

RESEARCH PAPER



Sex-specific maternal calcium requirements for the prevention of nonalcoholic fatty liver disease by altering the intestinal microbiota and lipid metabolism in the high-fat-diet-fed offspring mice

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ABSTRACT

The significance of maternal appropriate calcium intakes for energy metabolism in the offspring has been recognized. Nonalcoholic fatty liver disease (NAFLD) is considered as the hepatic manifestation of metabolic syndrome. So in this study, we proposed that there were long-term effects of maternal calcium status on the progress of NAFLD by altering the intestinal microbiota and lipid metabolism with attention to potential sex differences among the mouse offspring. Thirty-four-week female C57BL/6 J mice were subjected to obtain low, normal and high calcium reproductive diets throughout the gestation and lactation. After weaning, both the male and female mouse offspring were fed with the high-fat diet for 16 weeks, with the normal diet as control. Biochemical indicators in the plasma and hepatic tissue were measured using ELISA or enzymatic methods. The expression of lipid metabolism, inflammatory and fibrosis related genes was determined by RT-PCR. The intestinal microbiota was analyzed by 16S rRNA high-throughput sequencing. Maternal normal and low calcium intake could, respectively, inhibit the progress of high-fat diet induced NAFLD in the male and female mouse offspring, which was characterized by the least lipid droplets, inflammatory infiltration and fibrosis, the lowest concentrations of free fatty acids and triglyceride the lowest expression of genes involving in de novo lipogenesis and the highest expression of genes related to lipid oxidation and hydrolysis, inflammatory, and fibrosis. Pyrosequencing of 16S rRNA genes revealed that the male mouse offspring with maternal normal calcium intake and the female mouse offspring with maternal low calcium intake, after the high-fat diet feeding, had distinct intestinal microbiota, which was closer to those in mice with the normal diet feeding. Analysis of the functional features for the different microbiota was compatible with the expression of genes associated with lipogenesis, lipid oxidation and hydrolysis. Thus, there is a sex-specific manner for maternal calcium requirement to inhibit the progress of offspring NAFLD, that might be less for the female offspring and more for the male offspring.

ARTICLE HISTORY



KEYWORDS


Calcium; maternal; sex-specific; nonalcoholic fatty liver disease; intestinal; microbiota; lipid metabolism

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a growing cause of chronic liver injury by excessive fat deposition that has become a worldwide health concern with one billion individuals globally.¹ The clinical spectrum of NAFLD is comprised by a variety of diseases ranging from steatohepatitis, fibrosis, and even cirrhosis and hepatocellular carcinoma.² Despite the clinical and public health is important, there are few effective therapeutic prevention and clearly pathogenic mechanisms. Notably, many experimental evidence has suggested a complex interplay of multiple biological

processes involving in the occurrence and progress of NAFLD such as intestinal microbiota dysbiosis, heightened intestinal barrier permeability, abnormal lipid metabolism, metabolic endotoxemia, and various inflammatory processes.^{3–6} During the past decade, the disruptions of intestinal microbiota as the indicator of gut-liver-axis have received more attention on the progression of NAFLD owing to its roles on modulating the energy homeostasis, increasing the contents of short-chain fatty acids, endotoxemia, endogenous ethanol and other microbe products and affecting the choline and bile acids metabolism in the hepatic tissue.^{7–10}

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 Supplemental data for this article can be accessed on the publisher's website.

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Moreover, the results from the epidemiological and clinical studies have proved that the dysbiotic gut ecosystem might further disrupt the integrity of the intestinal barrier to exacerbate the lipid accumulation in the hepatic tissue to increase the risk of NAFLD.¹¹

The colonization and development of a stable intestinal flora in the infants are regulated by many elements, including maternal nutrition and diets, use of antibiotics, probiotics and other drugs, host's genetics, and etc. Recently, a growing body of evidence have demonstrated that not only over-nutrition or under-nutrition but also deficiency of micronutrients are critical in the colonization of intestinal microbiota, which is associated with the alterations of intestinal barrier function and elevated levels of bacterial endotoxin. Calcium, an essential mineral nutrient, plays an important role in many physiological processes including bone construction, weight reduction, energy metabolism, and hormone secretion as a second messenger and/or signal transduction.¹² Epidemiological and animal studies have demonstrated that the physiological effects of calcium are mediated primarily by the changes of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) under the condition of unsuitable calcium intake, which could induce the abnormal colonization of intestinal flora by modulating the intestinal pH, promoting the excretion of bile and fatty acids and affecting the intestinal stability.¹³ On the other hand, it can also exert coordinated regulatory effects on the adipocyte lipid metabolism and triacylglycerol storage by increasing the gene expressions on the lipogenic and de novo lipogenesis and inhibiting the lipolysis and thermogenesis to result in excessive lipid accumulation in the hepatic tissue and associated NAFLD.^{14,15} Recently, emerging evidence has shown that the first 1,000 d in life is a critical period for the colonization of intestinal flora and prevention of many chronic non-communicable diseases (NCD) such as NAFLD.¹⁶ Thus, keeping adequate calcium intake in this period of the life is of a great importance for a positive calcium balance, good bone density necessary for the skeletal consolidation, and reducing the occurrence of NCD in the later.¹⁷ However, the daily calcium intake is inadequacy among the most Chinese people including the pregnant woman based on comparison between

the actual intake and the Chinese Dietary Reference Intakes (DRIs).¹⁸ So one purpose of this study was to discuss whether this unsuitable intake of calcium in early life could influence the development of NAFLD in later life stage by altering the intestinal microbiota and lipid metabolism.

Up to now, there have been some researches that try to discuss the effects of maternal calcium supplementation on the healthy outcomes in the offspring. However, they mainly focused on offspring bone and body composition, and produced mixed results for different population and intervention. Ward KA et al. indicated that calcium supplementation of pregnant women with low calcium intakes could only accelerate the childhood trajectories of growth, bone, and body composition development among males aged 8–12 y, but result in slower growth among females.¹⁹ The above evidence implies that maternal calcium demand during the pregnancy and lactation is different depending on sex of the offspring, but the underlying mechanisms are not clear. This discovery provides new challenges for the existing maternal and child health care, and also gives powerful evidence for individualized nutrition, which is of great significance to improve the children's health. Therefore, the other important hypothesis of this study is that whether this unsuitable intake of calcium in early life can influence the development of NAFLD in later life stage in a sex-specific manner. In this present work, we investigated the effects of maternal insufficient and excessive calcium intake during the pregnancy and lactation on the development of NAFLD in response to a high-fat diet by examining the intestinal microbiota and lipid metabolism with attention to potential sex differences among the mouse offspring.

2. Materials and methods

2.1 Diets

Three types of reproductive diets with low (LC, 0.25%), normal (NC, 0.70%), and high calcium (HC, 1.20%) concentrations were designed according to the gestating and growing formula (D10012G) from the Research Diets, Inc (New Brunswick, NJ, USA). Meanwhile, a high-fat diet (HFD) (34.9% fat by weight, 60% kcal, No. H10060)

and a normal-fat diet (4.3% fat by weight, 10% kcal, No. H10010) with normal calcium content were designed based on the formula for mature rodents (D10012M) from Research Diets, Inc. All these diets were prepared by Beijing HFK Bioscience Co., Ltd. (<http://www.hfkbio.com/>) and stored at -20°C until use.

2.2 Animal experiments

Four-week-old C57BL/6J female mice were purchased from Beijing HFK Bioscience Co., Ltd. After 3 d of recovery, these mice were randomly divided into three groups ($n = 10/\text{group}$, $n = 5/\text{cage}$) and fed with the LC, NC, and HC reproductive diets, respectively, for 8 weeks. Then, the female mice were mated with the 12-week-old C57BL/6J male mice following two females to one male per cage and continued on their own diets throughout the whole gestation and lactation. The male and female mice offspring from maternal different calcium intervention groups (LC, NC, and HC) ($n = 8/\text{group}$, $n = 4/\text{cage}$) were both fed with the HFD diet for 16 weeks after weaning at 21 d old and named LC-HFD, NC-HFD, and HC-HFD groups, respectively. Meanwhile, the male and female pups from the NC group were still fed with the normal-fat diet after weaning as the NC-C group. The specific diagram for this study design was shown in Supplemental Figure 1.

In this feeding process, the body weight was recorded weekly, food intake was measured monthly, then the energy intake was calculated according to the food intake. All mice were fed at the Animal Facility in the Laboratory Animal Center of Fourth Medical Center of the PLA General Hospital of China according to the standard animal feeding methods with the 12 hour (h) light and 12 h dark cycle at $22\text{--}25^{\circ}\text{C}$ and humidity of 40–60%.

2.3 Sample collection

The feces samples from the male and female mice offspring were collected at the end of feeding procedure in the day before they were weaned and executed, and then stored at -80°C . The mouse offspring were anesthetized by intraperitoneal

injection of Avertin (2,2,2-tribromoethanol) (T-4840-2; Aldrich Chemical, Munich, Bavaria, Germany) (125 mg/kg) after 8-h fasting. The blood samples were obtained by heart puncture and then sacrificed by injection of an overdose of Avertin (500 mg/kg) to minimize the suffering. Immediately, the epididymal white adipose tissue (eWAT) and hepatic tissue were dissected free of the surrounding tissues, which were partly fixed by immersion in 10% buffered formalin, the remaining tissues were frozen in liquid N_2 and stored at -80°C cryogenic refrigerator until use.

All these sample collections were performed from 08:00 to 12:00 in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of National Administration Regulations on Laboratory Animals of China. The animal protocol used in this study was approved by the Ethics of Animal Experiments of Fourth Medical Center of PLA General Hospital in China.

2.4 Measurements of biochemical parameters in the plasma and hepatic tissue

The concentrations of calcium (Cat. no. C004-2-1, Nanjing Jiancheng Bioengineering Institute, China), parathyroid hormone (PTH) (Cat. no. H207-96t, Nanjing Jiancheng Bioengineering Institute, China), triglyceride (TG) (Cat. no. CH0101151, Sichuan Maker Science Tech. Co., LTD, China), total cholesterol (TC) (Cat. no. CH0101160, Sichuan Maker Science Tech. Co. LTD, China) in the plasma and/or hepatic tissue were, respectively, assayed by colorimetric or enzymatic methods. The levels of inflammatory cytokines TNF- α , IL-1 β , IL-4, and IL-10 in the plasma were also determined using ELISA kits, according to the manufacturer's guidelines (Invitrogen, ThermoFisher Scientific, CN).

2.5 Hepatic tissue histology

The hepatic tissues of the mouse offspring, fixed in 10% buffered formalin by immersion, were chosen to make the histology analysis. The hepatic histopathologies were evaluated by hematoxylin and eosin (H&E) staining, the accumulation of lipid drops in the hepatic tissue was stained with Oil red O for histopathological assessment. The degree

of fibrosis in the hepatic tissue was measured by Masson staining. Then all samples above were examined under light microscope at magnification of 400 \times . Then the Image-pro Plus 6 (IPP 6) was used to quantitatively analyze the amount of lipid droplets in each slice.

2.6 Gene expression analysis by RT-PCR in the hepatic tissue

Total RNA of the hepatic tissue was extracted using Trizol Reagents (Cat. No. 15596-206, Invitrogen, Carlsbad, CA, USA), and the cDNA was reversely transcribed by SuperScriptTM III First-Strand Synthesis System (Cat. No. 18080-051, Invitrogen, Carlsbad, CA, USA). Expressions of the target genes involved in lipid oxidation (PPAR- α , CPT-1a, CPT-1c, CPT2, PGC-1 α , UCP1, and PRDM16), hydrolysis (ATGL and HSL), lipogenesis (DGAT2, Plin2, Fasn, Acc1, LPL, and SREBP-1c), synthesis (PPAR- γ , C/EBP- α , and Fabp4), hepatic inflammation (TGF α , IL-1 β , ccl2, ccl4, IL4, and IL10), and fibrosis (TGF- β and Collagen) were measured using the quantitative RT-PCR with GAPDH as the

invariant internal control. The oligonucleotide primers for all these targeted genes were found in the Primer-Bank (<https://pga.mgh.harvard.edu/primerbank/>) following the NCBI gene ID, checked by the Primer-Blast (<http://www.ncbi.nlm.nih.gov/tools/primerblast/>) and composed by the Sangon Biotech (Shanghai) Co., Ltd, which were shown in Supplemental Table 1.

2.7 DNA extraction and 16S rRNA high-throughput sequencing

About 80-mg fecal samples of male and female mice offspring were used to extract the bacterial DNA using the QIAamp Fast DNA Stool Mini Kit (Cat. No. 51504, Qiagen, Germany) according to the manufacturer's protocol. The concentrations of bacterial DNA were measured using Nanodrop 2000 (Thermo Scientific, USA).

The final DNA purification and microbiota determination were measured by Shanghai Majorbio Bio-pharm Technology Co., Ltd. In this process, the V3-V4 region of the bacteria's 16S ribosomal RNA (rRNA) gene was amplified by

Table 1. Body weight and metabolic indicators in the plasma of male and female mice offspring under maternal different calcium intake.

	NC-C (n = 8)	NC-HFD (n = 8)	LC-HFD (n = 8)	HC-HFD (n = 8)	P
Male	9.68 \pm 1.09	10.00 \pm 1.62	10.10 \pm 1.95	9.28 \pm 1.93	.373
Initial body weight (g)					
Final body weight (g)	27.45 \pm 1.21	38.34 \pm 2.10*	43.77 \pm 2.90*#	44.63 \pm 3.42*#	<.001
Food intake (g/d)	2.33 \pm 0.11	2.21 \pm 0.19	2.07 \pm 0.24	2.25 \pm 0.13	.172
Energy intake (kcal/d)	8.96 \pm 0.43	11.52 \pm 0.98*	10.78 \pm 1.21*	11.73 \pm 0.68*	.021
Calcium (mmol/L)	2.06 \pm 0.66	2.19 \pm 0.65	2.42 \pm 0.62	2.22 \pm 0.53	.629
PTH (μ g/L)	1.25 \pm 0.079	1.19 \pm 0.12	1.45 \pm 0.076*#	1.28 \pm 0.094	.041
FFA (Emq/L)	6.09 \pm 1.84	13.74 \pm 2.10*	28.70 \pm 3.48*#	16.83 \pm 7.05*	<.001
TG (mmol/L)	0.17 \pm 0.069	0.27 \pm 0.098*	0.31 \pm 0.092*	0.37 \pm 0.13*#	.003
TC (mmol/L)	2.21 \pm 0.78	2.84 \pm 0.56*	3.69 \pm 0.91*#	4.05 \pm 0.79*#	<.001
IL6 (pg/mL)	10.42 \pm 1.01	11.28 \pm 1.17	11.12 \pm 1.36	11.64 \pm 0.37	.762
TNF- α (pg/mL)	15.88 \pm 0.95	16.32 \pm 0.26	16.67 \pm 0.40*	16.29 \pm 0.24	.045
IL-4 (pg/mL)	25.25 \pm 4.49	13.04 \pm 2.32*	10.85 \pm 1.54*#	9.11 \pm 0.82*#	<.001
IL-10 (pg/mL)	197.17 \pm 20.78	142.33 \pm 17.29*	102.58 \pm 14.17*#	108.65 \pm 14.57*#	.029
Female	8.89 \pm 0.90	8.77 \pm 0.78	9.18 \pm 1.03	8.74 \pm 0.82	.325
Initial body weight (g)					
Final body weight (g)	21.86 \pm 1.20	35.21 \pm 2.10*	29.38 \pm 1.90*#	34.87 \pm 3.40*	<.001
Food intake (g/d)	2.15 \pm 0.12	2.09 \pm 0.32	2.23 \pm 0.21	2.07 \pm 0.16	.802
Energy intake (kcal)	8.28 \pm 0.46	10.96 \pm 1.67*	11.69 \pm 1.10*	10.85 \pm 0.84*	.033
Calcium (mmol/L)	2.17 \pm 0.44	2.53 \pm 0.49	2.68 \pm 0.41	2.55 \pm 0.58	.133
PTH (μ g/L)	1.35 \pm 0.30	1.22 \pm 0.43	1.33 \pm 0.14	1.34 \pm 0.23	.214
FFA (Emq/L)	4.28 \pm 1.04	14.23 \pm 2.53*	9.17 \pm 1.91*#	13.82 \pm 2.45*	<.001
TG (mmol/L)	0.15 \pm 0.091	0.36 \pm 0.13*	0.24 \pm 0.054*#	0.33 \pm 0.11*	.003
TC (mmol/L)	1.74 \pm 0.56	3.35 \pm 0.54*	2.96 \pm 0.61*#	3.06 \pm 0.38*	<.001
IL-6 (pg/mL)	16.53 \pm 0.38	17.26 \pm 0.73*	17.33 \pm 0.28*	17.30 \pm 0.32*	.013
TNF- α (pg/mL)	20.32 \pm 0.23	21.50 \pm 0.19*	21.43 \pm 0.21*	21.30 \pm 0.14*	.034
IL-4 (pg/mL)	17.16 \pm 0.19	17.01 \pm 0.41	16.91 \pm 0.25	17.30 \pm 0.25	.051
IL-10 (pg/mL)	61.57 \pm 24.66	38.05 \pm 18.86*	42.79 \pm 11.18*	39.08 \pm 12.13*	.041

Four-week-old C57BL/6 J female mice were fed with low (LC, 0.25%), normal (NC, 0.70%), and high (HC, 1.20%) calcium reproductive diets, respectively, starting 2 months before the conception, which were continued throughout the whole pregnancy and lactation. After weaning, the pups were fed with a normal (NC-C) or high-fed diets (NC-HFD, LC-HFD and HC-HFD) for 16 weeks. All values are shown as means \pm SD, n = 8 in each group for both the male and female mouse offspring. *Compared with the NC-C group, P < .05. #Compared with the NC-HFD group, P < .05.

PCR with bar-code-indexed primers (338 F and 806 R) using Fastpfu Polymerase. Amplicons were purified by gel extraction (AxyPrep DNA Gel Extraction Kit, Axygen Bio-sciences, Union City, California, USA) and quantified using QuantiFluor-ST (Promega, USA). The purified amplification was then pooled in equimolar concentrations, and paired-end sequencing was performed using an Illumina MiSeq instrument (Illumina, San Diego, California, USA).

2.8 Microbial analysis

All the microbial data were analyzed on the free online platform of Majorbio I-Sanger Cloud Platform (www.i-sanger.com).²⁰ To clarify the similarities of intestinal flora composition among all the experimental groups (NC-C, NC-HFD, LC-HFD, and HC-HFD), the estimators of α -diversity (Shannon, Simpson, Ace and Chao indexes) were calculated based on rarefied OTU tables. Principal Coordinates Analyses (PCA) of unweighted UniFrac distance matrix is displayed as the β -diversity. Weighted UniFrac distance matrices were computed to detect global variations in the composition of microbial communities at the phylum and genus levels by one-way analysis of variance (ANOVA). PICRUST (phylogenetic investigation of communities by reconstruction of unobserved states, V1.0) was used to predict the 16S rRNA based high-throughput sequencing data for functional features from the phylogenetic information with an estimated accuracy of 0.8. The Cluster of Orthologous Groups (COG) database was obtained through the Green-gene ID corresponding to each OTU in the EGGNOG database (Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups). Then the predicted functional composition profiles were collapsed into level 2 of KEGG (Kyoto Encyclopedia of Genes and Genomes) database pathways. Pathways that were presented in <10% of the samples were not included in the comparison analysis. The correlations between the microbiota composition of the top 10 richness genus and the significantly different expression of lipid related genes among the mouse offspring with different maternal calcium intake during the pregnancy and lactation were determined by RDA/CCA analysis and the related heatmap figures.

2.9 Statistical analysis

All statistical analyses were conducted by SPSS 21.0. ANOVA was performed to compare the means of indexes among different groups with normally distributed data. The differences between these groups in data with the non-normal distribution were assessed using Mann–Whitney U-test or Wilcoxon Signed-Rank test. Then the Student–Newman–Keuls (SNK) test was used to determine where the differences existed between each two groups. Linear relationships between these variables were tested by Spearman's correlations. $P < .05$ was considered to be statistically significant.

3. Results

3.1 Body weight and energy intake in the male and female mice offspring

As shown in Table 1, the body weight and energy intake in the HFD groups (NC-HFD, LC-HFD, and HC-HFD) were significantly higher as compared to the NC-C group in both male and female mice offspring ($P < .05$). Further comparisons among these three HFD groups showed that the body weight in the LC-HFD and HC-HFD groups was heavier than those in the NC-HFD group ($P < .05$) with no changes of the energy intake in the male mouse offspring. While among the female mouse offspring, the body weight from the NC-HFD and HC-HFD groups was heavier than those in the LC-HFD group ($P < .05$) with no changes in energy intake.

3.2 The changes of biochemical parameters and inflammation-related cytokines in the plasma among the male and female mice offspring

As shown in Table 1, in the male mouse offspring, the contents of FFA, TC and TG in the HFD groups were higher as compared to the NC-C group, while the concentrations of IL-4 and IL10 were lower ($P < .05$). Further comparisons among these three HFD groups showed that FFA, TC, and TG in the LC-HFD or/and HC-HFD groups were higher, while the IL4 and IL10 were lower than those in the NC-HFD group ($P < .05$). Meanwhile, the concentrations of PTH and TNF α were higher in the LC-HFD group than those in the NC-C and NC-HFD groups.

In the female mouse offspring, the contents of FFA, TC, TG, TNF α , and IL-6 in the HFD groups were higher, while the concentrations of IL10 were lower as compared to the NC-C group ($P < .05$). Further comparison showed that FFA, TG, and TC in the LC-HFD group were lower than those in the NC-HFD group ($P < .05$).

3.3 Effects of maternal different calcium intake on the progress of HFD-induced hepatic steatosis in the male/female mouse offspring

In the male mouse offspring, the contents of hepatic TG (Figure 1(e)) among the HFD groups were higher as compared to the NC-C group. Further comparisons among the three HFD groups showed that hepatic TG in the LC-HFD or/and HC-HFD groups were higher than that in the NC-HFD group ($P < .05$). The histological analysis also showed the accumulation of lipid droplets, in the hepatic tissue

was more heavier in the LC-HFD and HC-HFD groups than that in the NC-HFD group ($P < .05$).²⁰ The inflammation and fibrosis has been clearly shown in Figures 2 and 3. In the mice fed with the HFD, local inflammation and fibrosis were more serious than those in the NC-C group (Figures 2(a) and 3(a)), with higher expressions of TNF- α (Figure 2(c)), IL-1 β (Figure 2(d)), ccl2 (Figure 2(e)), ccl4 (Figure 2(i)), TGF β (Figure 3 (b)) and Collagen (Figure 3(c)), and lower expressions of IL-4 (Figure 2(j)), and IL-10 (Figure 2(k)). Further comparisons among these three HFD groups proved that local inflammation and fibrosis were more serious in the LC-HFD and HC-HFD groups than those in the NC-HFD group, with the higher expressions of TNF- α , IL-1 β , ccl2, ccl4 and Collagen, and lower expressions of IL-4.

In the female mouse offspring, the concentration of hepatic TG (Figure 1(g)) among the HFD groups was higher as compared to the NC-C group

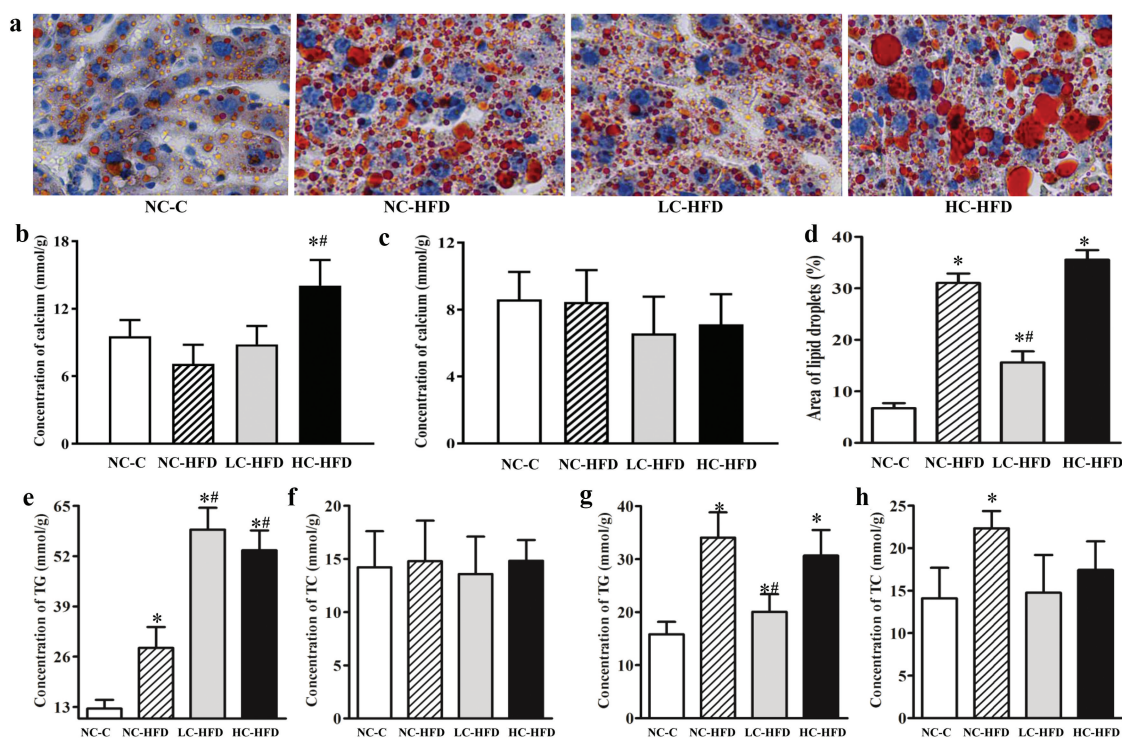


Figure 1. Maternal calcium intake during the pregnancy and lactation affected the lipid deposition and accumulation in the hepatic tissue for the male and female mouse offspring. Lipid deposition and accumulation in the hepatic tissue among the female mouse offspring (a and d) were shown by Oil red O staining and quantitatively analyzed using the Image-pro Plus. The concentrations of calcium, TG and TC in the hepatic tissue from the male (b, e, and f) and female mouse offspring (c, g, and h) were measured by colorimetric or enzymatic methods. Data are presented as means with standard deviation (SD), $n = 8$ mice/group. One-way analysis of variance (ANOVA) was performed to compare the indexes among different groups. Then, the Student–Newman–Keuls (SNK) test was used to determine where the differences exist between each two groups. *Compared to the NC-C group, $P < .05$; #compared to the NC-HFD group, $P < .05$.

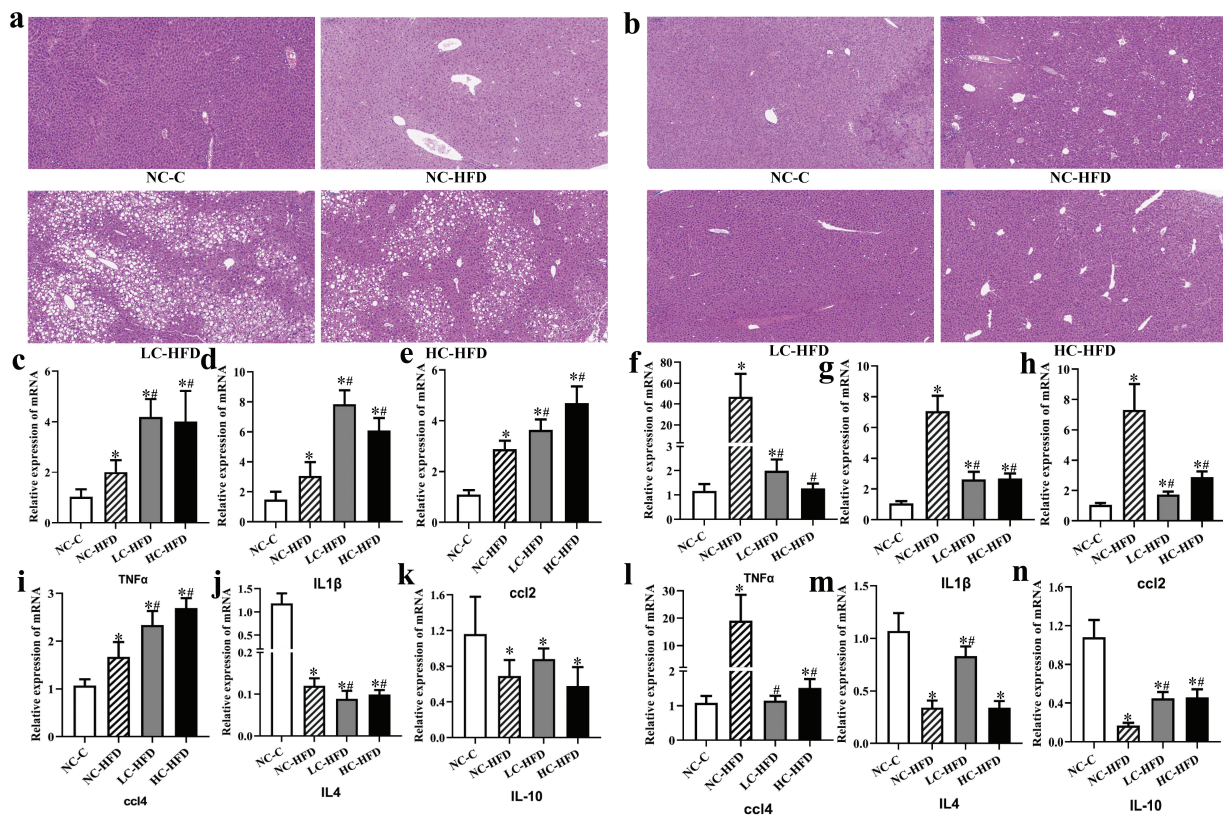


Figure 2. Effects of maternal different dietary calcium intake on the HFD-induced inflammatory infiltration and expression of related genes in the hepatic tissue of the male and female mouse offspring. The inflammatory infiltration in the hepatic tissue was shown by the H&E staining in the male (a) and female mouse offspring (b). The expression of proinflammatory genes (TNF- α , IL-1 β , ccl2, and ccl4) and anti-inflammatory genes (IL-4 and IL-10) in the hepatic tissue among the male (c, d, e, i, j, and k) and female mouse offspring (f, g, h, l, m, and n) was measured by quantitative RT-PCR. Data are presented as means with standard deviation (SD), $n = 8$ mice/group. One-way analysis of variance (ANOVA) was performed to compare the indexes among different groups. Then the Student–Newman–Keuls (SNK) test was used to determine where the differences exist between each two groups. *Compared to the NC-C group, $P < .05$; #compared to the NC-HFD group, $P < .05$.

($P < .05$). Further comparisons showed that the hepatic TG in the LC-HFD group was lower than those in the NC-HFD group ($P < .05$). The histological analysis also proved the accumulation of lipid droplets in the hepatic tissue was more serious in the NC-HFD and HC-HFD groups than that in the LC-HFD group (Figure 1(a,d)) ($P < .05$). The mice fed with HFD had more serious local inflammation and fibrosis compared to the NC-C group (Figures 2(b) and 3(d)), with the higher expressions of TNF- α (Figure 2(f)), IL-1 β (Figure 2(g)), ccl2 (Figure 2(h)), ccl4 (Figure 2(l)), TGF β (Figure 3(e)) and Collagen (Figure 3(f)) and lower expressions of IL-4 (Figure 2(m)), and IL-10 (Figure 2(n)). Further comparisons among the three HFD groups proved that local inflammation and fibrosis had been improved to a greater extent in the LC-HFD group than those in the NC-HFD group, with lower

expressions of TNF- α , IL-1 β , ccl2, ccl4, TGF β and Collagen, and lower expressions of IL-4, and IL-10.

3.4 Effects of maternal different calcium intake on the expressions of genes related with the lipid metabolism in the male/female mouse offspring

In the male mouse offspring, RT-PCR analysis confirmed that the expressions of genes related to lipid oxidation and hydrolysis such as PPAR- α , CPT-1 α , CPT-1c, PGC-1 α , UCP1, PRDM16, ATGL, and HSL were downregulated (Figure 4(a,b)); whereas the expression of genes involving in de novo lipogenesis including DGAT2, Plin2, Fasn, Acc1, LPL, SREBP-1c, PPAR- γ , and FABP4 were upregulated in the HFD groups as compared to the NC-C group ($P < .05$) (Figure 5(a,b)). Further analysis of these genes' expression in three HFD groups indicated

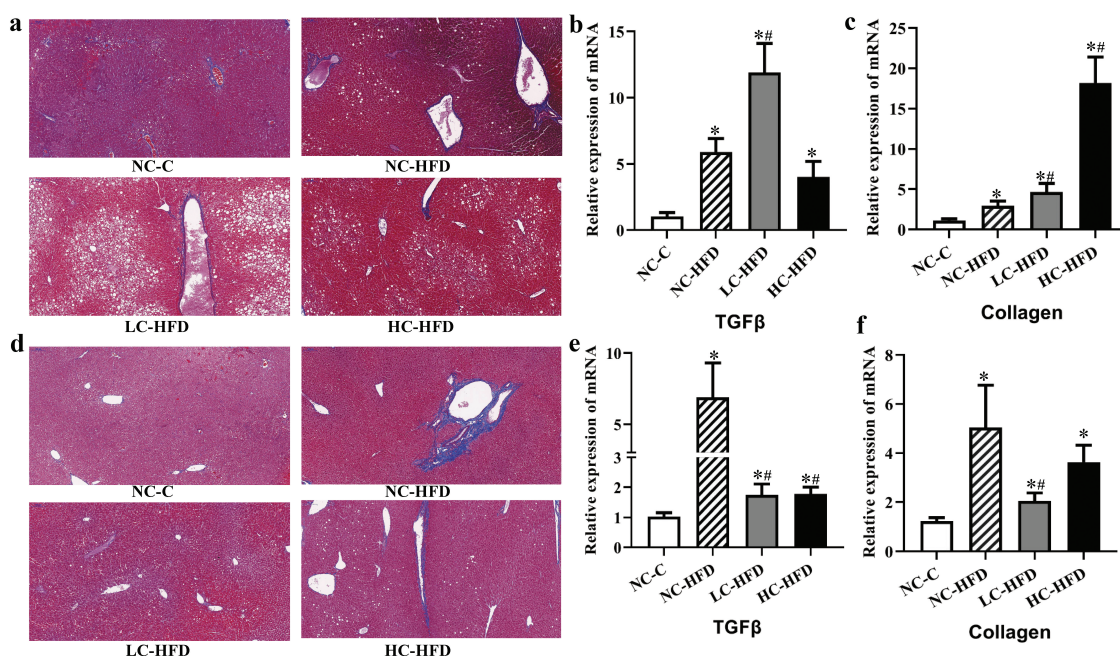


Figure 3. Effects of maternal different dietary calcium intake on the HFD-induced hepatic fibrosis and expression of related genes in the hepatic tissue of the male and female mouse offspring. The hepatic fibrosis in the hepatic tissue was shown by the Masson staining in the male (a) and female mouse offspring (d). The expression of genes associated with hepatic fibrosis (TGF β and Collagen) in the male (b and c) and female mouse offspring (e and f) was measured by the quantitative RT-PCR. Data are presented as means with standard deviation (SD), $n = 8$ mice/group. One-way analysis of variance (ANOVA) was performed to compare the indexes among different groups. Then the Student–Newman–Keuls (SNK) test was used to determine where the differences exist between each two groups. *Compared to the NC-C group, $P < .05$; #compared to the NC-HFD group, $P < .05$.

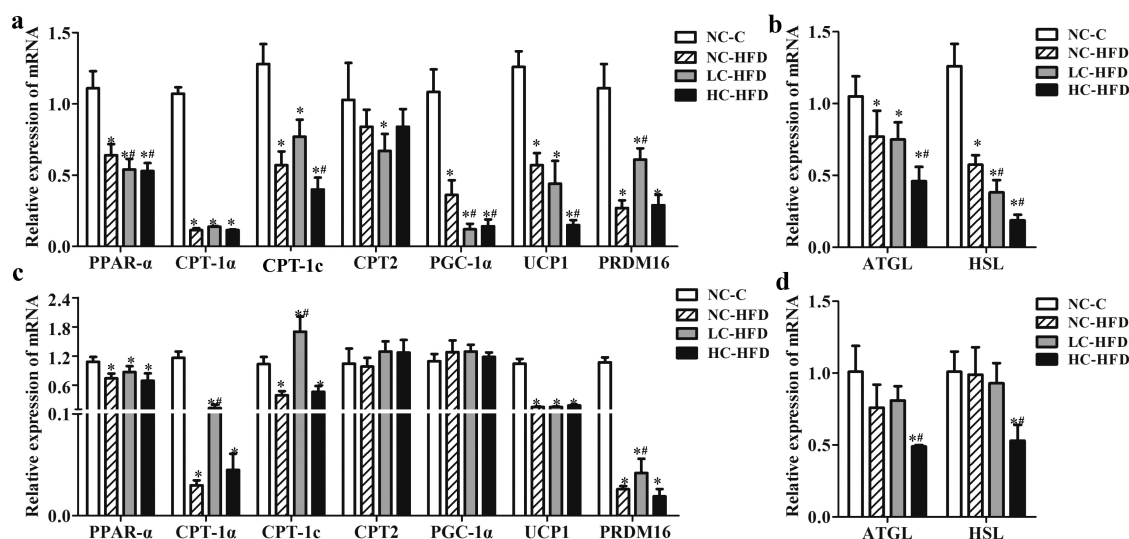


Figure 4. Effects of maternal different dietary calcium intake on the expression of genes involving in the lipid oxidation and hydrolysis in the hepatic tissue of the male and female mouse offspring. The expression of genes associated with lipid oxidation, lipolysis (PPAR- α , CPT-1 α , CPT-1c, CPT2, PGC-1 α , UCP1, and PRDM16) and hydrolysis (ATGL and HSL) in the hepatic tissue among the male (a, b) and female mouse offspring (c, d) was measured by the quantitative RT-PCR. Data are presented as means with standard deviation (SD), $n = 8$ mice/group. One-way analysis of variance (ANOVA) was performed to compare the indexes among different groups. Then the Student–Newman–Keuls (SNK) test was used to determine where the differences exist between each two groups. *Compared to the NC-C group, $P < .05$; #compared to the NC-HFD group, $P < .05$.

that there were lower expressions of PPAR- α , CPT-1c, PGC-1 α , UCP1, ATGL, and HSL, higher

expressions of DGAT2, Fasn, Acc1, LPL, SREBP-1c, PPAR- γ , and FABP4 in the LC-HFD or/and

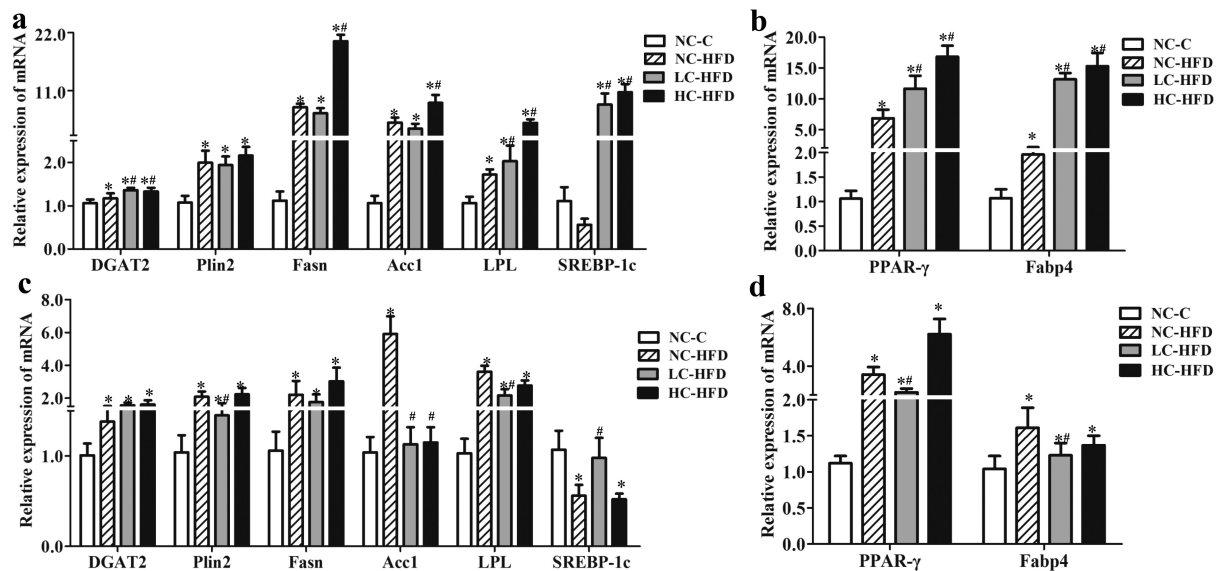


Figure 5. Effects of maternal different dietary calcium intake on the expression of genes involving in the lipogenesis and lipid synthesis in the hepatic tissue of the male and female mouse offspring. The expression of genes associated with lipid synthesis and lipogenesis (DGAT2, Plin2, Fasn, Acc1, LPL and SREBP-1c, PPAR- γ , C/EBP- α and Fabp4) in the hepatic tissue among the male (a, b) and female mouse offspring (c, d) was measured by the quantitative RT-PCR. Data are presented as mean with standard deviation (SD), $n = 8$ mice/group. One-way analysis of variance (ANOVA) was performed to compare the indexes among different groups. Then the Student–Newman–Keuls (SNK) test was used to determine where the differences exist between each two groups. *Compared to the NC-C group, $P < .05$; #compared to the NC-HFD group, $P < .05$.

HC-HFD groups than those in the NC-HFD group ($P < .05$).

In the female mouse offspring, the expression of PPAR- α , CPT-1 α , CPT-1c, UCP1, PRDM16, ATGL, and HSL was downregulated (Figure 4(c,d)); whereas the expression of DGAT2, Plin2, Fasn, Acc1, LPL, PPAR- γ , and FABP4 was upregulated in the HFD groups as compared to the NC-C group ($P < .05$) (Figure 5(c,d)). Further analysis of these genes' expression in three HFD groups indicated that, compared to the NC-HFD group, there was an increased expression of CPT-1 α , CPT-1c and PRDM16, and decreased expression of Plin2, Acc1, LPL, PPAR- γ , and FABP4 in the LC-HFD group ($P < .05$).

3.5 Gut microbiota dysbiosis among the male and female mice offspring affected by maternal different calcium intake

The bacterial community relative abundance (Ace and Chao indexes) and diversity (Shannon and Simpson indexes), measured by numbers of observed OTUs, were compared among the mouse offspring in maternal different calcium intake groups. We confirmed Shannon index was increased and Simpson was decreased in three HFD

groups as compared with the NC-C group in the male mouse offspring, which were also affected by maternal insufficient calcium intake (LC-HFD) as compared to the NC-HFD group ($P < .05$) (Figure 6 (e,f)). However, Chao index among the male mouse offspring and all α -diversity indexes (ACE, Shannon and Simpson) in the female mouse offspring were not significantly different among the four groups (Figures 6(d) and 7(d–f)). The beta diversity index as an unweighted UniFrac-based principal analysis (PCA) revealed that the overall microbial composition of HFD groups could be deviated from the NC-C group at the phylum and genus levels, either in the male or female mouse offspring ($P < .05$). Further analysis found that the clusters in the male mouse offspring from the LC-HFD and HC-HFD groups were significantly different from those in the NC-HFD fed mice at the genus level ($P < .05$; Figure 6(a,b)). While in the female mouse offspring, the clusters in samples from the LC-HFD group was significantly different from those in the NC-HFD and HC-HFD groups at the genus level ($P < .05$; Figure 7(a,b)).

To investigate the specific changes of microbiota and differences between the male and female mouse offspring affected by maternal different dietary

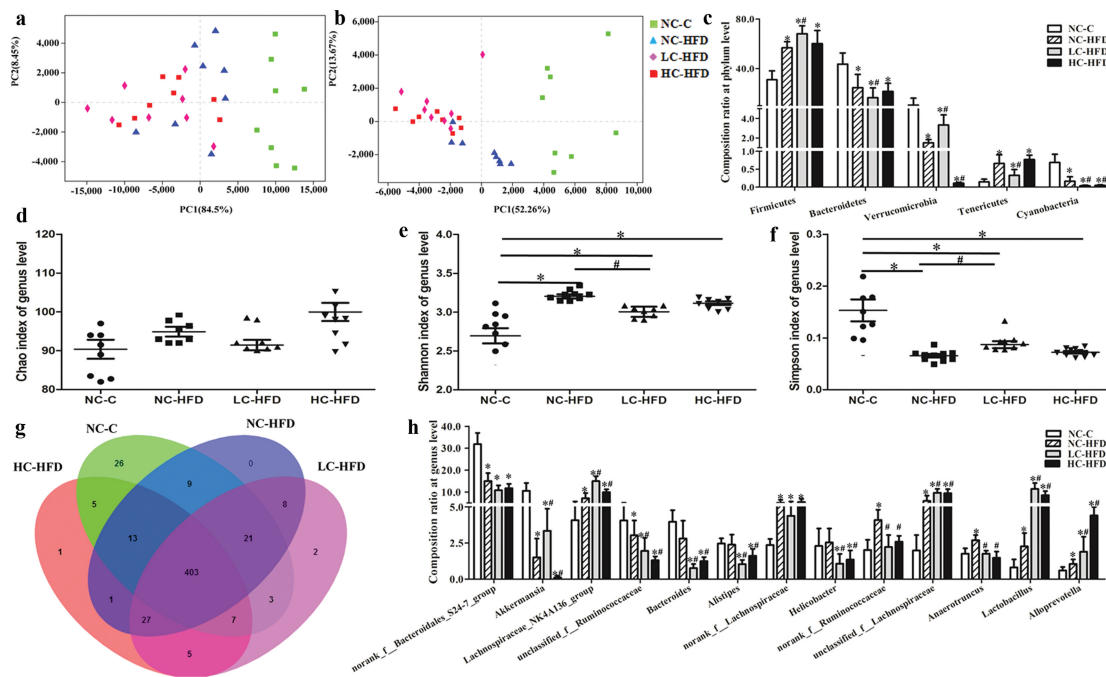


Figure 6. Microbiota composition and diversity of the male mouse offspring affected by maternal different calcium intake during pregnancy and lactation. Bacterial communities at the phylum and genus levels based on weighted UniFrac distance were shown using the PCA analysis (a and b). Estimate of bacterial richness was assessed by Chao index at the genus level (d). Estimates of bacterial diversity were assessed by Shannon and Simpson indexes of genus level (e and f). Different fecal microbiota compositions were, respectively, shown at the levels of the phylum (c) and genus (g, h). Data are presented as median and quartile, $n = 8$ mice/group. The differences of the data among the different calcium intake groups during the pregnancy and lactation were assessed using Mann–Whitney U-test and Wilcoxon signed-rank test. *Compared to the NC-C group, $P < .05$; # compared to the NC-HFD group, $P < .05$.

calcium intake, we assessed the gut microbiota compositions at the phylum and genus levels. In the male mouse offspring, at the phylum level, the proportions of *Firmicutes*, and *Tenericutes* were increased, and those of *Bacteroidetes*, *Verrucomicrobia*, and *Cyanobacteria* were decreased in the HFD groups as compared to the NC-C group ($P < .05$). Meanwhile, there were much higher contents of *Firmicutes* and lower percents of *Bacteroidetes*, *Verrucomicrobia* and *Cyanobacteria* in the LC-HFD or/and HC-HFD groups than those in the NC-HFD group (Figure 6(c)). Different bacterial groups at the genus level showed the effects of maternal insufficient (LC-HFD group) or/and excessive calcium intake (HC-HFD group) on the offspring, characterized by the reductions in proportions of *unclassified_f_Ruminococcaceae*, *Bacteroides*, *Alistipes*, *Helicobacter*, *norank_f_Ruminococcaceae*, and *Anaerotruncus*, and increases in proportions of *Akkermansia*, *Lachnospiraceae_NK4A136_group*, *unclassified_f_Lachnospiraceae*, *Lactobacillus* and *Alloprevotella* as compared with the NC-HFD group ($P < .05$) (Figure 6(g,h)).

In the female mouse offspring, at the phylum level, the proportions of *Firmicutes*, *Saccharibacteria*, and *Deferribacteres* were over-represented, and those of *Bacteroidetes* and *Verrucomicrobia* were decreased in the HFD groups relative to the NC-C group ($P < .05$). Further analysis showed there were much higher contents of *Proteobacteria* and *Deferribacteres* and lower proportions of *Firmicutes*, *Verrucomicrobia* and *Saccharibacteria* in the HC-HFD than those in the NC-HFD group, while in the LC-HFD group, the proportions of *Verrucomicrobia* and *Deferribacteres* were lower (Figure 7(c)). At the genus level, we observed 13 bacterial taxa that displayed different abundance among the four groups. Compared to the NC-HFD and/or HC-HFD groups, there were significant reductions in the proportions of *Helicobacter*, *Blautia*, *Oscillibacter*, *Ruminiclostridium* and *Mucispirillum*, and increases in the proportions of *Bacteroides*, *Akkermansia*, *Lachnospiraceae_NK4A136_group* and *Alloprevotella* in the LC-HFD group (Figure 7(g,h)).

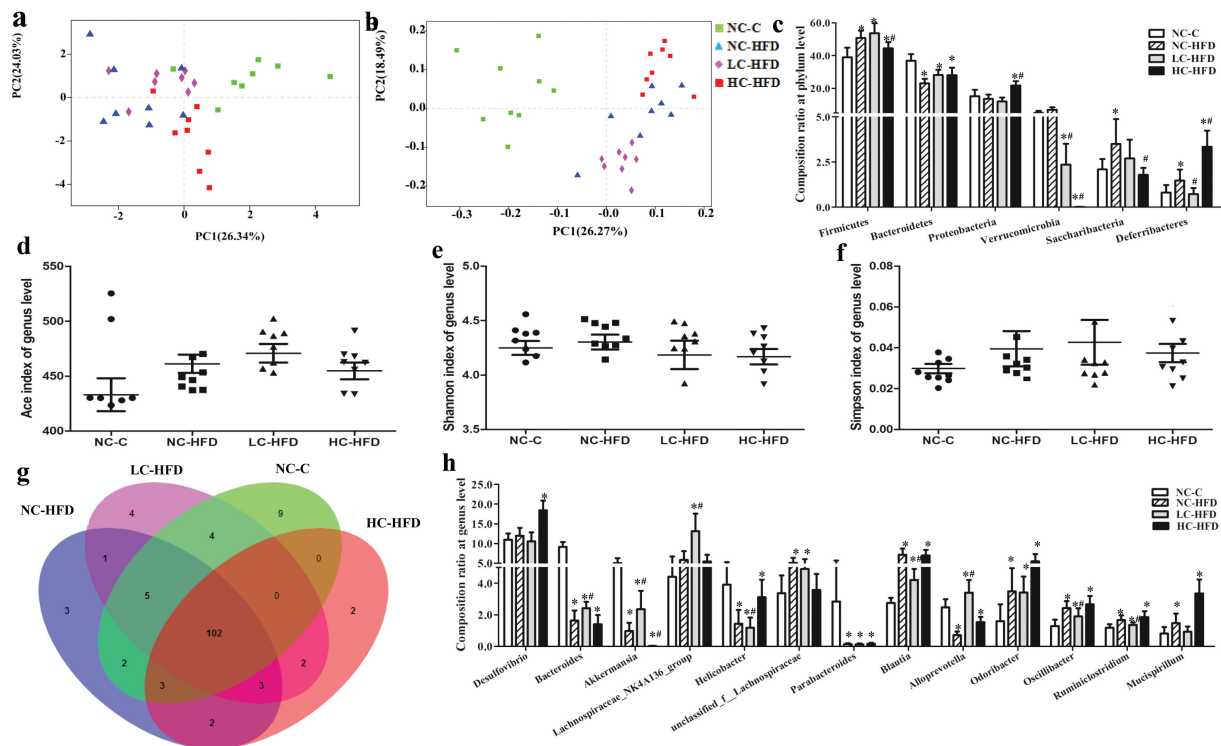


Figure 7. Microbiota composition and diversity of the female mouse offspring affected by maternal different calcium intake during the pregnancy and lactation. Bacterial communities at the phylum and genus levels based on weighted UniFrac distance were shown using the PCA analysis (a and b). Estimate of bacterial richness was assessed by Ace index at the genus level (d). Estimates of bacterial diversity were assessed by Shannon and Simpson indexes of genus level (e and f). Different fecal microbiota compositions were, respectively, shown at the levels of the phylum (c) and genus (g, h). Data are presented as median and quartile, $n = 8$ mice/group. The differences of the data among the different calcium intake groups during the pregnancy and lactation were assessed using Mann-Whitney U-test and Wilcoxon signed-rank test. *Compared to the NC-C group, $P < .05$; # compared to the NC-HFD group, $P < .05$.

3.6 Functional features of the gut microbiota isolated from the male and female mouse offspring affected by maternal different calcium intake

To better understand the important role of the gut microbiota in the male and female mouse offspring affected by maternal different calcium intake, PICRUST1.0 program was used to predict our 16S rRNA based high-throughput sequencing data and further analyze the data in the context of COG database and KEGG (level 2) categories. Using the above methods, we obtained a microbial COG profile, KEGG and correlated the microbial functional features with the important genes found in the mouse offspring (Figure 4), which may predict the metabolic functions enriched in our samples.

In the male mouse offspring, the top 10 vigorous functional features of COG profile included carbohydrate transport and metabolism, general function prediction only, amino acid transport and metabolism, Transcription; cell wall/membrane/envelope

biogenesis, replication, recombination and repair, translation, ribosomal structure and biogenesis, signal transduction mechanisms, energy production and conversion, and inorganic ion transport and metabolism (Figure 8(a,c)). While, in the female mouse offspring, the major function features were carbohydrate transport and metabolism, general function prediction only, amino acid transport and metabolism, transcription, cell wall/membrane/envelope biogenesis, replication, recombination and repair, signal transduction mechanisms, translation, ribosomal structure and biogenesis, energy production and conversion and inorganic ion transport and metabolism (Figure 8(b,d)).

Multiple KEGG (level 2) categories were disturbed in the mouse offspring affected by maternal different dietary calcium. In the male mouse offspring, the pathways enriched in HFD groups (LC-HFD, NC-HFD, and HC-HFD) highlighted carbohydrate metabolism, amino acid metabolism, energy metabolism, lipid metabolism, sorting and

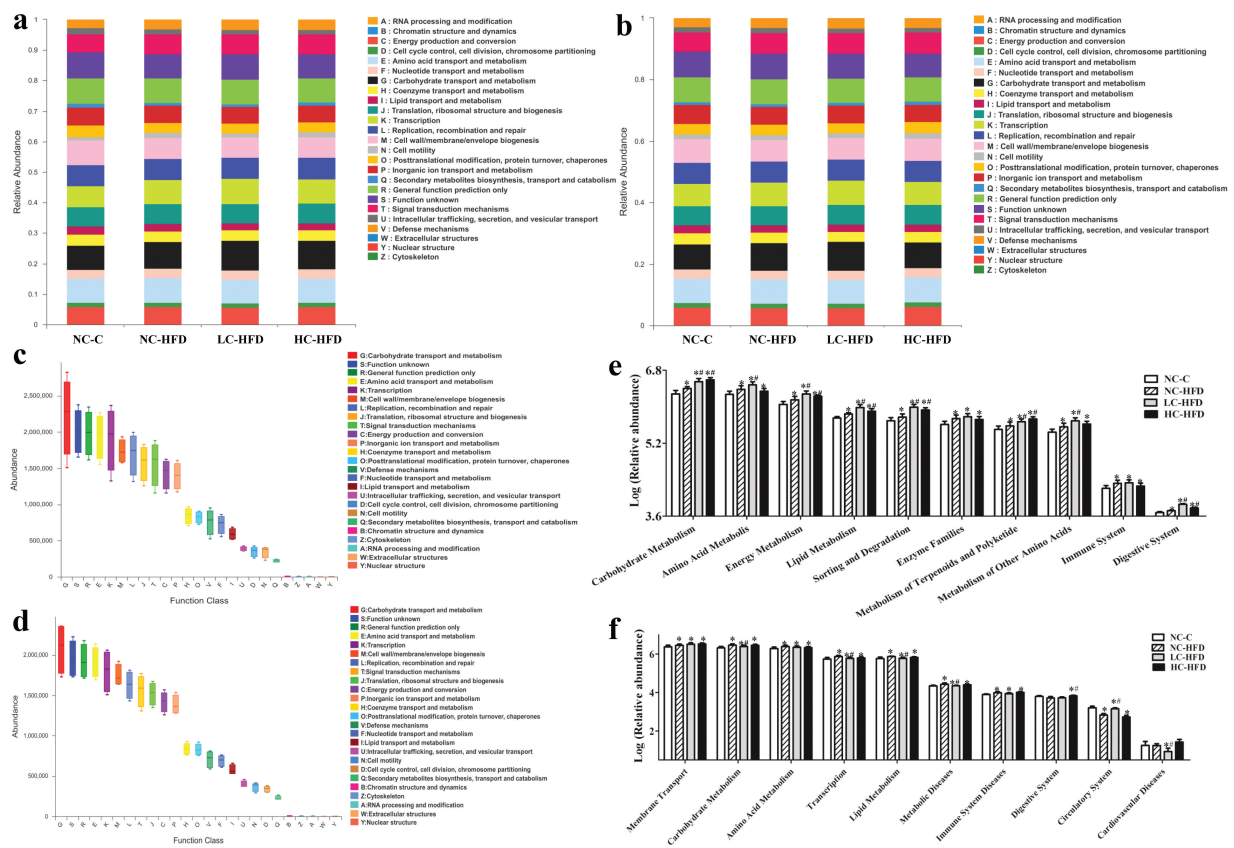


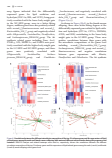
Figure 8. The microbial functional features among the male and female mouse offspring with maternal different calcium intake during the pregnancy and lactation The Cluster of Orthologous Groups (COG) and KEGG (Kyoto Encyclopedia of Genes and Genomes) database pathways were, respectively, used to predict the microbial functional features among the male (a, c, and e) and female mouse offspring (b, d, and f) with maternal different calcium intake during the pregnancy and lactation. One-way analysis of variance (ANOVA) was performed to compare the KEGG pathways among the different groups. Then, the Student–Newman–Keuls (SNK) test was used to determine where the differences existed between each two groups. *Compared to the NC-C group, $P < .05$; #Compared to the NC-HFD group, $P < .05$.

degradation, enzyme families, metabolism of terpenoids and polyketide, metabolism of other amino acids, immune system and digestive system relative to the NC-C group. Further comparison showed that comparing with those in the NC-HFD group, the pathways involving in carbohydrate metabolism, energy metabolism, lipid metabolism, sorting and degradation, metabolism of terpenoids and polyketide, and digestive system were higher in the LC-HFD and HC-HFD groups (Figure 8(e)). The gut microbiome of the HFD groups among the female mouse offspring was characterized by overrepresentation of pathways involved in membrane transport, carbohydrate metabolism, amino acid metabolism, transcription, lipid metabolism, metabolic diseases, immune system diseases, and low-representation of pathways in the circulatory system than those in the NC-C group. Strikingly,

bacterial invasion in the LC-HFD group was more significantly lower in the predicted pathways for carbohydrate metabolism, transcription, lipid metabolism, metabolic diseases, cardiovascular diseases, and higher level of pathways in the circulatory system than those in the NC-HFD group, in which the pathways in the LC-HFD were similar to those in the NC-C group.

3.7 Relationships between the microbiota community structure and the characteristics of lipid metabolism

Spearman correlation test was performed to evaluate the relationships between the different maternal dietary calcium status-associated genera and the characteristics of lipid metabolism. In the male mouse offspring, RDA/CCA analysis and related heat-



related genes including *Fasn*, *Acc1*, *PPAR-γ*, *LPL*, and *Plin2* were positively correlated with the higher body weight gain in the NC-HFD and HC-HFD groups, and further these genes were positively correlated with *Blautia*, *Desulfovibrio* and *Odoribacter*, and negatively correlated with *norank_f_Bacteroidales_S24_7_group*, *Lachnospiraceae_NK4A136_group* and *norank_f_Ruminococcaceae*.

4. Discussion

NAFLD, which is defined as the associations of hepatic steatosis, hepatocellular injury, inflammation and fibrosis, is associated with many metabolic syndromes such as obesity, insulin resistance, type 2 diabetes, and other chronic non-communicable diseases.^{21,22} So understanding the prevention and mechanisms of NAFLD is of the utmost importance. Recently, many evidences have shown that the disorder of the gut microbiota, and resulted disturbance in intestinal barrier function, could lead to the increment of hepatic fat accumulation.²³ Interestingly, Le Roy T. and et al. also proved that the gut microbiota dysbiosis contributed to the development of NAFLD through affecting the fatty acid oxidation and lipogenesis by comparing the germ-free (GF) and gut microbiota transplant mice.²⁴ Thus, these results demonstrated that the “gut-liver axis” is exerted to maintain the homeostasis of the gut microbiota and hepatic lipid metabolism.^{25,26} In our study, compared with the normal-fat diet feeding, high-fat diet feeding could result in the NAFLD by altering the gut bacterial communities and lipid metabolism both in the male and female mice, which is completely consistent with the previous researches.^{23–26}

The onset and progression of NAFLD are closely associated with the insufficient (or excessive) intake of specific nutrients, such as marine n-3 fatty acids, soybean oil-derived n-6 fatty acids, sugars, folate and vitamin B12, and certain dietary patterns that composed of highly processed foods, sweets, and sugar-sweetened beverages as well as high-fat diets.^{27–30} However, there were few researches to discuss the associations between NAFLD and the dietary supplementation of mineral substance. Calcium, as the fifth most abundant element in

human body with more than 99% residing in the skeleton, plays a central role in a wide range of essential functions including bone formation, muscle contraction, neurotransmitter release and lipid metabolism.³¹ It has been demonstrated that many functions of the liver could be regulated by the homeostasis between the intracellular and extracellular Ca^{2+} , which can be affected by inappropriate calcium intake through the modulation of regulatory enzyme activity.^{32,33} During the pregnancy and lactation, maternal calcium metabolism should adapt to the demand created by the fetus and placenta, which could be drawn together with other minerals to mineralize the development of fetal skeleton.³⁴ So it is a priority for mothers-to-be to obtain a balanced profile of calcium to ensure optimal fetal growth and development and productivity later in the life.³⁵ Our previous epidemiological and animal experiments have confirmed that maternal calcium intake could promote the growth and development of the infants, while imbalance in maternal calcium intake would aggravate the high-fat induced obesity of the adult male mouse offspring through modulating on the gut microbiota and lipid metabolism.²⁰ In addition, our animal study found that maternal insufficient or excessive calcium status during pregnancy and lactation programmed an abnormal expression of hepatic and adipose genes in the offspring, leading to dyslipidemia and hepatic lipid accumulation with the normal diets.³⁶ In the current study, maternal inappropriate calcium supplementation has long-term effects on the progress of high-fat induced NAFLD among the mouse offspring by altering the intestinal microbiota and lipid metabolism. Specifically, maternal normal and low calcium intake could respectively have the greatest inhibition on the progress of NAFLD in the male and female mouse offspring, which was characterized by the least lipid droplets, inflammatory infiltration and fibrosis, the lowest concentrations of free fatty acids and triglycerides, the lowest expression of genes involving in de novo lipogenesis and the highest expression of genes related to lipolysis. Pyrosequencing of 16S rRNA genes also revealed that the male mouse offspring in the NC-HFD group and the female mouse offspring in the LC-HFD group had distinct intestinal microbiota, which were closer to those in the NC-C group.

Analysis of the functional features for the different microbiota was compatible with the gene expressions in *de novo* lipogenesis, lipid oxidation and hydrolysis.

To be noteworthy, our study indicated a significant sex-specific differences for the maternal calcium demand to inhibit the progress of NAFLD between the male and female mice offspring, showing that less calcium might be needed during maternal pregnancy and lactation for beneficial effects in the female than male offspring. Our findings of sex differences in response to maternal calcium supplementation on the progress of NAFLD by HFD induction have similarities to those seen in the previous clinical investigations, which all demonstrate different maternal calcium needs to promote health between the male and female offspring. Kate AW et al. found that maternal calcium supplementation affected childhood growth and bone development in a sex-specific manner, resulting in slower growth among females compared to placebo and accelerated growth among males at age 8–12 y in the rural African with daily extremely low intake.¹⁹ In contrast, the results from longitudinal follow-up trial were shown that pre-pubertal males aged 8–12 y who received calcium supplements for a year and who were followed to the end of growth have gone into their pubertal growth spurt earlier and so reached peak velocities for height and bone development at an earlier age than those who were in the placebo group.^{37,38} However, there is no clear and definite evidence that pregnant women with different genders of babies should be given different amount of calcium supplementation during the pregnancy and lactation. In this study, male and female offspring mice were conducted to discuss the effects of maternal calcium supplementation during pregnancy and lactation on offspring adult health, compensating for the short-term and uncontrollable for epidemiological study.

All these results proved the hypothesis that long-term effects of maternal calcium status on the progress of NAFLD could be regulated by altering the intestinal microbiota and lipid metabolism with attention to potential sex differences among the mouse offspring. As we all know, sex, an important biological variable, can markedly impact diverse physiological and pathological processes, as well as response to treatment in various diseases including heart disease,

hypertension, obesity, and etc.³⁹. Recently, more attentions have been paid to discuss the sex differences in many situations which demonstrate male and female infants respond differentially to same environmental stimuli, with different growth and neurodevelopmental trajectories.^{40–43} Male infants are also more likely to be disadvantaged when subjected to adversity. It has been demonstrated that animal milk composition differs depending on the offspring sex, suggesting that the tailoring of early life nutrition may be one mechanism to maximize health protection and development to infants of both sexes. Thus, the early life nutritional care may be different and optimism to minimize the “male disadvantage”.⁴³ It is active calcium transport from the mother placenta to fetus during pregnancy that meets fetal more requirements for skeletal formation and mineralization through epithelial calcium channels by regulating many placental calcium binding proteins and plasma membrane calcium ATPase.^{44,45} Both “male disadvantage” and higher bone mineral density imply that male mice need more calcium during the early life and can not adapt to low-calcium environment for their adult health. Our findings are modest, yet significant, and need to be proved by the birth cohort study in the future.

5. Conclusions

Insufficient or excessive intake of calcium during maternal pregnancy and lactation adversely affected the expression of genes associated with lipid hydrolysis, oxidation, synthesis and lipogenesis, and gut microbiota, resulting in more severity of NAFLD, and the effects of maternal calcium were sex specific for the offspring mice. Less calcium might be needed during maternal pregnancy and lactation for the beneficial effects on high-fat diet associated NAFLD in the female than the male offspring.

Acknowledgments

The authors would like to thank KY and DZ for animal feeding and Shanghai Majorbio Bio-pharm Technology Co., Ltd for microbial community analysis service.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by National Natural Science Foundation of China (#81602859 to PL) and Research Funds of Profession Quota Budget from Beijing Municipal Science and Technology Commission (#2019 and 2020-bjsekyjs to KQ).

Author contributors

PL and KQ participated in the study design, acquisition, analysis and interpretation of the data. XLC and XYC performed the mRNA extraction from the hepatic tissue. RW and TT did the whole RT-PCR experiments. KY and DZ carried out the animal feeding, XF performed the bioinformatics of the microbial community analysis. PL and KQ drafted the manuscript, which all authors had commented on.

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