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Alterations of Bile Acids and Gut Microbiota in Obesity Induced by High Fat Diet in Rat Model

Hong Lin^{†,‡}, Yanpeng An[†], Huiru Tang^{*†}, Yulan Wang^{*§}

[†]State Key Laboratory of Genetic Engineering, Collaborative Innovation Center for Genetics and Development, Metabolomics and Systems Biology Laboratory, School of Life Sciences, Fudan University, Shanghai 200433, China.

[‡]Shanghai Metabolome Institute (SMI)-Wuhan, Wuhan, 430000, China

[§]Singapore Phenome Center, Lee Kong Chian School of Medicine, School of Biological Sciences, Nanyang Technological University, Singapore

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*To whom the correspondences should be addressed: Yulan Wang, E-mail: yulan.wang@ntu.edu.sg and Huiru Tang, E-mail: huiru_tang@fudan.edu.cn.

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ABSTRACT

Obesity has become a worldwide health issue and has attracted much public attention. In the current study, we aim to elucidate the roles of bile acids and their associations with gut microbiota during obesity development, employing high fat diet (HFD)-induced obesity in rat model. We collected feces and plasma, liver tissues and segments of intestinal tissues and developed bile acids quantification method by employing ultraperformance liquid chromatography coupled with mass spectrometry detection (UPLC-MS) strategy. We then assessed bile acids fluxes in the biological matrixes collected. We found that irrespective of dietary regimes, taurine-conjugated bile acids were the dominant species in the liver whereas unconjugated bile acids were in plasma. However, HFD caused slight increases in the total bile acids pool and particularly the increases in the levels of deoxycholic acid (DCA) (138.67 ± 37.225 nmol/L in control group, 242.61 ± 43.16 nmol/L in HFD group, $p=0.014$) and taurodeoxycholic acid (TDCA) (2.8 ± 0.247 nmol/g in control group, 4.5 ± 0.386 nmol/g in HFD group, $p=0.0018$) in plasma and liver tissues respectively, which were consistent with the increased levels of DCA in intestinal tissues and feces. These changes are correlated to an increase in abundance of genera *Blautia*, *Coproccoccus*, *Intestinimonas*, *Lactococcus*, *Roseburia* and *Ruminococcus*. Our investigation revealed the fluxes of bile acids and their association with gut microbiota during obesity development and explicated unfavorable impact of HFD on health.

KEY WORDS

Bile acids; High fat diet; Deoxycholic acid; Obesity; Gut microbiota; UPLC-MS.

INTRODUCTION

Obesity has become a worldwide health issue and has attracted much public attention, especially in western and developed countries.^{1,2} World Health Organization reported (2016) that 39% of people aged 18 and over are overweight. An increasing number of evidence illustrated that obesity is a metabolic disease and characterized as low-grade chronic inflammation, which was caused by excess nutrient uptake and insufficient energy expenditure.³⁻⁵ In addition, obesity is one of the important risk factors for other metabolic diseases, such as insulin resistance, type 2 diabetes, fatty liver disease, atherosclerosis, hypertension, stroke.^{4,6,7}

Apart from inflammatory induced obesity, gut microbiota have been recognized as an important factor contributing to obesity. The disparity of the distal gut microbiome between obese and lean individuals (both mice and human) suggested that obesity is related to the relative proportion of two preponderant bacteria phyla, *Bacteroidetes* and *Firmicutes*. The microbiome from obese population had greater capacity of energy harvest than that from lean population.⁸ The gut microbiota influence host disease states through gut microbiota-host co-metabolites. High-fat diet induces the increase of specific gut microbiota producing lipopolysaccharide (LPS), and the concentration of the plasma LPS is closely related to obesity. The high level of plasma LPS is reported to cause obesity accompanied with inflammation, insulin resistance, type 2 diabetes and atherosclerosis.^{9,10} Short chain fatty acids (SCFAs, including butyrate, propionate and acetate) in intestine are gut microbiota derived metabolites that not only act as source of energy for enterocytes, but also could reduce inflammation.¹¹ Furthermore, SCFAs-mediated activation of GPR43 (G-coupled receptor 43) could suppress insulin signaling in adipocytes, decrease fat accumulation in adipose tissues and maintain the body energy homeostasis.¹²

Bile acids are another class of metabolites representing gut microbiota-host co-metabolism. Bile acids in mammalian intestine could be classified into two types according to their origins: 1, the primary bile acids (cholic acid and chenodeoxycholic acid in humans and cholic acid, α -muricholic acid, β -muricholic acid in rodents) which are generated in liver and transported to intestine through the enterohepatic circulation; 2, the secondary bile acids which come from the modification of gut microbiota from the primary bile acids, and they dominate in total fecal bile acids pool. The secondary bile acids (including DCA, lithocholic acid, ursodesoxycholic acid, ω -muricholic acid, and their conjugated counterparts) are produced through expressions of bile salt hydrolases, hydroxysteroid dehydrogenase (HSDH), 7 α / β -dehydroxylation. Gut microbiome can affect bile acids distribution through regulating the expression of farnesoid X receptor (FXR), which is activated by bile acids *via* negative feedback mechanism.¹³⁻¹⁷ Conversely, some bile acids have bacteriostatic action, affecting gut microbiota population.¹⁸ The administration of cholic acid altered the composition of the intestinal microbiota, which was similar to the changes induced by high-fat diet.¹⁸ Mechanisms of the three way interactions between gut microbiota-bile acids-metabolism of host have also been investigated. Some bile acids have strong hydrophobicity, and perturbations of their concentrations would alter bacterial membrane permeability, therefore

leading to disturbed gut microbiota.^{18,19} In addition, bile acids were reported to facilitate the formation of RNA secondary structure and could cause DNA damage, which activated the related DNA repair enzymes in both mammalian cells and bacteria.²⁰

Gut microbiota structure is dynamic and could be affected by many factors. Previous studies have shown the impact of different dietary interventions on the alterations of gut microbiome and subsequent fecal metabolites in both human and mice model.^{21,22} Recently, the diurnal cycle has been demonstrated to affect the dynamic compositions of gut microbiome and the fecal bile acids and the same study also concluded that time-restricted feeding helped prevent occurrence of obesity and related metabolic diseases.²³ Our previous research has reported that the development of obesity was accompanied by the alteration of gut microbiome and fecal metabolites,²⁴ in particular, bile acids. However, only the total bile acids were detected. This is because bile acids are structurally similar molecules and displayed in nuclear magnetic resonance (NMR) spectra as a heavily overlapped signal. The ultraperformance liquid chromatography coupled with mass spectrometry detection (UPLC-MS) technique could detect different bile acids with great sensitivity.

In the present research, we aim to investigate the impact of high-fat diet induced changes in rat model with emphasis on the interplay between bile acids and gut microbiota. We first of all optimized the UPLC-MS technique for bile acids quantification and then investigated dynamic changes of bile acids and gut microbiota in response to high-fat diet. We comprehensively monitored bile acids from their generation to circulation and re-absorption in rat fed with high-fat diet. To our knowledge, it is the first study that focused on the investigation of bile acids in enteric-hepatic cycle and their interplay with microbiome during the development of high-fat diet induced obesity. Our work provided details of “life-cycle” of bile acids and their modulation by gut microbiome during the progress of obesity. In addition, our work revealed potential function of specific strain of microbiota on bile acid modulation.

MATERIALS AND METHODS

Standard Substance and Reagents

All of the 28 bile acid standard compounds were purchased from Steraloids Inc. (Newport, Rhode Island, United States). Full name of 28 bile acids and their corresponding abbreviations are listed in supporting information (Table S1, supporting information). HPLC grade methanol and acetonitrile were purchased from Sigma-Aldrich Inc. (St. Louis, MO, United States). 1 mM mixed bile acid stock solution and 0.4 mg/ml deuterated bile acid stock solution, used as internal standard, were prepared in methanol : water = 9 : 1 (v/v) and stored at -20°C. Calibration standard solutions were obtained by diluting the stock solution to ten levels ranging from 3 µmol/L to 0.2 nmol/L into charcoal stripped liver extract solution with added internal standard (0.3 µmol/L). The calibration curve generated from this preparation takes matrix effect into consideration.

Animal experiment and Sample Collection

Animal experiment was conducted as previously described.²⁵ Twenty-four SPF-grade male Sprague–Dawley rats (6-weeks old, weighted 177.8 ± 18.6 g) were divided into two groups randomly. One group of rats was fed with normal diet and the other with high-fat diet for the duration of 81 days. Fecal samples of rats were collected at time points of day 7, 28, 56 and 81 and these were carried out in individual cage in the morning to avoid contamination. The rats were sacrificed by neck dislocation under isoflurane anesthesia at the end of experiment. The liver, jejunum, ileum, cecum, colon tissues were also collected immediately after sacrifice from the middle part of these regions, respectively. Plasma samples were collected after centrifugation (16000 g, 4°C, 15 mins) in a standard manner with sodium heparin as anticoagulant. All samples were snap-frozen with liquid nitrogen immediately and stored at –80°C until analysis.

Sample preparation for UPLC-MS/MS Analysis

Bile acids were extracted using the previously published procedure with some minor modifications.²⁶ The tissues of liver (10 mg ~ 15mg) and different intestinal segments (about 30 mg) were added with 1 mL extraction solvent (methanol : water (v:v) = 2 : 1, 0.005% HCOOH) and 50 µL internal standard and followed with homogenization using tissulyzer (QIAGEN, TissueLyser II, Hilden, Germany) at 20 Hz for 90 s. The fecal sample (10 mg) were mixed with the same extraction solvent and was subjected to rapid frozen-thawed cycle in liquid nitrogen three times, and followed by homogenization using tissulyser. The extracted supernatant was obtained by centrifugation at 10000 g, 4°C for 15 mins. Finally, samples was filtered using Nylon 66 Syringe filters (Jin Teng, Φ13 mm, 0.22 µm, Tianjin, China) before UPLC-MS/MS analysis. 50 µL plasma was mixed with 500 µL precooled methanol and 5 µL internal standard solution, the mixture was vortexed and centrifuged at 10000 g, 4°C for 15 mins. The supernatant was then evaporated to dryness and re-dissolved in 100 µL extraction solvent.

UPLC-MS/MS Analysis

The UPLC-MS/MS system was made up of an Agilent 1290 UPLC coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with an Agilent Jet Stream electrospray ionization (ESI) source (Agilent Technologies, Inc. Santa Clara, CA, USA). Samples (1 µL) were separated on Kinetex® Core-Shell 2.6 µm C18 column (100 × 2.1mm, 2.6 µm, Phenomenex Inc. Torrance, USA) equipped with Kinetex 2.6 µm Minibore Security Guard Ultra Cartridges (Phenomenex Inc. Torrance, USA) at 45 °C. Mobile phases consisted of A (water with 0.005% HCOOH, v/v) and B (acetonitrile with 0.005% HCOOH,v/v). The elution gradient was set step wise as follows: 1. 23% B to 33% B for 2 mins; 2. 33% B to 34% B for 4 mins; 3. 34% B to 70% B for 5 mins. The flow rate was 0.6 mL/min. MS detection of bile acids were conducted in negative mode. Fragmentor and product ion for every bile acid were optimized through direct infusion of available bile acid standards to improve detective sensitivity. Due to the low proportion of some bile acids in bio-samples, we chose $[M - H]^-$ as product ion to promote sensitivity under multiple reaction monitoring (MRM) scan mode. Data acquisition and analysis were performed with Mass Hunter

software (Agilent Technologies, Inc. Santa Clara, CA, USA).

Method Validation

Method validation was conducted according to the FDA Guidelines for Bioanalytical Method Validation.²⁷

Linearity: Linearity of each bile acid calibration curve was evaluated by correlation coefficient (R^2), which was generated by least-squares linear regression analysis using the MRM peak area of each bile acid over MRM peak area of the internal standards. Notably, since some of bile acids internal standards were not commercially available, the internal standard with similar structures to these bile acids was used.

LOQ(limit of quantification): LOQ was defined as the concentration of analyte when the ratio of signal to noise is equal to 10. In this assay, we performed five replicates.

Inter- and Intra-assay Precision and Accuracy: The stability and robustness of the method was evaluated by measuring inter- and intra-assay precision and accuracy. We prepared quality control (QC) samples with directly detective molar of 2 femtomole (fmol), 4 fmol, 300 fmol, 1500 fmol and analyzed repeatedly for 5 times every day for consecutive 3 days. Precision and accuracy for inter- and intra- assay were obtained by comparing the measured value to real value. We could achieve precision of 20% and accuracy of 80-120%, which are within the accepted values of FDA guidelines.

Recovery: Extraction efficiency of the method was evaluated using six replicate liver tissues. Each of the liver samples was divided into five sections, and four of those were spiked with appropriate amount calibration solution before and after extraction process (final concentration 0.2 $\mu\text{mol/L}$ and 2 $\mu\text{mol/L}$), respectively. One section was used to determine endogenous concentrations of bile acids. Then recovery could be calculated as follows:

$$\text{Recovery} = \frac{C_{\text{before}} \times V_{\text{before}} - C_{\text{endog}} \times V_{\text{endog}}}{C_{\text{after}} \times V_{\text{after}} - C_{\text{endog}} \times V_{\text{endog}}}$$

Data Analysis

Bile acid concentrations were compared statistically using the Student's t-test when the data were of normality and homoscedasticity, otherwise by Kruskal-Wallis test with MATLAB 7.1 software (MATLAB 7.1, Mathworks Inc., USA). Concentrations of fecal bile acids were normalized to dry stool weight, while the hepatic bile acids and intestinal bile acids were normalized to wet tissue weight, plasma bile acids were normalized to the volume of plasma sample. To explore the interplay of bile salts and structurally changed bacterial community under dietary perturbation, correlation between the fecal bile acid concentrations and abundance of gut microbiota was analyzed based on the Pearson's Correlation Coefficients. The heatmap showing the correlation matrix between bile acids and gut bacteria was generated with gplots package of R software (R, Version 3.1.2) and strong correlation would be affirmed at 95% confidence interval with two-tailed t-test. The data of gut bacteria was reported previously by our group.²⁴

RESULTS

Sensitivity and throughput of bile acids detection: In this research, we established a rapid and high-throughput method for quantification of bile acids. The detection sensitivity for analytes was evaluated by LOQ; the lower of LOQs value, the better the sensitivity is. LOQs of T- β -MCA, THCA, α -MCA, β -MCA, HCA, CA, UDCA, HDCA, CDCA, DCA, iso-DCA, iso-LCA, LCA from our method were lower than the best of reported data.²⁸ Some LOQs of bile acids were similar to the best literature value, such as, T- α -MCA, TUDCA, ω -MCA, GCA, GUDCA, GHDCa, TDCA, ACA, GDCA and TLCA. While few bile acids showed higher values of LOQs compared with the literature values and these include TCA, GCDCA and GLCA.^{28,29} The detailed information was listed in Table S1 (supporting information). Additionally, detection time of present method (11 mins; Figure S1, supporting information) was far shorter than that of reported data (21mins and 26mins).^{28,29} Therefore, this method was high sensitive and high throughput.

Assessments of the bile acids quantification method

All bile acids achieved excellent linearity with correlation coefficients in the range of 0.9989-0.9999. Most of bile acids could be extracted from biological samples with an acceptable recovery (80% - 120%, relative standard deviation (RSD) < 20%). This is with exception of several taurine conjugated bile acids that have low pKa values (2-3), and hence are susceptible to the pH value of extraction solvent (Table S2, supporting information). The quantitative validation suggested that the method is stable and accurate (Table S3 and Table S4, supporting information). Inter-day accuracy ranged from 93.08% to 115.2%, with precision from 1.39% to 16.98%, and intra-day accuracy ranged from 87.7% to 112%, with precision from 0.41% to 15.29% at four concentration levels.

The HFD induced changes in hepatic and plasma bile acids

To explore the influence of HFD feeding on bile acids circulating in the enterohepatic circle, we analyzed species of bile acids and their concentrations in liver, plasma and different intestinal segment tissues obtained from control and HFD-induced obese rats, which has been previously established.²⁴ We found that HFD induced slight increase in the levels of total bile acid pool in liver and plasma (Figure S2, supporting information). In addition, HFD did not induce major changes in the proportions of conjugated relative to unconjugated bile acid in liver and plasma, suggesting that bile acids flux in the enterohepatic circle of HFD-induced obese rats followed the similar trend as the rats fed with control diet. For example, taurine-conjugated bile acids were the predominant bile acids in liver and among these, levels of TCA were the highest (Figure 1A). However, majority of bile acids in plasma were unconjugated (55.34%) and only 40.7% were taurine-conjugated bile acids (Figure 1B). Close inspection of changes of individual bile acids showed that the level of hepatic TUDCA was lower in rats fed by HFD compared with normal diet (16 nmol/g in control group, 7.73 nmol/g in HFD group, $p=0.0002$); whereas the level of hepatic

TDCA was higher in HFD group (2.8 nmol/g in control group, 4.5 nmol/g in HFD group, $p=0.0018$) (Figure 1C). HFD intervention induced higher levels of DCA (138.67 nmol/L in control group, 242.61 nmol/L in HFD group, $p=0.014$) and lower levels of iso-DCA (51.98 nmol/L in control group, 47.15 nmol/L in HFD group, $p=0.037$) in plasma of rats fed with 81-day HFD (Figure 1D, Table S8, supporting information).

The HFD induced changes in bile acids in intestinal tissues

The most profound impact of HFD displayed on the distribution of bile acids in jejunum, ileum, cecum and colon tissues (Figure 2 A&B). It was observed that glycine-conjugated bile acids predominated in jejunum and ileum whereas negligent levels of glycine-conjugated bile acids presented in cecum and colon. Unconjugated bile acids were presented in high levels in ileum, cecum and colon. HFD induced significant decreases in the levels of glycine-conjugated bile acids in jejunum and ileum, but increases in the levels of unconjugated bile acids in colon (Figure 2 A&B). Specifically, reduction in the levels of GCA, GCDCA, TUDCA, GUDCA, GHDCa, GDCA and HDCA in jejunum tissue and GHDCa, HDCA in cecum tissue were associated with HFD intervention with only one taurine-conjugated bile acid, T- α -MCA reduced in jejunum. We also observed increased levels of few bile acids, such as DCA, β -MCA in colon tissue of HFD rats (Figure 2 C&D).

The HFD induced changes in fecal bile acids

To investigate the dynamic changes of fecal bile acids induced by HFD intervention, the compositions of fecal bile acids from HFD and those from control groups at day 7, 28, 56 and 81 after HFD intervention were analyzed. Twenty bile acids were quantified. The levels of both taurine-conjugated and glycine-conjugated bile acids were decreased significantly in HFD group from day 28 onwards (Figure 3A&B). Specially, T- α -MCA, T- β -MCA, TCA, TUDCA and GCA in fecal extract were decreased significantly (Figure S3, Table S6, supporting information). However, no significant change was observed for the unconjugated bile acids when comparing HFD group with control group (Figure 3C). It is worth noting that the levels of fecal DCA in HFD group increased significantly comparing to the group with control diet from day 28 onwards (Figure 3D). On the contrary, the levels of UDCA decreased significantly from day 28 onwards in HFD group compared with the control (Figure 3E). Additionally, the levels of fecal HDCA, iso-LCA, LCA also showed obvious decreases from day 28 onwards in HFD group (Figure S3, Table S6, supporting information). To visualize the variations of all fecal bile acids, we mapped the relative changes of each bile acids with and without HFD at day 7, day 28, day 56, day 81, calculated as $(C_{\text{HFD}} - C_{\text{control}}) / C_{\text{control}}$ (Figure 4). Clearly, most of conjugated bile acids in feces were decreased in HFD rats, while fecal unconjugated bile acids with different functions had different change tendency (Figure 4).

Correlations between fecal bile acids and abundance of gut microbiota

Correlation coefficients between the fecal bile acid concentrations and significantly changed bacteria at genus-level showed to be significant at all time points (Figure 5). Several bile acids

correlated with specific bacterial genera. For instance, the concentrations of T- α -MCA, T- β -MCA, TUDCA in fecal samples negatively correlated with *Lachnospiraceae_unclassified*, *Blautia*, *Roseburia*, *Intestinimonas*, *Coprococcus*, *Erysipelotrichaceae_unculture*, *Pseudobutyrvibrio*, *Lactococcus*, *Deffluviitaleaceae_unclassified*. On the contrary, amount of fecal DCA was positively correlated with abundance of *Oscillibacter*, *Ruminococcus*, *Blautia*, *Coprococcus*, *Intestinimonas*, *Lactococcus*, *Roseburia* whilst negatively correlated with *S24-7_norank* (*unidentified genera bacteria*). The Pearson correlation coefficients of other genera bacteria and the concentrations of all the bile acids were listed in Table S7 (supporting information).

DISCUSSION

Obesity has become endemic issue worldwide. Research has shown that both HFD-induced and genetic obesity are accompanied by the alterations in gut microbiota and energy harvesting capacity of host.^{10,30,31} Gut microbiome affecting on host metabolism can often be mediated by microbiota related metabolites. Previously, contributions of SCFAs, as co-metabolites between gut microbiome and host, to obesity have received much attention.^{12,32} However, contributions of bile acids, as another category of cometabolites between host-microbiota, to obesity have been largely overlooked. There are relative few studies demonstrating that a positive correlation of plasma bile acids has been associated with BMI in the population.³³ In addition, the levels of CDCA and DCA have been correlated with activities of appetite regulation enzymes and energy metabolism enzymes, such as gastrointestinal hormones, peptide YY, glucagon-like peptide-1 (GLP-1) and ghrelin after meal in human model.³⁴ However, concurrent information on changes of gut microbiota is lacking. Nevertheless, these findings underscored the importance of gut microbiome modulations through bile acids played important roles in obesity. Hence quantification of bile acids with high sensitivity and throughput is essential for further investigation of roles of bile acids in obesity. In the current investigation, we developed a sensitive and rapid UPLC-MS/MS method for quantitative detection of 28 bile acids in biological samples. Method validation was conducted according to the FDA guidelines. We subsequently applied the validated method to investigate flux of bile acids in the enterohepatic circulation systems, i.e. liver, intestinal tissue, plasma and feces, and their associations with gut microbiota, with the view to understand the roles of these bile acids and their associations with gut microbiota during the development of obesity in HFD rat model (Figure 6).

Our obese rat model was successfully constructed by feeding rats with HFD for the duration of 81 days. The body weight gains of the HFD group became more obvious with proceeding of the experiment as previously reported.²⁵ The epididymal and perirenal fat masses of the HFD-fed rats showed significant increase compared to the rats fed with controlled diet. In addition, the rats in the HFD group suffered from liver steatosis at the end of experiment.²⁴

One of the important findings in the current investigation was that HFD feeding increased the levels of DCA and reduced the levels of UDCA in enterohepatic circulation (Figure 1, Figure 3 D&E). DCA and UDCA are secondary bile acids produced by microbes in intestine, and they are

conjugated with taurine to produce TDCA and TUDCA in liver. Therefore, the levels of TDCA and TUDCA in liver are closely related to the levels of DCA and UDCA in feces. In our study, the levels of hepatic TDCA, plasma DCA and colonic DCA increased significantly in the HFD group, which is consistent with the increased levels of fecal DCA in the HFD group (Figure 3&4). It has been demonstrated that production of DCA is *via* catalysis of the 7 α -dehydroxylase that presents in bacteria including species of *Eubacterium* and *Clostridium* genus.²⁰ Therefore the observed increase in fecal DCA concentration could be related to the enhanced population of 7 α -dehydroxylase containing bacteria. Our correlation analysis between dynamic fecal bile acids and gut microbiota suggested that DCA is positively correlated with genera *Blautia* (p=2.76E-05), *Coprococcus* (p=0.00099), *Intestinimonas* (p=3.25E-06), *Lactococcus* (p=1.51E-06), *Roseburia* (p=6.43E-08), *Ruminococcus* (p=0.0084) (Figure 5). Among these bacteria, some species in *Ruminococcaceae* and *Blautia* are known to produce 7 α -dehydroxylase, contributing to the increases in the levels of fecal DCA in cirrhosis patients.³⁵ Clearly, further investigation on the roles other DCA-correlated bacteria are warranted.

The high level of DCA has been demonstrated to induce adverse effects on health. This is because DCA, when it is higher than physiological level, causes DNA damage³⁶ and contributes to obesity-associated hepatocellular carcinoma.³⁷ In addition, higher intestinal DCA concentrations induced by HFD could disrupt intestinal epithelial integrity due to hydrophobic nature of DCA.¹⁴ It is also because of hydrophobic nature of DCA that can perturb plasma membrane structure and causing ligand-independent activation of EGFR in primary rat hepatocytes, leading to cell proliferation.³⁸ The high levels of UDCA have been shown to mitigate DCA-induced barrier dysfunction and suppress DCA-induced cell apoptosis *via* a series of signal transduction pathway.³⁹ Thus, in our investigation, the overall increased levels of fecal DCA and decreased levels of fecal UDCA induced by HFD pose adverse effects on health. This unfavorable effect is believed to be due to, at least in part, a shifting of healthy organisms to some harmful organisms as the results of HFD feeding.

HFD-feeding elevated the total amount of hepatic, intestinal and plasma bile acids slightly in rats (Figure S2, supporting information). The increased levels of bile acids pool could counteract a range of metabolic consequences associated with high-fat diet induced obese. This notion is supported by previous study showing that a larger bile acid pool acts as signaling molecules and stimulates the activity of G-protein-coupled receptor 5 (TGR5) that, in turn, increases energy expenditure to alleviate the obesity and diabetes.^{8,40} The activated TGR5 signaling has been demonstrated to promote mitochondrial fission and beige remodeling of white adipose tissue under multiple environmental conditions, including cold shock and prolonged high-fat diet feeding.⁴¹ Administration of bile acids, particularly TCA, is able to activate TGR5 and enhances energy expenditure in brown adipose tissue.⁴² In our study, the levels of hepatic TCA increased in rats fed with high-fat diet (FigureS2, supporting information), implying more activated TGR5 in HFD group than control group. The bile acids are also nature ligands for FXR, activating FXR.

The activated FXR can suppress the activity of cholesterol 7 α -hydroxylase, reducing bile acids production. In addition, the activation of intestinal FXR has been demonstrated to reduce diet-induced weight gain, body-wide inflammation and hepatic glucose production and to enhance thermogenesis and browning of white adipose tissue.⁴³ Therefore, increased levels of bile acid pool observed in HFD rats in the current investigation demonstrated the effort to counteract unfavorable effects associated with the development of obesity to a certain extent.

We also discovered that the levels of conjugated bile acids in feces were decreased in HFD group, specifically, the levels of T- α -MCA, GCA, TUDCA and GCDCA (Figure 3&Figure 4). The conjugated bile acids act as emulsifier, aiding the absorption of fat ingested, which explains the reduced levels of conjugated bile acid in feces. In addition, the reduced levels of conjugated bile acids are contributed by gut microbiota that is capable of hydrolysis of bile acids, producing a range of secondary bile acids.^{36,44} In our investigation, the relative abundance of enteric anaerobic bacteria *Bacteroides* were negatively correlated with the concentrations of fecal conjugated bile acids (TCA and GCA) (Figure5), suggesting that *Bacteroides* could contribute to the reduction of fecal conjugated bile acids in HFD group. This notion was supported by previous research.⁴⁵

In conclusion, the present study demonstrated the alternations of bile acids and gut microbiota in high fat diet induced obese in rat model, particularly, the levels of DCA in enterohepatic circulation and in feces. The correlations between fecal bile acids and intestinal microbiota offered important clues to exploring specific strains involved in bile salt hydrolases and 7- α -dehydroxylation. These findings allowed us to realize the roles of bile acids and gut microbiota in the development of obesity, providing the novel view for understanding the obesity-related metabolic diseases.

Notes

The authors declare no conflict of interest.

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Supplementary information

Figure S1. The UPLC-MS/MS chromatogram of 28 bile acids standard solution, including 15 unconjugated, 6 glycine-conjugated, 7 taurine-conjugated bile acids and 5 deuterium (d)-labeled bile acids as internal standard (peak signals shown in red).

Figure S2. Plasma bile acids, hepatic bile acids, intestinal bile acids and hepatic TCA in control and HFD groups. Data are shown as mean $\pm$ s.e.m.

Figure S3. The dynamic changes of fecal bile acids at day 7, day 28, day 56, day 81 in two different treated groups. The red lines and black lines denoted the data from rats fed with HFD and normal diet respectively. The unit of concentration is nmol/g dried stool weight. The data are shown as means $\pm$ s.e.m.

Figure S4. The fluxes of bile acids in organs involved enterohepatic circulation. Blue color denoted as decrease whereas red as increase in rat fed with HFD.

Table S1. List of 28 bile acid standard compounds and their linearity, and the LOQ obtained from our method and optimal result reported in literature.

Table S2. The calibration curve, correlation coefficient and recovery of each bile acid obtained from liver tissues by taking matrix effect into consideration

Table S3. The intra- and inter-day accuracy (%), precision (%) of quantification method at three direct detection levels.

Table S4. Retention time (RT) and the precision (intra-day and inter-day RSDs) of each bile acid at three direct detection levels.

Table S5. The bile acids detected in liver of rats fed with HFD and normal control diet

Table S6. The profile of fecal bile acids from two groups at day 7, day 28, day 56, day 81 after dietary intervention. The data were expressed as mean±s.e.m. (nmol/g).

Table S7. The significant ($p < 0.05$) Pearson' correlation coefficients between abundance of bacteria and the concentrations of bile acids.

Table S8. The bile acids detected in plasma of rats fed with HFD and normal control diet

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Figure captions

Figure 1. Bile acid compositions in (A) liver and (B) serum from rats fed with normal diet for 81 days. (C) Concentrations of TUDCA and TDCA in liver from both control (n=12) and HFD (n=12) groups. (D) Concentrations of DCA and iso-DCA in plasma from both control and HFD groups. Data are collected with UPLC-MS/MS method and shown as mean $\pm$ s.e.m in histogram C and D. * represents p value < 0.05, p-values were from the Student's t-test or Kruskal-Wallis test.

Figure 2. (A) Total glycine-conjugated bile acids and (B) total unconjugated bile acids in different intestinal segment tissues (jejunum, ileum, cecum, colon) of rats fed with normal diet or HFD for 81 days (n=12). Data are collected with UPLC-MS/MS method and shown as mean $\pm$ s.e.m. (C) Changes of conjugated bile acids and (D) unconjugated bile acids from intestinal tissues with statistical difference. * represents p < 0.05 , p-values were from the Student's t-test or Kruskal-Wallis test.

Figure 3. The amount (nmol/g dried weight of stool) of (A) taurine-conjugated bile acids, (B) glycine-conjugated bile acids, (C) unconjugated bile acids, (D) DCA and (E) UDCA in control and HFD groups at day 7, day 28, day 56, day 81 after diet intervention (n=12). Data are collected with UPLC-MS/MS method and shown as mean $\pm$ s.e.m. * represents p value < 0.05, p-values were from the Student's t-test or Kruskal-Wallis test.

Figure 4. The heatmap showed the relative variations of fecal bile acids, represented as $(C_{HFD}-C_{Control})/C_{Control}$ at day 7, day 28, day 56, day 81 compared HFD group with control group (n=12). Relative variation ratios are within range (A) from -1 to 1; (B) from -1.5 to 1.5; (C) from -15 to 15.

Figure 5. The heatmap showed Pearson' correlation coefficient with p<0.05 between several significant changed fecal bile acids and genera bacteria. Red cells represent positive correlation and blue cells represent negative correlation.

Figure 6. The biotransformation of bile acids in rodent. Four squares respectively indicates the level of specific bile acid in fecal samples at day 7, day 28, day 56, day 81; while the circle indicates the level of hepatic bile acids. Data are collected with UPLC-MS/MS method. Red denotes a significant increase in HFD group (n=12) compared with control group (n=12) whilst blue shows the decrease, black indicates no change and white means no detected.

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