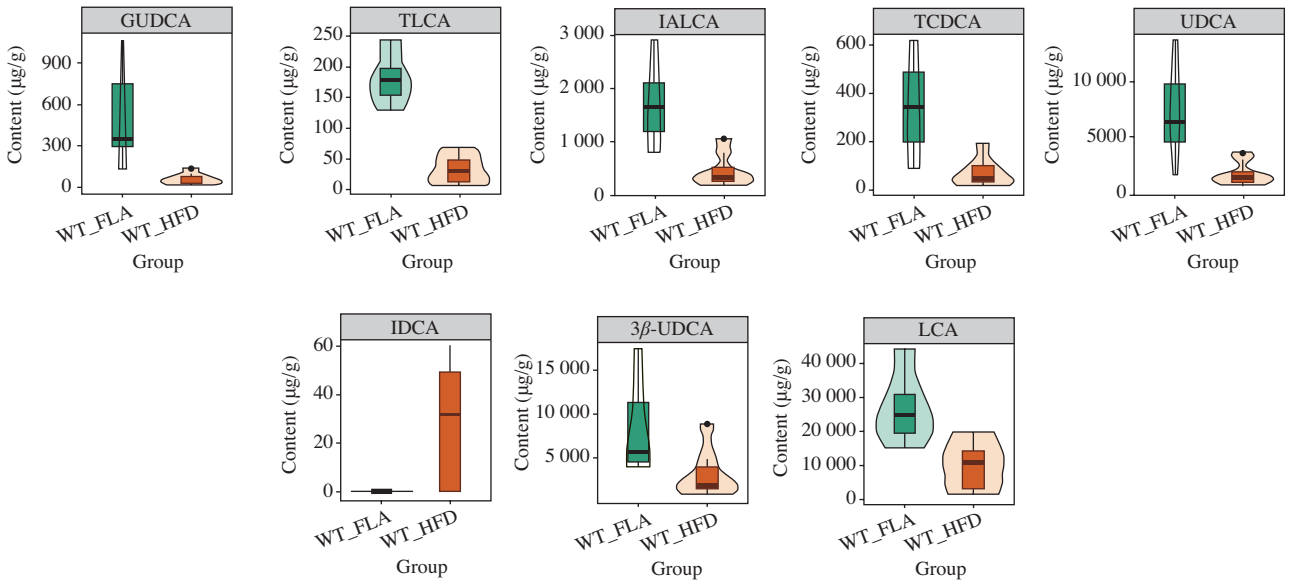

Fig. 6 (*Continued*)

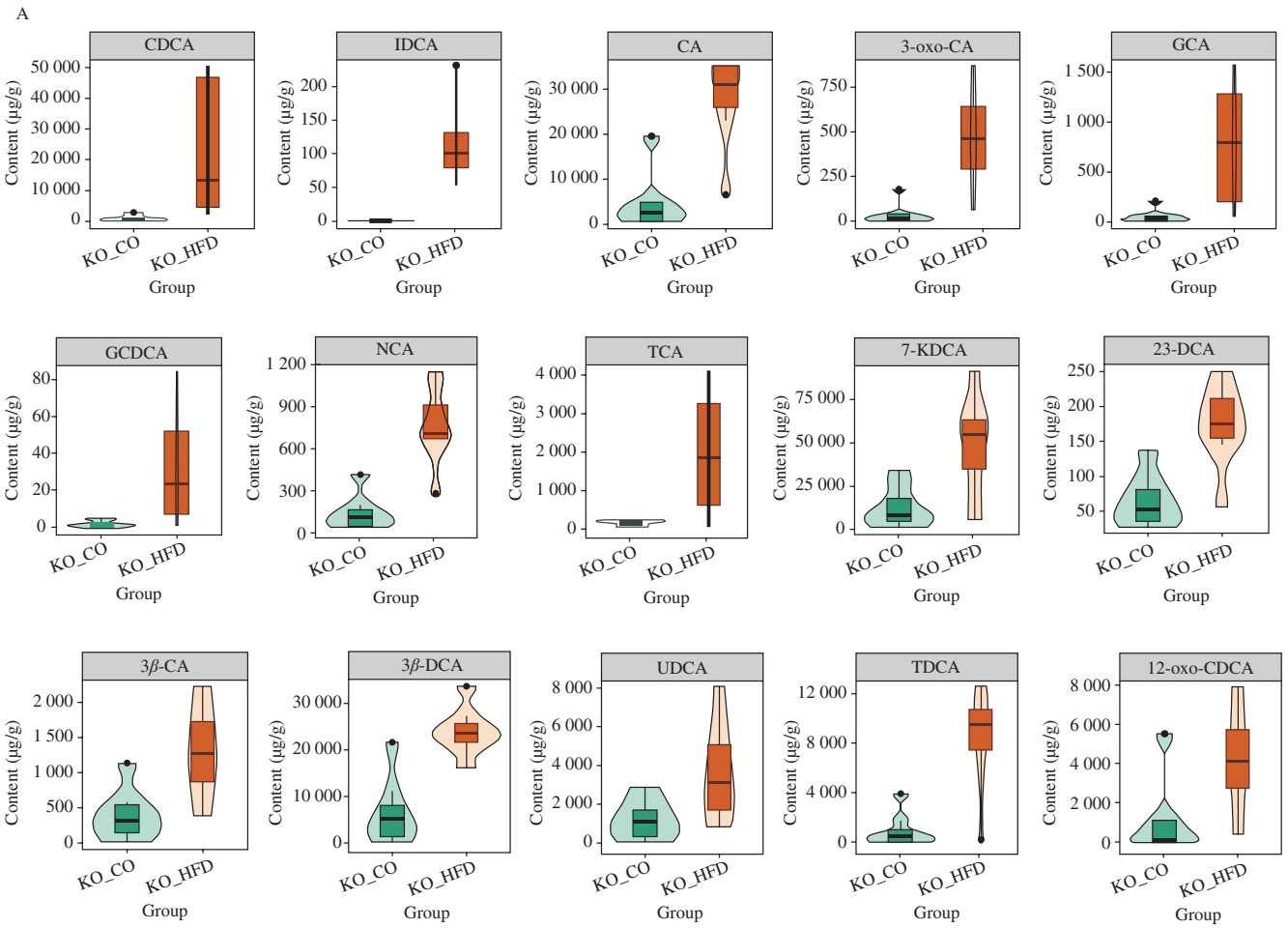

Fig. 7 Violin plots comparing BA differences among three groups of *TGR5*$^{-/-}$ mice using OPLS-DA. (A) KO_CO *vs.* KO_HFD; (B) KO_FLA *vs.* KO_HFD. KO_CO, *TGR5* gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, *TGR5* gene knockout type flaxseed powder intervention group.

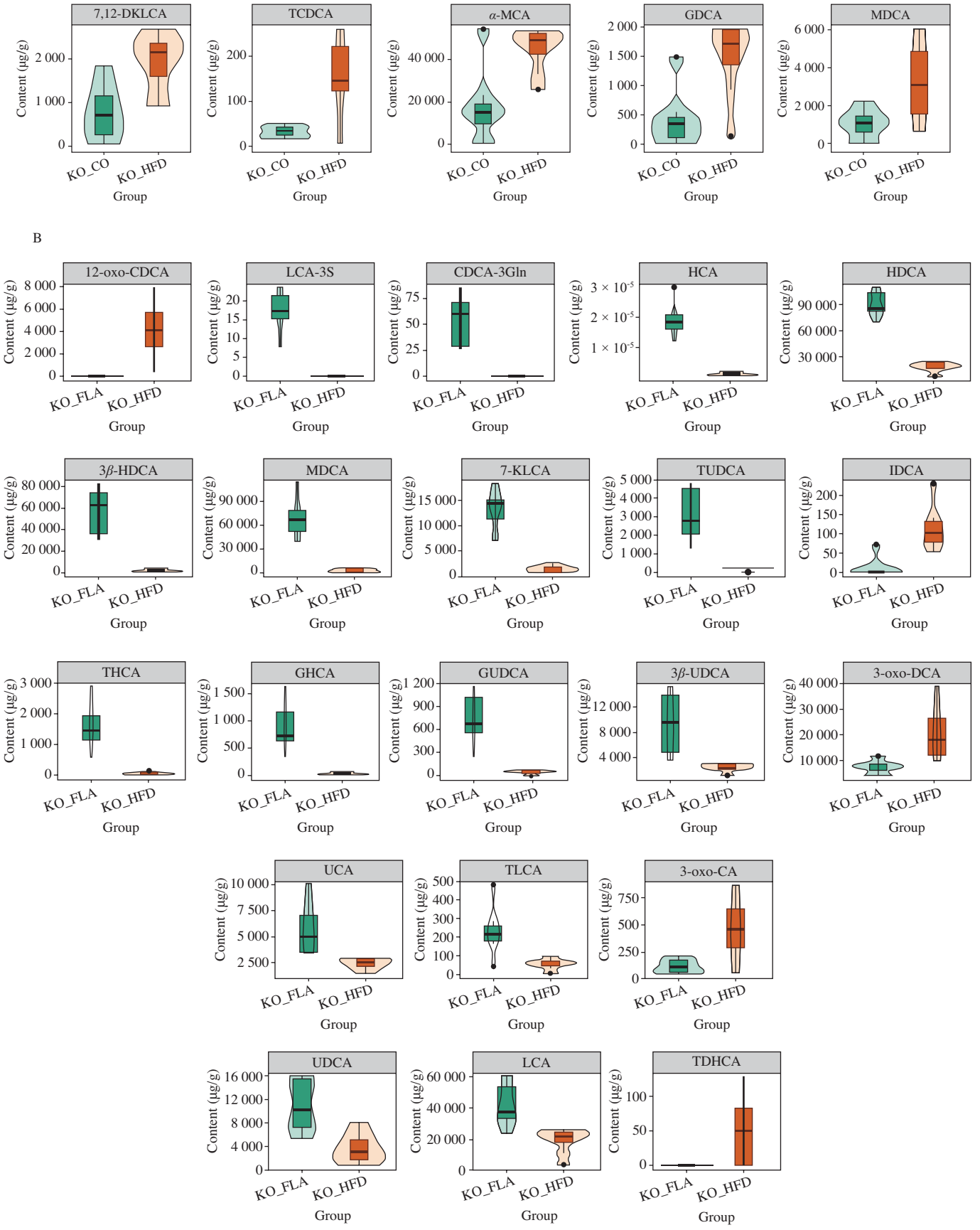

Fig. 7 (*Continued*)

Further analysis of α-diversity was conducted using the Chao1 index, Faith's PD index, Good's coverage index, Simpson index, Shannon index, Pielou's evenness index, and observed species index, with separate analyses for WT and *TGR5*$^{-/-}$ mice (Fig. S4). The results showed no significant differences in the Chao1 index, observed species index, Simpson index and Shannon index, Faith's PD index, and Good's coverage index between the gut microbiota of WT and *TGR5*$^{-/-}$ mice. However, Pielou's evenness index was significantly different in both WT and *TGR5*$^{-/-}$ type mice intestinal flora ($P = 0.025$ and $P = 0.039$). Since Pielou's evenness index was related to species evenness, it suggested that FLA intervention could increase the species evenness of gut microbiota.

To reveal the differences in gut microbiota among the FLA intervention group, the CO group, and the HFD group, the study employed PCoA based on weighted UniFrac to analyze changes in gut microbiota structure. As shown in Fig. S5A, the microbial composition of the FLA group in three sets of WT mice was similar to that of the CO group but distinct from the HFD group. PC1 axis explained 28.2% of the total variation in the microbial community, while 16.5% of the variation in the microbial community was explained by PC2 axis. As shown in Fig. S5B, the composition of the FLA microbiome differed from that of the HFD and CO groups in *TGR5*$^{-/-}$ mice. PC1 axis explained 11.8% of the total variation in the microbial community, while the PC2 axis accounted for 8.9% of the variation. The study further compared the microbial composition between the WT_CO and KO_CO groups, WT_HFD and KO_HFD groups, and WT_FLA and KO_FLA groups in pairs. As shown in Figs. S5C–E, the results indicated that the microbial composition of the WT_CO group was similar to that of the KO_CO group, as well as the WT_HFD group to the KO_HFD group. However, there was a more pronounced distinction between the microbiome composition of the WT_FLA and KO_FLA groups, suggesting interconnections and influences among the FLA intervention, the gut flora, and the TGR5 receptor.

At the phylum level, as shown in Fig. 8A, the 10 phyla of bacteria present in the gut contents of WT mice were Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia, Deferribacteres,

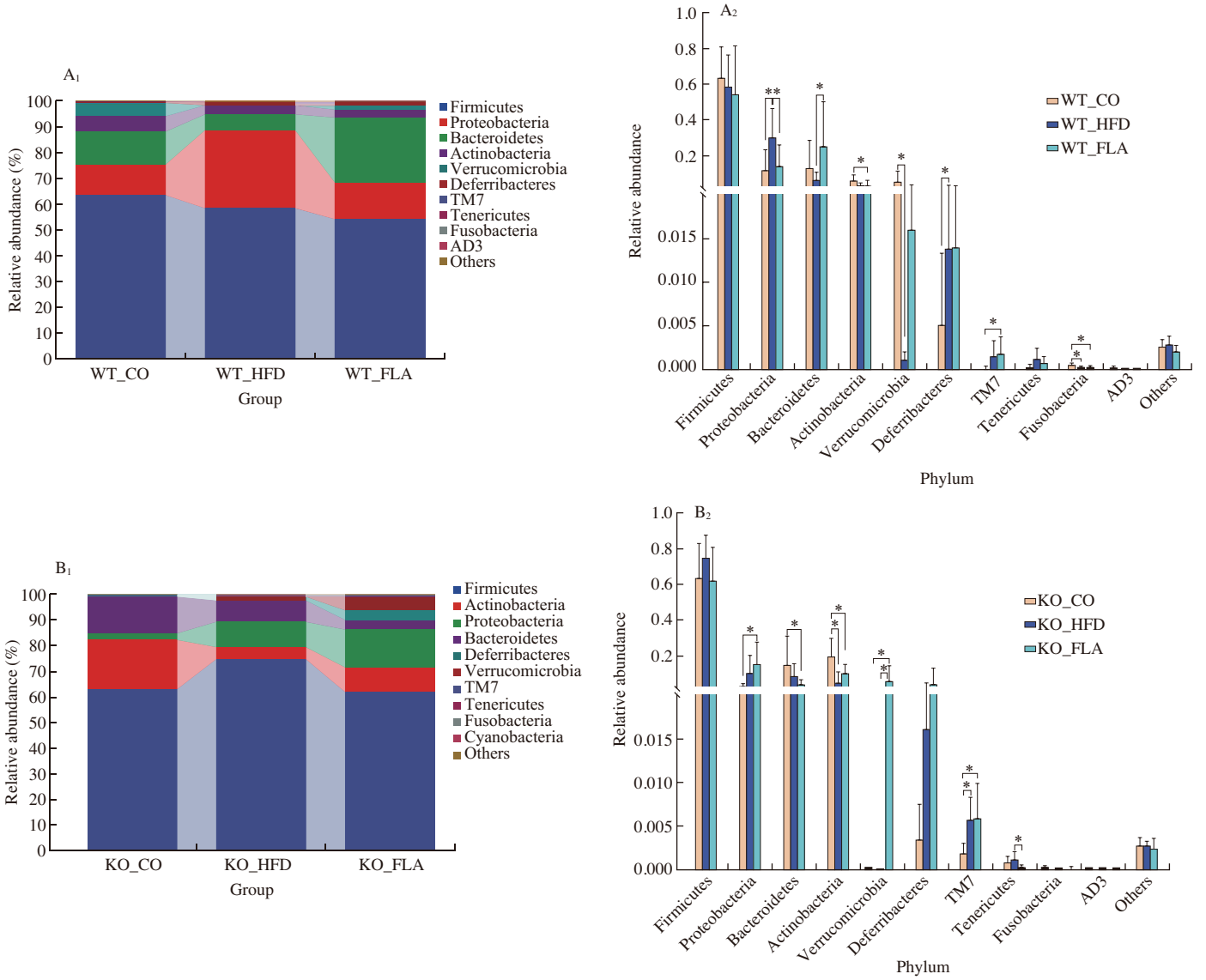

Fig. 8 Composition of gut microbiota at phylum level in (A) WT and (B) *TGR5*$^{-/-}$ mice. WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, wild-type flaxseed powder intervention group; KO_CO, *TGR5* gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, *TGR5* gene knockout type flaxseed powder intervention group. Data were expressed as mean ± SD ($n = 8$). *$P < 0.05$.

TM7, Tenericutes, Fusobacteria, and AD3. Among these, Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria were the main dominant phyla. Compared with the WT_HFD group, the fecal Bacteroidetes phylum was significantly higher in the WT_FLA group of mice, and the Proteobacteria phylum was significantly lower ($P < 0.05$). Additionally, the relative abundance of Verrucomicrobia was much lower in the WT_HFD group than in the WT_CO group ($P < 0.05$). However, FLA intervention effectively regulated the relative abundance of Verrucomicrobia in the WT_FLA group of mice, maintaining it at a level similar to that of the WT_CO group.

Comparative analysis among the KO_CO group, KO_HFD group, and KO_FLA group of *TGR5*$^{-/-}$ mice showed that the 10 bacterial phyla present in the intestinal contents of the mice were similar to those in WT mice, but the dominant order of the microbiota changed, the consistent intervention effect of FLA observed in WT mice was not seen (Fig. 8B). Compared with KO_HFD, FLA intervention did not significantly improve any of the other bacterial phyla except for significantly reducing Verrucomicrobia in the KO_FLA group. This result further indicated that the knockout of the *TGR5* gene affected the regulatory effect of FLA on the gut microbiota. In order to further elucidate the connection between the TGR5 receptor and the gut microbiota, the study compared the differences between WT mice and *TGR5*$^{-/-}$ mice fed the same formulated diet. The Proteobacteria and Verrucomicrobia phyla in the feces of both KO_CO and KO_HFD groups were significantly lower compared to WT_CO and WT_HFD groups ($P < 0.05$ or 0.01), while the TM7 phylum in KO_CO and KO_HFD groups was significantly higher compared to the WT_CO and WT_HFD groups ($P < 0.01$) (Figs. 9A and B). However, the differences in the Proteobacteria and Verrucomicrobia phyla between the *TGR5*$^{-/-}$ and WT mice under FLA intervention did not consistent with the above results (Fig. 9C).

The above results indicated that at the phylum level, FLA intervention had a significant regulatory effect on the gut microbiota of NAFLD mice, the knockout of the *TGR5* gene affected the regulatory effect of FLA on the gut microbiota, and that FLA intervention might improve NAFLD in mice through a combined effect on the gut microbiota and the TGR5 receptor.

At the genus level (Fig. S6), the top 10 bacterial genera in the gut contents of WT mice were *Lactobacillus*, *Allobaculum*, *Oscillospira*, *Alistipes*, *Akkermansia*, *Shigella*, *Mucispirillum*, *Bacteroides*, *Bifidobacterium*, and *Sutterella*. While the top 10 genera of intestinal flora in *TGR5*$^{-/-}$ mice were *Allobaculum*, *Lactobacillus*, *Bifidobacterium*, *Mucispirillum*, *Akkermansia*, *Oscillospira*, *Adlercreutzia*, *Bacteroides*, *Desulfovibrio*, and *Ruminococcus*. As shown in Fig. 10, compared to the WT_CO group, the WT_HFD group had significantly higher levels of *Adlercreutzia*, *Ruminococcus*, *Desulfovibrio*, *Lactobacillus*, *Dehalobacterium*, *Streptococcus*, and *Halomonas* in the feces of mice ($P < 0.05$, 0.01, or 0.001). However, the genera *Lactococcus*, *Bifidobacterium*, *Akkermansia*, *Shigella*, *Sutterella*, and Clostridiaceae_*Clostridium* were significantly reduced in the WT_HFD group ($P < 0.05$). Compared to the WT_HFD group, the relative abundances of *Bifidobacterium*, *Akkermansia*, *Alistipes*, *Bacteroides*, Clostridiaceae_*Clostridium*, and *Odoribacter* in the gut microbiota of WT_FLA mice significantly increased, while the relative abundances of *Allobaculum*, *Desulfovibrio*, *Lactococcus*, *Dehalobacterium*, and *Halomonas* were significantly lower than those in WT_HFD mice ($P < 0.05$, 0.01, or 0.001). In *TGR5*$^{-/-}$ mice, compared to the KO_CO group, the relative abundances of *Desulfovibrio*, *Lactococcus*, Clostridiaceae_*Clostridium*, *Dehalobacterium*, and *Halomonas* in the feces of the KO_HFD group significantly increased ($P < 0.05$ or 0.01), while the relative abundances of *Bifidobacterium*, *Bacteroides*, and *Sutterella* significantly decreased in the KO_HFD group ($P < 0.05$, 0.01, or 0.001). Compared to the KO_HFD group, the KO_FLA group showed a significant increase in the relative abundances of *Bifidobacterium*, *Akkermansia*, *Bacteroides*, *Adlercreutzia*, *Ruminococcus*, and *Coprococcus* ($P < 0.05$ or 0.01), and a significant decrease in the relative abundances of *Shigella*, *Desulfovibrio*, *Lactococcus*, *Dehalobacterium*, and *Butyricimonas* ($P < 0.05$, 0.01 or 0.001).

Our study further compared the changes in the dominant bacteria of the intestinal flora at the genus level in WT and *TGR5*$^{-/-}$ mice under the same formulated diet. As shown in Fig. 11, the relative abundance of *Akkermansia*, *Sutterella*, Clostridiaceae_*Clostridium*, and *Helicobacter* genera in the gut microbiota of KO_CO group was significantly lower than that of WT_CO group, while the relative abundance of *Bifidobacterium* and *Desulfovibrio* in KO_CO group was significantly higher than WT_CO group ($P < 0.05$). Furthermore, the relative abundances of the *Oscillospira*, *Akkermansia*, *Alistipes*, *Lactococcus*, and *Helicobacter* genera in the gut microbiota of KO_HFD group were significantly lower than those in WT_HFD group, while the relative abundance of the *Desulfovibrio* genus in the KO_HFD group was significantly higher than those in WT_HFD group ($P < 0.01$). The differential microbiota between KO_FLA group and WT_FLA group increased compared with the difference between KO_HFD group and WT_HFD group, as well as the difference between KO_HFD group and WT_HFD group, with the relative abundance of *Allobaculum*, *Bifidobacterium*, *Adlercreutzia*, and [*Ruminococcus*] significantly higher in the gut microbiota of KO_FLA group compared to WT_FLA group ($P < 0.05$ or 0.01), while the relative abundance of *Oscillospira*, *Alistipes*, *Shigella*, *Parabacteroides*, Clostridiaceae_*Clostridium*, *Butyricimonas*, and *Turicibacter* was significantly lower in the KO_FLA group than in the WT_FLA group ($P < 0.05$).

The above results suggested that at the genus level, FLA intervention could significantly regulate the gut microbiota composition of NAFLD mice and improve the microbial community structure. Additionally, the *TGR5* gene knockout caused alterations in the dominant flora, and FLA intervention significantly regulated its relative abundance. In the comparison of FLA intervention effects between WT and *TGR5*$^{-/-}$ mice, we found that the role of FLA in regulating key microbial populations remained consistent, such as *Bifidobacterium*, *Akkermansia*, *Bacteroides*, and *Desulfovibrio.*

3.6 Correlation analysis between gut microbiota and BAs

To investigate whether changes in intestinal BAs were related to changes in the gut microbiota, Spearman correlation analysis was performed between the significantly different fecal BAs and the significantly different fecal microbiota among the three groups of WT and *TGR5*$^{-/-}$ mice. In WT mice, there was a relatively close correlation between gut microbiota and changes in BAs. Specifically, g_*Allobaculum* showed a strong positive correlation with GUDCA, TLCA, UDCA, TUDCA, and TCDCA, and a strong negative correlation with HDCA, LCA, 3β-UDCA, and 3β-HDCA. g_*Alistipes* showed a positive correlation with THCA, GHCA, MDCA, HCA, 3β-HDCA, and HDCA. g_*Bacteroides* showed a positive correlation with

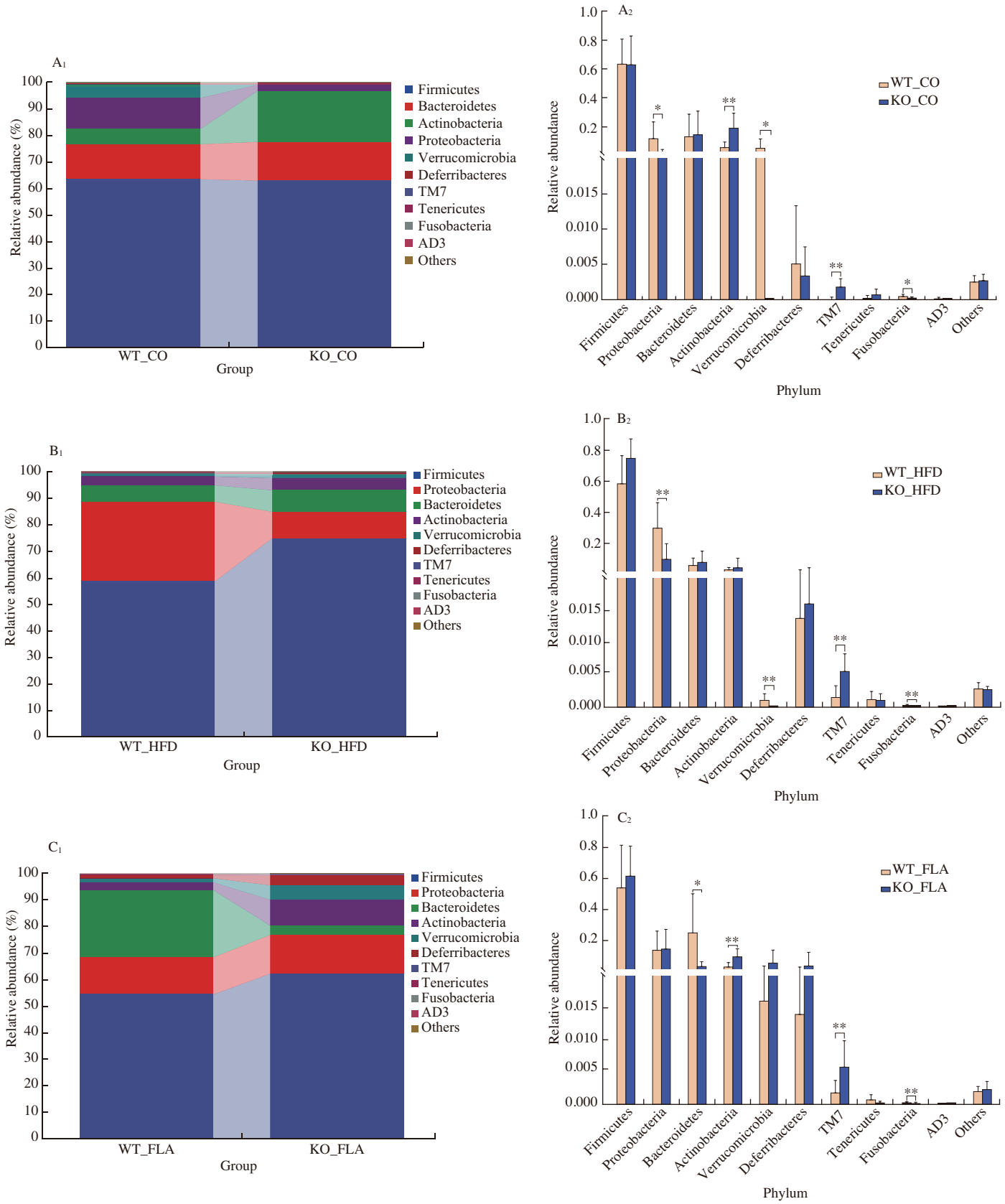

Fig. 9 Composition and comparison of gut microbiota at phylum level between WT and *TGR5*$^{-/-}$ mice. (A) WT_CO *vs.* KO_CO; (B) WT_HFD *vs.* KO_HFD; (C) WT_FLA *vs.* KO_FLA. WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, wild-type flaxseed powder intervention group; KO_CO, *TGR5* gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, *TGR5* gene knockout type flaxseed powder intervention group. Data were expressed as mean ± SD (n = 8). *P < 0.05, **P < 0.01.

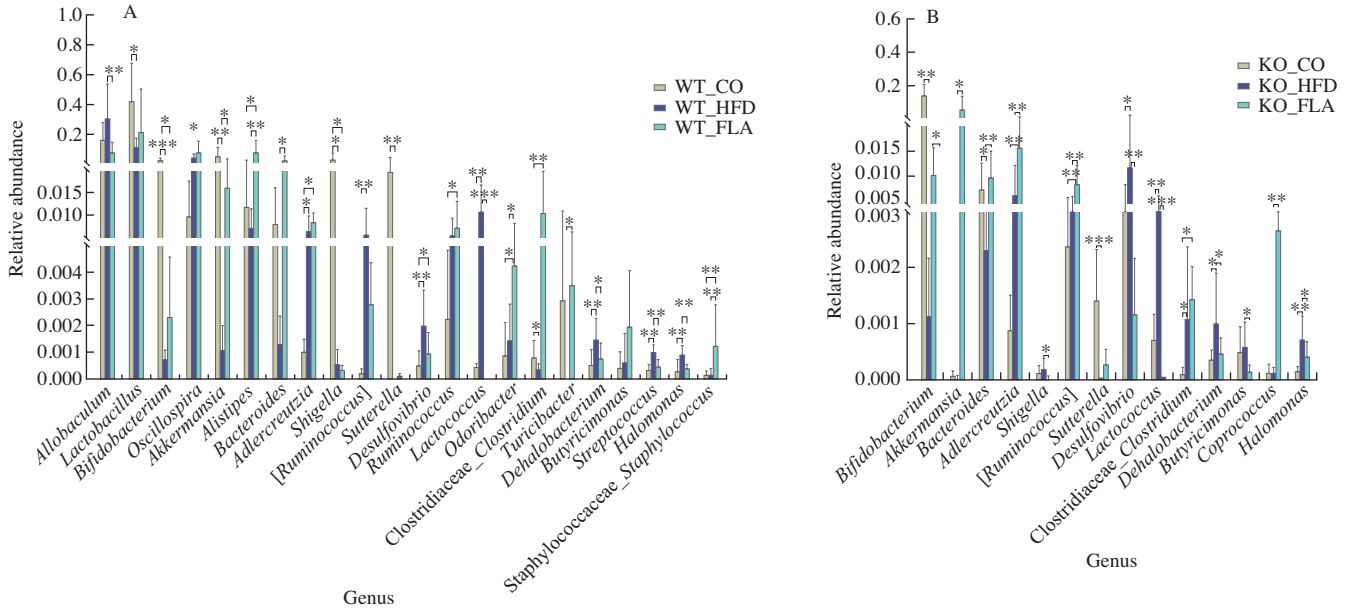

Fig. 10 Composition and comparison of gut microbiota at genus level in (A) WT and (B) *TGR5*$^{-/-}$ mice. WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, wild-type flaxseed powder intervention group; KO_CO, *TGR5* gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, *TGR5* gene knockout type flaxseed powder intervention group. Data were expressed as mean ± SD (n = 8). *P < 0.05, **P < 0.01, ***P < 0.001.

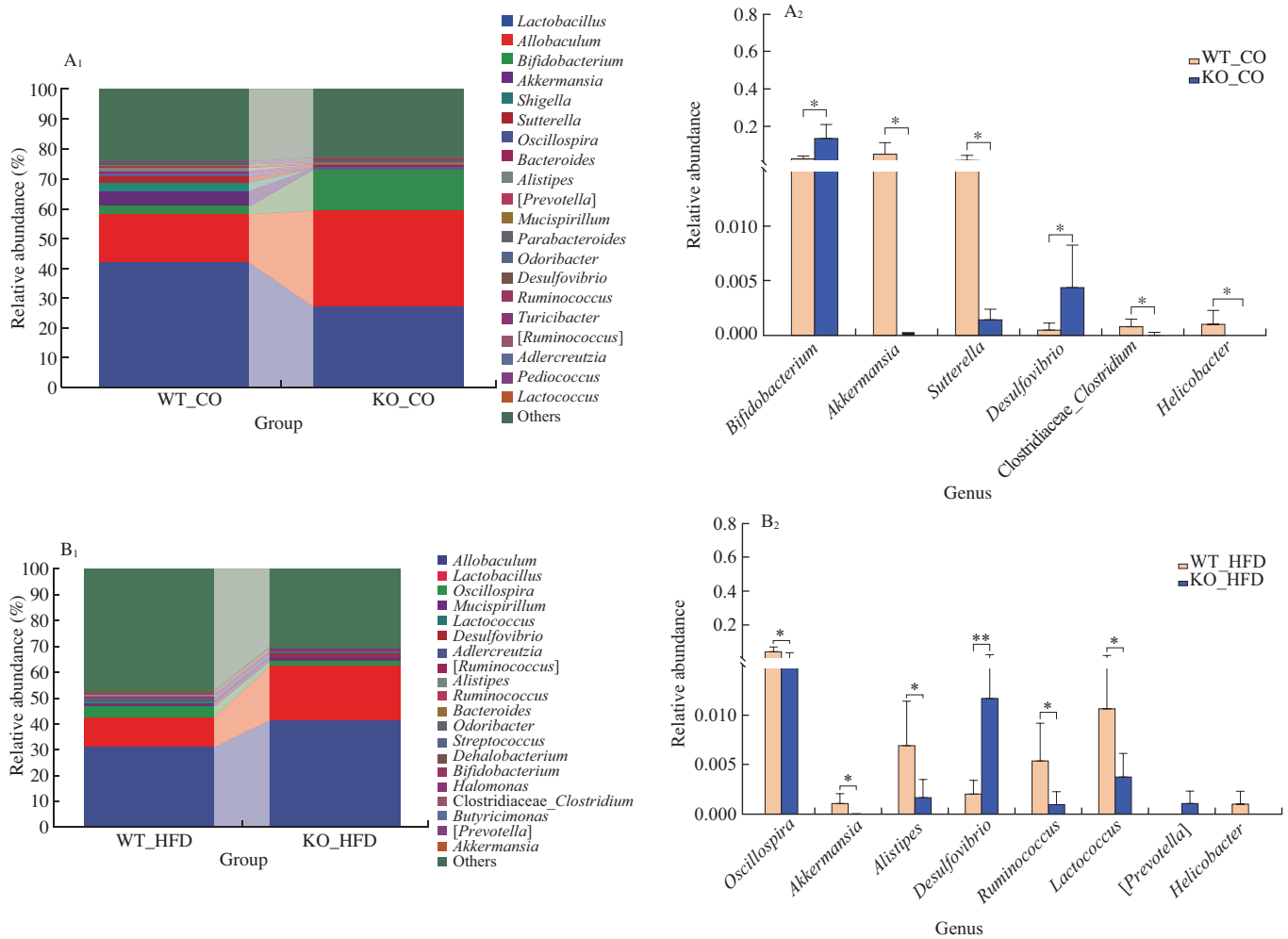

Fig. 11 Composition and comparison of gut microbiota at genus level between WT and *TGR5*$^{-/-}$ mice. (A) WT_CO *vs*. KO_CO; (B) WT_HFD *vs*. KO_HFD; (C) WT_FLA *vs*. KO_FLA. WT_CO, wild-type control group; WT_HFD, wild-type high-fat model group; WT_FLA, wild-type flaxseed powder intervention group; KO_CO, *TGR5* gene knockout type control group; KO_HFD, *TGR5* gene knockout type high-fat model group; KO_FLA, *TGR5* gene knockout type flaxseed powder intervention group. Data were expressed as mean ± SD (n = 8). *P < 0.05, **P < 0.01.

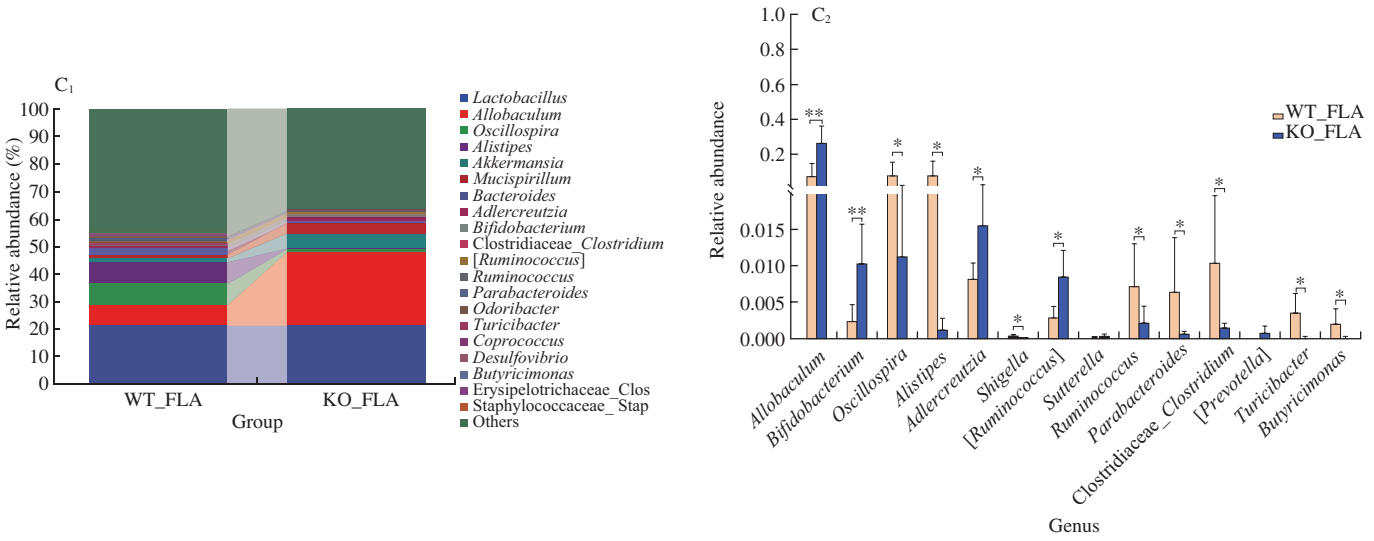

Fig. 11 (*Continued*)

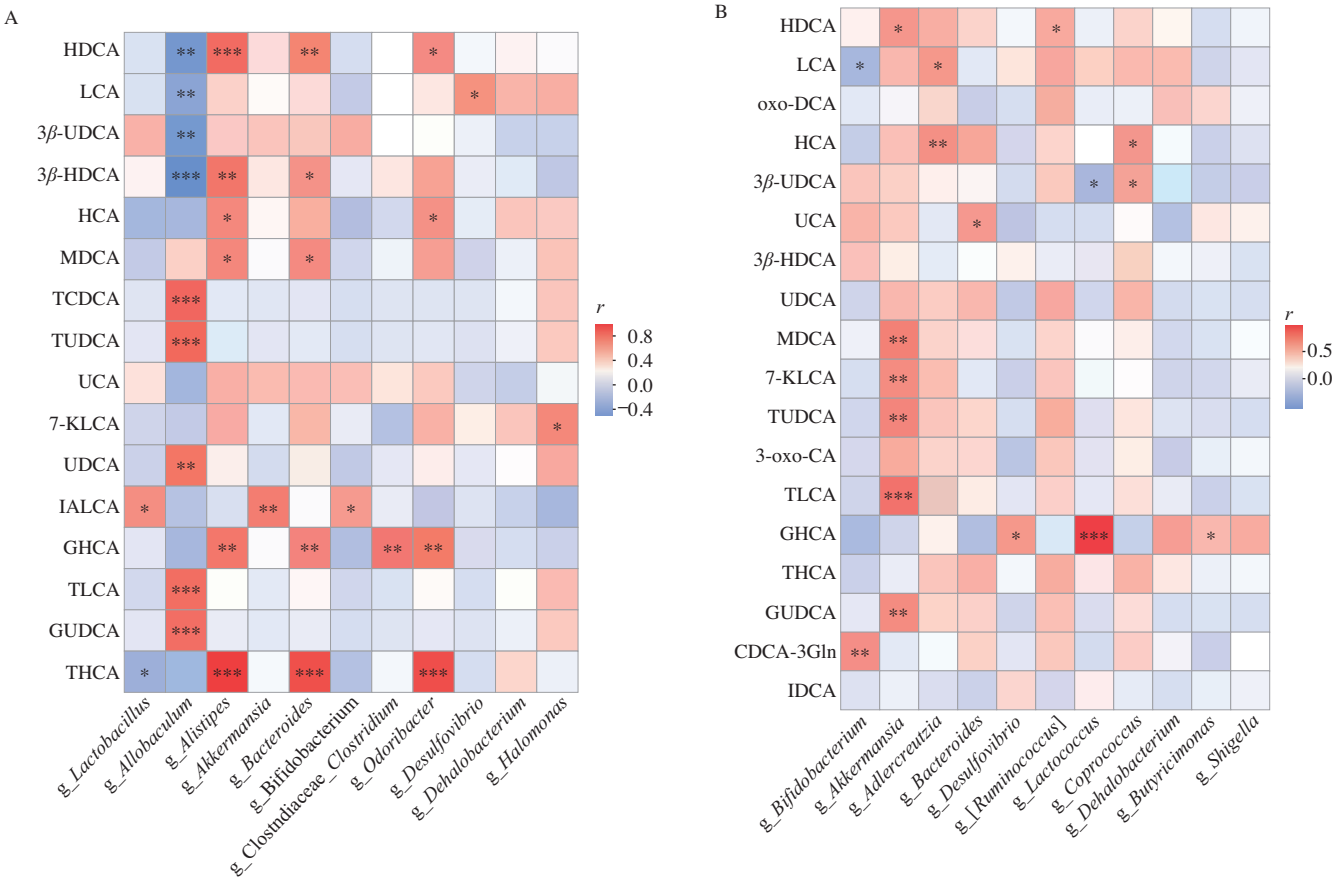

Fig. 12 Heatmap of Spearman correlation coefficients (*r*) between fecal BAs and microbiota in (A) WT and (B) *TGR5*$^{-/-}$ mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

THCA, GHCA, HDCA, MDCA, and 3*β*-HDCA. g_*Desulfovibrio* showed a positive correlation with LCA. g_*Odoribacter* showed a positive correlation with THCA, GHCA, HCA, and HDCA (Fig. 12A). In *TGR5*$^{-/-}$mice, Spearman correlation analysis showed that a negative correlation between g_*Bifidobacterium* and LCA, a strong positive correlation between g_*Akkermansia* and TLCA, GUDCA, TUDCA, 7-KLCA, and MDCA, and a strong correlation between g_*Lactococcus* and GHCA (Fig. 12B). It could be seen that the correlation between gut microbiota and BA changes in *TGR5*$^{-/-}$ mice was weaker than that in WT mice, indicating that *TGR5* gene knockout weakened the relationship between gut microbiota and BAs.

4. Discussion

The results of this study showed that in WT mice, a high-fat diet induced NAFLD model mice exhibited increased body weight and body organ fat content, and a significant elevation of blood lipid levels, accompanied by a significant elevation in the level of the inflammatory factor TNF-α. Liver pathology sections showed severe hepatic steatosis, hepatocyte swelling, vacuolar degeneration, the appearance of inflammatory cells in the NAFLD model mice. However, compared with the NAFLD model mice, FLA intervention improved the mice's body weight, body fat, liver lesions, and serum levels of TG, TC, LDL-C, and TNF-α, in addition, NAS scores had been reduced. This suggested that FLA intervention had beneficial effects on lipid-lowering, anti-inflammatory processes, and improvement of liver fat degeneration.

The above findings were similar to those obtained in other flaxseed studies, FLA was considered an excellent supplementary food for reducing inflammation and hepatic steatosis in NAFLD patients due to its functional components[26-27]. The findings of Yang et al.[26] indicated that FLA intervention exerted lipid-lowering and anti-inflammatory effects by influencing intestinal BA metabolism and significantly altering functional BAs, thereby activating the intestinal FXR and TGR5 signaling pathways. To more clearly elucidate the roles of FXR and TGR5 receptors in the intestine, this study knocked out the *TGR5* gene in WT mice, this study knocked out the *TGR5* gene in WT mice, and observed lipid and inflammatory alterations, as well as the expression of key proteins of the metabolic pathway, changes in intestinal flora and BAs in mice, in order to further explore the mechanism of FLA's action in ameliorating NAFLD.

The research results showed that *TGR5* gene knockout exacerbated the severity of NAFLD in mice, specifically evidenced by increased body weight, body fat, blood lipid levels, inflammation levels, and higher NAS scores in *TGR5*$^{-/-}$ mice compared to WT mice. Compared with the WT_HFD group, the serum TNF-α levels in the WT_FLA and WT_CO groups were significantly reduced ($P < 0.05$). However, the inflammation levels in the KO_FLA, KO_CO, and KO_HFD groups of mice remained high. FLA intervention did not reduce inflammation levels in *TGR5*$^{-/-}$ mice, but *TGR5* gene knockout did not impair the ability of FLA to improve blood lipid levels. The reduction in serum TC, TG, and LDL-C levels in the KO-FLA group was similar to that in the WT-FLA group. In addition, FLA intervention caused a decrease in NF-κB, TLR4, and CYP7A1 protein expression in the livers of mice in the WT_FLA group compared to the WT_HFD group. However, there was no significant difference in the liver tissues of mice in the KO_FLA group compared to the KO_HFD group ($P > 0.05$). This was similar to the results of previous studies in which *TGR5*$^{-/-}$ mice exhibited more severe hepatic necrosis and inflammation compared to WT mice in a LPS-induced inflammation model[13,28]. The above results indicated that *TGR5* gene knockout blocked the anti-inflammatory effect of FLA. The *TGR5* gene was a key protein in the anti-inflammatory pathway of FLA. FLA intervention reduced inflammation in NAFLD by inhibiting NF-κB and TLR4 through intestinal TGR5 activation. The regulation of liver inflammation by FLA was mainly related to TGR5 activation.

TGR5 was a BA-specific G-protein-coupled receptor that activated various intracellular signaling pathways when interacting with BA, especially in non-parenchymal cells[6,29]. It had been confirmed that *TGR5* gene knockout was more susceptible to cholestasis and inflammatory liver injury[13,30-31]. It's a recognized fact that activating TGR5 inhibited the macrophage's generation of pro-inflammatory cytokines[32]. Early research indicated that TGR5 was highly expressed in macrophages, and these cells, along with the secretion of their pro-inflammatory cytokines, were closely related to the progression of NAFLD, suggesting that TGR5 might be involved in the regulation of macrophages in NAFLD. Macrophages from *TGR5*$^{-/-}$ mice were more susceptible to LPS stimulation compared to those from WT mice[33]. TGR5 alleviated liver ischemia and reperfusion injury by inhibiting the TLR4-NF-κB pathway, which might play a key role in mitigating associated inflammation[34]. During the inflammatory response, the activation of TGR5 reduced inflammation by inhibiting the transcription of NF-κB, leading to a decreased expression of inflammatory cytokines such as TNF-α, IL-6, and monocyte chemoattractant protein-1, as well as inhibition of phagocytosis and immune cell migration[28].

The gut microbiota consisted of four phyla, Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria, which accounted for nearly 90% of the gut microbial community, with Firmicutes and Bacteroidetes covering nearly 70% of the bacteria in the gut[35]. Changes in their relative abundance were considered to be two major bacterial branches that altered the genetic composition and metabolic activity of the mammalian gut microbiota[36]. In patients with obesity and NASH, the Firmicutes/Bacteroidetes (F/B) ratio increased[37-38], dietary interventions could significantly reduce the F/B ratio, thereby improving gut dysbiosis and metabolic disorders[39]. The results of this study showed that the relative abundance of Bacteroidetes and Verrucomicrobia in the WT_FLA group increased, whereas the relative abundance of Firmicutes phylum and Proteobacteria phylum decreased, and that the FLA intervention significantly reduced the F/B ratio compared with that in the WT_HFD group. Multiple gut microbiome analysis studies had confirmed that the Bacteroidetes and Firmicutes had high bile salt hydrolase (BSH) activity[40-41]. Supporting evidence from another study showed that the relative abundance of *Lactobacillus* and *Bifidobacterium* was positively correlated with the secondary/primary BAs ratio, whereas the abundance of Firmicutes was negatively correlated with the secondary/primary BAs ratio[42]. In addition, Proteobacteria, as a disease-specific gut microbiota associated with NAFLD, was significantly elevated in NAFLD/NASH populations[43-44], and was related to liver inflammation[45], lipid and endotoxin biosynthesis[46]. The Verrucomicrobia was a group of bacteria closely related to human metabolism. The relative abundance of Verrucomicrobia in the gut microbiota increased before and after the improvement of liver steatosis in model mice[47]. Compared to KO_HFD, the FLA intervention significantly increased Verrucomicrobia in the KO_FLA group, but no similar results were observed for other bacterial phylum as in WT mice. Interestingly, in the gut microbiota of *TGR5*$^{-/-}$ mice, the phylum TM7 was higher in all three groups of knockout mice fed different diets compared to WT mice ($P < 0.01$). TM7 was closely associated with obesity, with its relative abundance increased in obese patients[48].

At the genus level, compared to the WT_HFD group, the WT_FLA group of mice showed a significant increase in the relative abundance of the genera *Akkermansia*, *Bifidobacterium*, *Alistipes*, *Bacteroides*, Clostridiaceae_*Clostridium*, and *Odoribacter* in their gut microbiota, while the relative abundance of *Desulfovibrio* significantly decreased

($P < 0.05$ or 0.01). *Akkermansia* had been observed in both humans and animals and had been found to be associated with weight loss and improvements in metabolic disorders[49], which also correlated with its ability to enhance intestinal protection and improved intestinal barrier function[50-51]. In addition, studies had found a negative correlation between obesity and the genus *Alistipes*[52], and further research suggested a negative correlation between *Alistipes* and TG[53]. In human hypercholesterolemic patients, *Bifidobacterium* and *Bacteroides* had been shown to be negatively correlated with serum TC[54]. The genus *Bacteroides* was known to produce short-chain fatty acids (SCFAs), which primarily inhibited *de novo* lipogenesis, hepatic steatosis, and inflammatory infiltration, thereby reducing serum levels of TG, TC, and free fatty acid (FFA)[55]. *Clostridium* (clusters XIVa and XI) belonged to the phylum Firmicutes, responsible for the conversion from primary to secondary BAs, specifically generating DCA through 7α-dehydroxylation in the large intestine[56]. The family Desulfovibrionaceae were endotoxin-producing bacteria, associated with gut permeability and NAFLD/NASH[57]. FLA intervention significantly reduced the relative abundance of this bacterium in the intestines of WT mice. Under the same formulated feed, the beneficial bacteria, including *Akkermansia*, *Sutterella*, and Clostridiaceae_*Clostridium*, were significantly lower in the feces of *TGR5*$^{-/-}$ mice compared to WT mice, while the relative abundance of the harmful bacteria *Desulfovibrio* significantly increased ($P < 0.05$ or 0.01). Furthermore, in the comparison of FLA's intervention effects on WT and TGR5$^{-/-}$ mice, we found that the effects of FLA in regulating the key flora remained consistent, and FLA intervention significantly increased the levels of *Bifidobacterium*, *Akkermansia*, and *Bacteroides* in the feces of both WT and *TGR5*$^{-/-}$ mice, while significantly decreasing the levels of *Desulfovibrio*.

From the above 16S rRNA gene sequencing results, it was not difficult to see that FLA intervention significantly improved the gut microbiota of NAFLD mice, and further affected the phenotype of NAFLD mice. Moreover, the altered microbiota by FLA exhibited rich BSH activity. Bacteria such as *Lactobacillus*, *Bifidobacterium*, *Clostridium*, and *Bacteroides* with functional BSH could deconjugate BAs[40,58]. By deconjugating BAs, BSH altered their hydrophobicity, which could induce the expression of key genes related to lipid metabolism and transport[59]. The improvement effect of FLA on liver lipids and inflammation in NAFLD mice was closely related to the gut microbiota associated with BAs metabolism, whereas *TGR5* knockdown led to alterations in the dominant intestinal flora of mice, and FLA intervention significantly modulated their relative abundance as well as the composition of intestinal bacterial genera, and improved the structure of the microbiota. Thus, it was speculated that the beneficial effect of FLA on NAFLD mice could be attributed to the regulation of gut dysbiosis in mice.

More and more evidence suggested that BAs had transcended the realm of surfactants in digestion, becoming signaling molecules that regulate various biological functions, including glucose and lipid metabolism, energy homeostasis, liver regeneration, and liver repair[60-61]. BAs' physiological function primarily resulted from the activation of BA receptors FXR and TGR5, and the activation of intestinal TGR5 was inseparable from the high expression of BAs as ligands[61-62]. In the differential analysis of BAs, we found an increase in 6α-hydroxylated BAs, for example, HCA, HDCA, GHCA, THCA, and GUDCA in FLA-fed mice compared with HFD-fed mice in *TGR5*$^{-/-}$ mice, and similar changes were observed in WT mice. The synthesis of HCA and HDCA in humans had not been fully understood. However, they had been reported as novel biomarkers for metabolic disorders, obesity, and diabetes[63-64]. A previous study had shown that the HCA species levels were negatively correlated with body mass index and HOMA-IR[65]. In the meantime, studies found that HCAs reduced body weight gain and improved glucose metabolism by secreting glucagon-like peptide-1 (GLP-1) via activating functional TGR5 signaling[66-67]. In addition, we found unconjugated primary UDCA and its conjugated primary TUDCA and GUCDA increased in FLA-fed mice, and similar results were observed for unconjugated secondary LCA and its conjugated primary TLCA. All in all, the results indicated that *TGR5* gene knockout might not have a significant impact on BAs. This finding was consistent with previous studies, where the mRNA expression of various cytochromes and proteins involved in BA synthesis and transport was higher in the liver of *TGR5*$^{-/-}$ mice than that in WT mice, particularly the rate-limiting enzyme for BA synthesis, Cyp7a1. However, the BA pool did not increase[68]. Taken together, these results indicated that FLA induced production of 6α-hydroxylated BAs.

The metabolism of HCA and HDCA was closely related to gut microbiota. The similar increase in caecal HCA and HDCA between WT and *TGR5*$^{-/-}$ mice reflected the conservative transformation of bacterial BA between the two genotypes. Our 16S rRNA analysis revealed that FLA-enriched bacteria belonged to *Bacteroides*, *Bifidobacterium*, *Clostridium*, and *Akkemansia* genus, which were closely correlated to the levels of HCA, HDCA, THCA, and GHCA in the cecum. More recently, several studies also suggested that 6α-hydroxylated BAs in serum or ileum content were associated with gut microbiota, especially *Clostridia* species[67,69]. A recent study had showed that *TGR5*$^{-/-}$ mice could lead to a modification of gut microbiota composition, which could impact the effects of Western diet on *TGR5*$^{-/-}$ physiology[70]. This was consistent with our research findings. We speculated that FLA supplementation maintained the physiological levels of key bacteria to maintain its abundance in the intestine and was responsible for the production of 6α-hydroxylated BA. Makki et al.[67] and Zheng et al.[66] had demonstrated that 6α-hydroxylated BAs possessed beneficial effects by stimulating TGR5 activity in mice.

In summary, *TGR5* gene knockout blocked FLA's inhibition of key proteins NF-κB and TLR4 in the inflammation regulation pathway, indicating that FLA's regulation of liver inflammation through the BA metabolism pathway was mainly related to TGR5 activation. It also affected FLA's inhibition of CYP7A1, suggesting that FLA's regulation of liver lipids might be related to the synergistic effect of FXR and TGR5. Additionally, we revealed that FLA regulated the gut microbiota by enriching bacteria involved in the production of 6α-hydroxy BAs, activating the gut-liver-bile acid metabolic pathway specific receptor TGR5, thereby reducing liver fat and improving inflammation.

There were still some limitations in this study. First, we did not analyze BAs in the serum and liver tissues of mice, and FLA might also play a role by affecting BAs and their receptors in serum and liver tissues. On the other hand, the intestinal BA profile could better reflect the role of the gut microbiota[71]. Secondly, the conclusion that FLA exerted its anti-inflammatory effect by activating the TGR5 receptor had been confirmed, but whether its lipid-lowering efficacy

was through the synergistic effect of FXR and TGR5 remained to be verified, and further knockdown of FXR and TGR5 receptors in the intestine and liver, respectively, was needed to further clarify its mechanism of action.

5. Conclusion

In summary, FLA intervention improved body weight, body fat, liver lesions, and serum levels of TG, TC, LDL-C, and TNF-α in NAFLD model mice, reducing the NAS score, suggesting that FLA intervention had the health benefits of lipid-lowering, anti-inflammatory effects, and improving liver steatosis. FLA intervention altered the relative abundance of gut microbiota with BA metabolic enzymes, which in turn regulated intestinal BA composition, especially the proportion of 6*α*-hydroxylated BA, thereby activating the intestinal BA-specific receptor TGR5 and exerting a role in lowering hepatic steatosis and ameliorating inflammation.

Conflict of interest

Wang Liao, Hui Xia, Shaokang Wang, and Guiju Sun are editorial board members for *Food Science and Human Wellness* and was not involved in the editorial review or the decision to publish this article. The authors report there are no competing interests to declare.

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Ethics approval statement

The animal studies were complied with all relevant ethical regulations and approved by the Institutional Animal Care and Use Committee of Southeast University. The animal ethics approval number is 2019-0725-017 for Animal Experiment 1 and 2020-0330-011 f or Animal Experiments 2 and 3.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250459.

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