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The Impact of Apple Pectin on Gut Microbiota and Bile Acid Homeostasis in mice treated with an alternating Low-Fat/High-Fat Diet

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ABSTRACT: Dietary components play a critical role in modulating gut microbiota composition. Pectin has a positive regulatory effect on the disorder of gut microbiota-metabolites caused by a high-fat diet. A cyclic low-fat/high-fat diet (CLHFD), the alternating cycle between short-term low-fat and high-fat diets (HFD), capitalizes on the rapid adaptability of gut microbiota to short-term HFD interventions. Building on this plasticity, the effect of CLHFD on the composition of the gut microbiota and bile acids (BAs) with and without apple pectin (AP) treatment have attracted our attention. The experimental results showed that CLHFD has the higher *Acetatifactor* abundance than low-fat diet LFD, while AP selectively enriched *Duncaniella* and *Bacteroides* under CLHFD. Notably, AP upregulated hepatic CYP7A1 expression and mitigated CLHFD-induced BA metabolic abnormalities. Our results position AP as a microbiota-modulating supplement to buffer the metabolic stress of dietary shifts, with potential applications in personalized nutrition programs.

Key word: apple pectin; a cyclic low-fat/high-fat diet ; gut microbiota; bile acids

1. Introduction

The gut microbiota homeostasis is of significant importance as a 'microbial organ' within the human body. Its relationship with the host health is remarkably intricate, exerting a profound impact on the development and function of the immune system^[1]. Emerging evidence indicates that dietary components have the ability to directly or indirectly regulate the structure of the microbiota^[2]. The gut-liver axis is an important pathway connecting the host's gut microbiota and liver metabolic signals, and its balanced state is crucial for maintaining the health of the body^[3]. High-fat diet (HFD) is one of the key factors leading to the imbalance of the gut-liver axis^[4]. It can disrupt the stability of the gut microbiota and cause disorders in bile acid (BA) metabolism, resulting in liver inflammation and other health problems^[5]. Critically, specific gut

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bacteria (e.g., *Clostridium*, *Enterococcus*) secrete BAs -metabolizing enzymes, such as bile saline hydrolase (BSH) and 7 α -dehydroxylases [6]. These enzymes catalyze reactions (including dehydroxylation, dehydrogenation, desulfurization, etc.) that convert primary BAs into secondary BAs. This process transforms liver-synthesized BAs into metabolites with distinct structures, thereby affecting the composition of the BA pool. Substantial experimental data confirm that chronic HFD induces dysbiosis of gut microbiota and BA homeostasis, leading to endotoxemia and systemic inflammation [7]. While HFD is an established driver of obesity and metabolic syndrome, we hypothesize that a cyclic low-fat/high-fat (CLHFD) dietary regimen, the alternating cycle between short-term low-fat and high-fat diets, may leverage microbial plasticity to stabilize BA pool dynamics and subsequently improve fat digestion [8].

Dietary fibre is a pivotal substrate for colony metabolism due to its indigestible nature, while pectin, a class of polysaccharide fibre with a complex structure, has garnered significant attention due to its distinctive immunomodulatory capabilities[9]. Pectin, a heteropolysaccharide that is widely found in plant cell walls, has traditionally been used primarily as a food additive. Recent studies have demonstrated that pectin, as a prebiotic, its structural specificity (e.g., Degree of Methylation (DM) and compartmentalised distribution pattern) directly determines its fermentation behaviour and physiological effects in the metabolic syndrome[10]. Moreover, pectin exerted a substantial influence on the differentiation and function of intestinal T cells by modulating the composition of gut microbiota and its metabolites (e.g., short-chain fatty acids) [11]. Importantly, pectin enriches specific bacterial genera (e.g., *Mycobacterium_avium*, *Akkermansia*), which may participate in the remodelling of the BA pool via metabolic pathways such as 7 α -dehydroxylase [12]. In pigs fed with 5% apple pectin (AP), cecal ursodeoxycholic acid and hyocholic acid showed a decreasing tendency [13].

This study systematically explored the effects of CLHFD on liver lipid metabolism and gut microbiota in mice, and further explored the mechanism by which AP supplement in the CLHFD model improves liver lipid deposition by regulating the gut microbiota-BA metabolism axis. The establishment of a time-series analysis model revealed the spatiotemporal regulation between pectin-mediated microbiota remodelling and BA metabolism reprogramming. This provides a theoretical foundation for the development of precise nutritional intervention strategies based on dietary fibre. The findings of this study will enhance our understanding of the early response mechanisms underlying dietary component-microbiota interactions, offering novel insights for stage-specific prevention and control of metabolic diseases.

2. Material and methods

2.1 Material

As shown in Table 1, AP was commercial pectin, composed of the following monosaccharides, with a molecular weight of 154.6 ± 0.37 kDa.

Table 1. The composition and molecular weight of apple pectin

mol%	Fuc	Rha	Ara	Gal	Glc	Man	Xyl	GalA	GlcA	Mw/ kDa	Mw/Mn
AP	0.11 ± 0.01	1.56 ± 0.01	2.63 ± 0.10	14.92 ± 0.01	6.29 ± 0.53	0.51 ± 0.04	4.56 ± 0.04	69.42 ± 0.09	n.d.	154.6 ± 0.37	3.264 ± 0.79

2.2 Mice

Mice (male, C57BL/6J strain, aged 5–6 weeks) used in this study were obtained from Charles River Laboratories (Beijing, China). They were maintained in specific pathogen-free (SPF) facilities at the South China Agricultural University under controlled environmental conditions: a 12-hour light/dark cycle, 65% relative humidity, and a temperature of (25 ± 2) °C. All animal experiments were conducted in strict compliance with the guidelines established by the China Animal Center and were approved by the South China Agricultural University Animal Ethics Committee (Guangzhou, China; approval no. 2021b171). Mice were provided with an AIN-93G standard diet (supplied by Changzhou SYSE Biotechnology Co., Ltd.). Furthermore, 3% (w/w) AP was incorporated into both low-fat (10% of energy from fat, D12450B) and high-fat (60% of energy from fat, D12492) diets.

2.3. Dietary intervention and experimental procedure

After a one-week acclimatization period on a low-fat diet, mice were randomly assigned to four groups ($n = 8$): the low-fat diet group (LF), the high-fat diet group (HF), the cyclic low-&high-fat diet group (LHF), and the cyclic low-&high-fat diet with 3% (w/w) AP supplementation group (LHAP). Notably, the addition of 3% (w/w) AP in the LHAP group diet provided an equivalent amount of cellulose (3%, w/w) as that present in the LF, HF, and LHF group diets. Both the LHF and LHAP groups underwent dietary alternation every 3 days between low-fat and high-fat diets within 6-day cycles, with this pattern repeated for 5 consecutive cycles. Throughout the experiment, body weight was measured at regular intervals, and fecal samples were collected periodically for gut microbiota and BA analysis. At the conclusion of the experiment, mice were euthanized under carbon dioxide anesthesia followed by cervical dislocation. Blood was immediately collected via cardiac puncture, and the animals were dissected. During dissection, a portion of the liver was fixed in 10% neutral buffered formalin for subsequent histopathological examination, while the remaining tissue was stored in enzyme-free centrifuge tubes. Additionally, all animal samples were preserved at -80 °C for further analysis.

2.4. Measurement of biochemical parameters

The blood samples were allowed to clot at room temperature for two hours, after which they were subjected to a centrifugation process at $3500 \times g$ for 15 minutes at 4°C using a high-speed cryo-centrifuge. The upper serum layer was then extracted with a pipette and transferred into pre-labelled centrifuge tubes. Serum concentrations of total cholesterol (TC) and triglycerides (TG) were then quantified using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). A quantity of liver and faeces was homogenised with pH 7.4 phosphate-buffered saline (PBS), respectively, and the resultant upper layer was subjected to centrifugation in order to prepare tissue extracts. These extracts were then used to determine the content of lipopolysaccharides (LPS) by means of enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.5. H&E and Oil Red O staining

The liver samples were removed from the fixative and processed for histological staining. Hematoxylin and eosin (H&E) staining was performed to assess general liver morphology. In this method, the basic dye hematoxylin binds to nuclear DNA, staining nuclei blue-violet, while the acidic dye eosin stains cytoplasmic and extracellular matrix components pink. The stained sections clearly revealed the hepatic lobule architecture, including hepatocyte cords, the central vein, and confluent area, with distinct nuclear (blue) and cytoplasmic (pink) staining.

For lipid visualization, Oil Red O (ORO) staining was conducted. Liver sections were first treated with 70% ethanol, then stained with Oil Red O for 5–10 minutes, followed by hematoxylin counterstaining of nuclei. Under microscopy, lipid droplets appeared bright red or orange-red, while nuclei were stained blue, allowing qualitative assessment of hepatic lipid accumulation.

2.6. Real-time qRT-PCR

The total RNA was extracted from liver tissue using TRIzol reagent (Thermo Fisher Scientific, USA) and reverse-transcribed into cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Quantitative real-time PCR (qRT-PCR) analysis was conducted to determine the relative concentration of target mRNAs using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA) on a CFX96TMOptics Module thermal cycler system (Bio-Rad, Singapore). The amplification protocol consisted of an initial incubation at 50°C for 2 minutes, followed by denaturation at 95°C for 2 minutes. Subsequently, 40 cycles of amplification were performed under the following conditions: 95°C for 15 seconds and 60°C for 1 minute. Gene expression levels were quantified relative to β -actin mRNA levels using the comparative Ct method ($2^{-\Delta\Delta\text{Ct}}$). Notably, mRNA normalization was performed independently for short-term

and long-term experiments. Initially, the CT values of target genes were normalized to the CT values of β -actin across all tested samples. Thereafter, in both experimental durations, the Δ CT values of experimental groups were normalized to the mean Δ CT value of the control group. Primers were synthesized by Shanghai Kinko Bio, and their specificity and amplification efficiency were validated via PCR amplification and melting curve analysis. The primer sequences specific to the target genes are summarized in Table 2.

Table 2. List of primers used in qRT-PCR analysis

Genes	Primer sequence (5' to 3')
SREBP-1c	Forward: ATCTCCTAGAGCGAGCGTTG
	Reverse: TATTTAGCAACTGCAGATATCCAAG
PPAR γ	Forward: CAGTGGGAGAGAGGACAGA
	Reverse: AACCATTGGGTCAGCTCTTG
FAS	Forward: CCCTTGATGAAGAGGGATCA
	Reverse: GAACAAGGCGTTAGGGTTGA
SCD1	Forward: GCTGGGCAGGAAGTAGTGAG
	Reverse: GAAGGCATGGAAGGTTCAAA
PGC-1 α	Forward: ATGTGTCGCCTTCTTGCTCT
	Reverse: ATCTACTGCCTGGGGACCTT
CYP7A1	Forward: AGCAACTAAACAACCTGCCAGTACTA
	Reverse: GTCCGGATATTCAAGGATGCA
β -actin	Forward: TCATCACTATTGGCAACGAGCG
	Reverse: CGGATGTCAACGTCACACTTCA

2.7. 16S rRNA gene sequencing and data analysis

As stipulated by the manufacturer's guidelines, total bacterial DNA was extracted from the faecal samples using the MagPure Fecal DNA Kit (MAGEN, Guangzhou, China). The PCR reaction system was configured with 30 ng of genomic DNA samples of satisfactory quality and the corresponding fusion primers. The PCR amplification was then carried out by setting up the relevant PCR parameters. Subsequent to this, the PCR products were purified to complete the library construction. Subsequently, the primers 338F (5'-ACTCCTACGGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGTWTCTAAT-3') were utilised to amplify the V3 region of the 16S rRNA gene. The range and concentration of the libraries were examined using an Agilent 2100 Bioanalyzer. The qualified libraries were then subjected to sequencing on UW's proprietary DNBSEQ platform. The downstream data underwent a filtration process to remove low-quality reads, leaving only the high-quality clean data available for subsequent analysis. The reads were then spliced into tags through the utilisation of the overlap relationship between the reads. Following this, the tags were clustered into operational taxonomic units (OTUs) based on a specified similarity level. Principal Component Analysis (PCA) assessed inter-group variations in microbial community structure. Statistical significance of taxonomic composition and structural differences was evaluated using Student's t-test and LEfSe (LDA Effect Size) analysis.

2.8. BAs analysis

In this study, liquid chromatography-mass spectrometry (LC-MS) was employed to determine the composition of BAs in fecal samples. The standards for BAs included cholic acid (CA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), α -methylcholic acid (α MCA), β -methylcholic acid (β MCA), and taurine sodium salt hydrate (TCA). Additional relevant acids were taurine chenodeoxycholic acid sodium salt (TCDCA), taurine deoxycholic acid (TUDCA), deoxycholic acid (DCA), lithocholic acid (LCA), murine deoxycholic acid (MDCA), and hypodeoxycholic acid (HDCA). Taurine, chenodeoxycholic acid-d4 sodium salt (TDCA-d4), chenodeoxycholic acid-d4 (CDCA-d4), and lithocholic acid-d4 (LCA-d4) were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). These compounds were dissolved in HPLC-grade methanol. The same sample extraction and detection method were used as described previously^[14]. Briefly, a series of daily fecal samples were homogenized in ice-cold methanol containing internal standards (0.1 μ M TDCA-d4, CDCA-d4, and LCA-d4). The samples were subsequently centrifuged at $18,000 \times g$ for 15 minutes at 4°C. The supernatant was then diluted with 0.22 μ M TDCA-d4 (containing 0.1 μ M TDCA-d4, CDCA-d4, and LCA-d4) for 3 minutes. The resulting solution was filtered through a 0.22 μ m filter, transferred to an amber vial, and stored for subsequent analysis by ultra-performance liquid chromatography-triple quadrupole tandem mass spectrometry (UPLC-QQQ-MS/MS).

2.9. Statistical analysis

The data are presented as mean $\pm$ SEM to reflect sample variability. Statistical analyses were conducted using Prism 8.0 (GraphPad Software Inc., USA). Group differences were evaluated via one-way ANOVA, with statistical significance defined as $P < 0.05$. In cases where ANOVA results were significant, post-hoc tests were employed to determine specific differences among groups. For a comprehensive interpretation, P-values should be considered in conjunction with effect sizes and confidence intervals.

3. Results

3.1. AP had an impact on liver pathology of CLHFD mice

HFD have been extensively studied and are known to cause various metabolic syndromes such as obesity and non-alcoholic fatty liver disease^[15]. Body weight and liver pathology are important indicators of the pathological damage caused by a HFD to the body. As shown in Figure 1B, after 30 days of dietary intervention, mice in the HF group exhibited significantly greater weight gain compared to the LF group. The LHF group showed intermediate weight gain, higher than LF but lower than HF, while there was no difference between the LHF group and the LHAP group. The liver index in the HF group did not differ from the LF and LHF groups, but the LHAP group was lower than the LF group and the LHF group (Figure 1C). This finding was also observed in the liver pathological sections (Figure 1D). H&E staining showed scattered fat vacuoles

(mild steatosis) in a few lobules in the HF group, which was not the case in the LHF group. Oil Red O staining revealed a significant increase in both the number and size of lipid droplets in the HF group compared to the LF group, indicating mild hepatic steatosis [16]. This steatotic phenotype was less pronounced in the LHF group than in the HF group, while the LHAP group exhibited lipid droplet profiles comparable to those of the LF controls. Analogously, three homogalacturonan-type pectins could reverse the trend of hepatocyte swelling and steatosis in the liver of the model group after intervention, among which AP had the best effect [17].

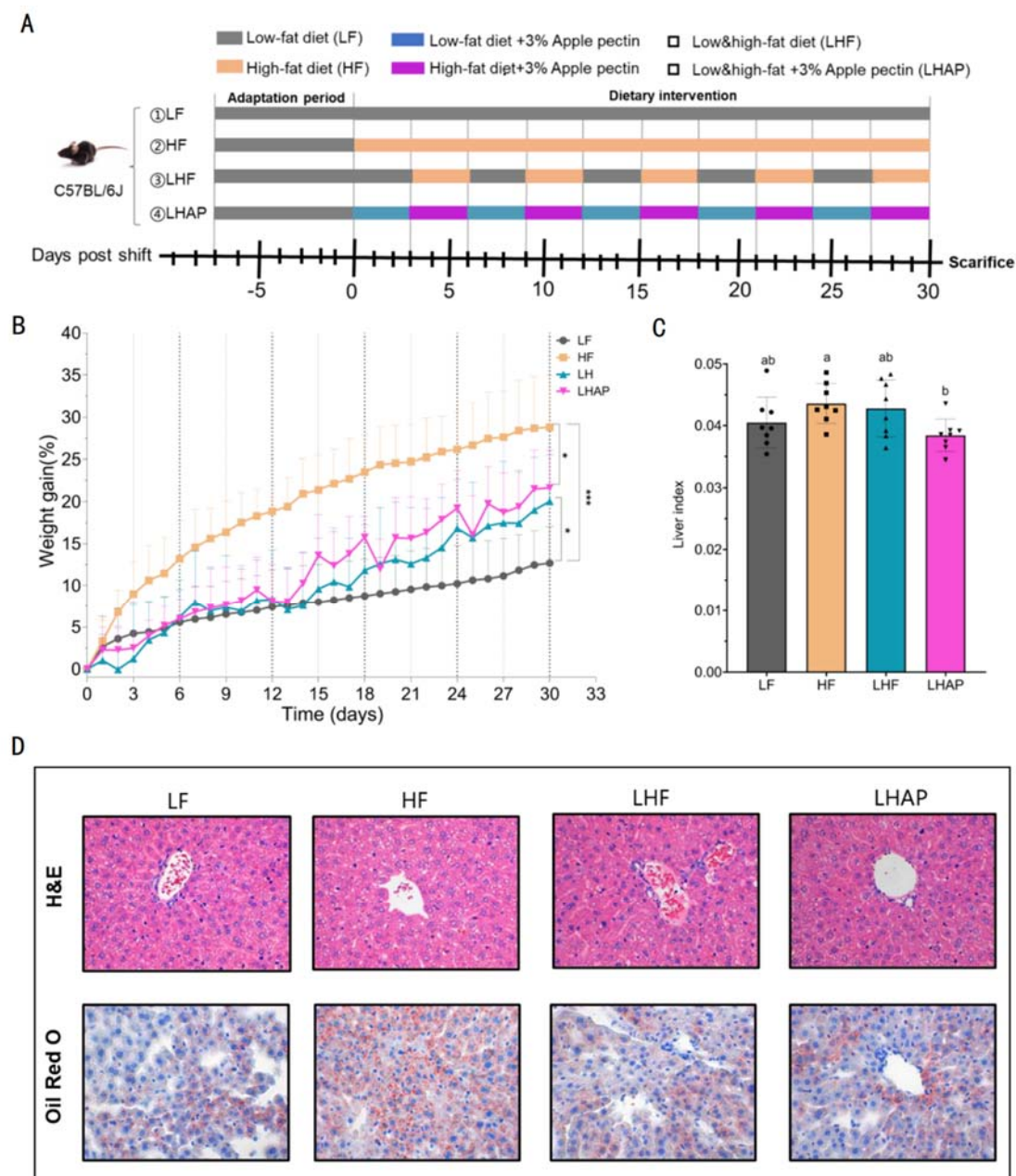


Figure 1. AP had an impact on liver pathology of CLHFD mice. Animal experiment design (A), daily body weight gain (%) (B), liver index (defined as the ratio of liver weight to mouse body weight) (C), histopathological results from H&E staining and Oil Red O staining (Magnification of 400x) of the mouse livers (D). Significant differences in the scatter plot were denoted as different letters (a and b) among the groups ($P < 0.05$). LF: Low-fat diet group, HF: High-fat diet group, LHF: the cyclic low-&high-fat diet group, LHAP: the cyclic low-&high-fat diet with 3% (w/w) apple pectin supplementation group.

3.2. AP modulated liver lipid metabolism and alter fecal and serum levels of LPS in CLHFD mice

Long-term HFD causes abnormalities in hepatic lipid metabolism, including the accumulation of TG and TC in the liver [18]. As shown in Figure 2A, the hepatic TG level in the HF and LHF groups were higher than that in the LF group. The TG level of mice in the LHAP group was lower than that in the LHF group and tended to be at the level of LF group. The TC level of mice in the HF group was higher than that of mice in the other three groups, and the LHF group was higher than the LF group (Figure 2B). The TG level of mice in the LHAP group was lower than that of mice in the LHF group. The results showed that compared with CLHFD, AP could enhance the cholesterol metabolism capacity.

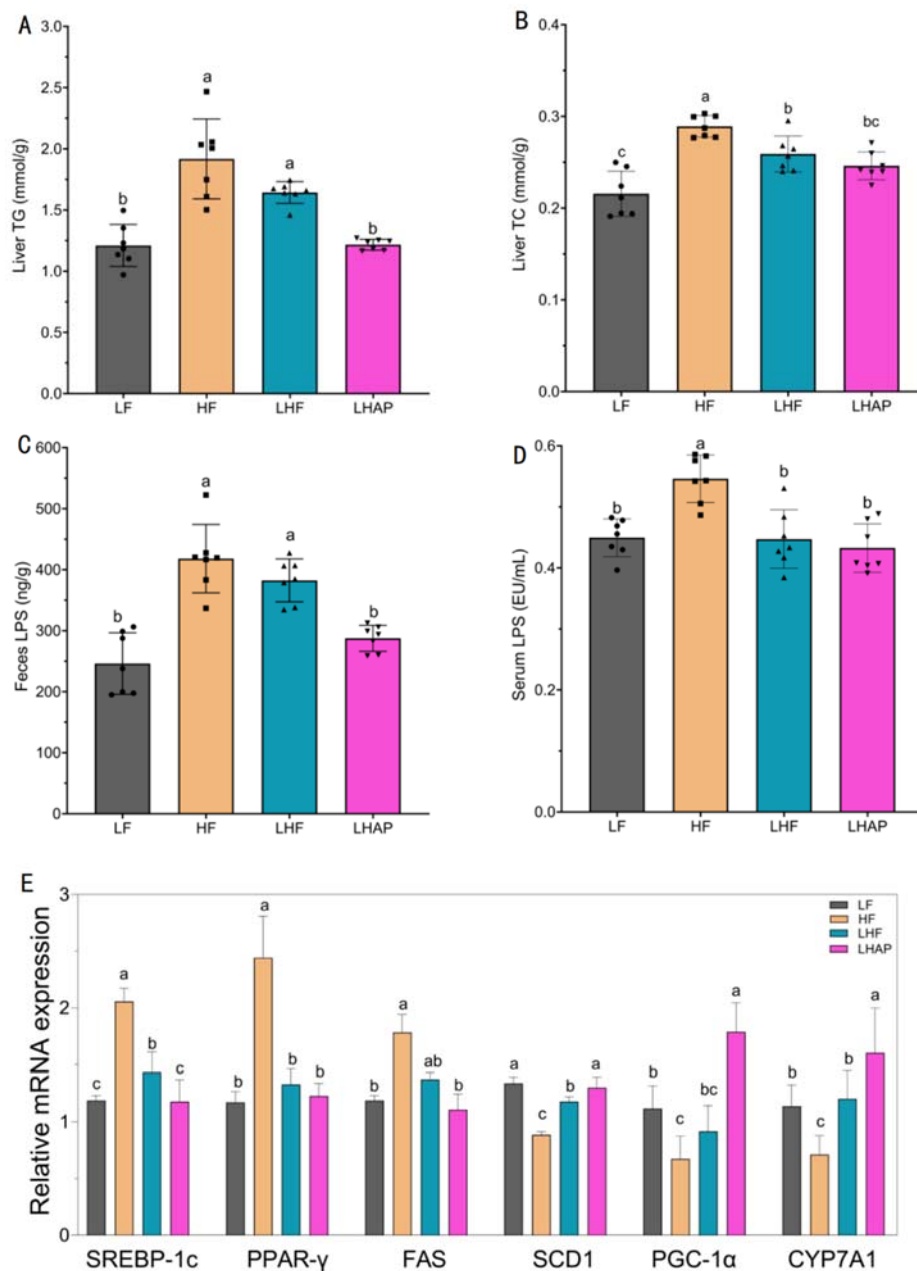


Figure 2. AP modulated liver lipid metabolism and alter fecal and serum levels of LPS in CLHFD mice. Effects of AP combined with cyclic LHF on liver lipid content (total cholesterol(A) and total triglycerides(B)), gut microbiota-associated inflammatory factors (mouse fecal lipopolysaccharides(C) and serum lipopolysaccharides(D)), and the relative expression of genes(E) related to oxidative stress, lipid metabolism, and BA metabolism. Significant differences in the scatter plot were denoted as different letters (a, b and c) among the groups ($P < 0.05$)

HFD are often accompanied by abnormally elevated levels of the inflammatory factor LPS (which is a component of the outer wall of the cell wall of Gram-negative bacteria and consists mainly of lipids and polysaccharides), which activates the immune cells and produces inflammatory cytokines that can cause damage to the organism^[19]. Interestingly, although the duration of dietary intervention in this study was only 30 days, it was found that faecal LPS levels were higher in the HF and LHF groups than in the LF group, while the fecal LPS level in the LHAP group was lower than that in the LHF group and was close to the LF group. Significantly, serum LPS levels were higher in the HF group than in the LF group, while the LHAP and LHF groups did not differ from the LF group (Figure 2C-D). Similarly, apple-derived pectin significantly decreased HFD-induced LPS appearance in serum^[20].

3.3. AP regulated liver-related functional gene expression in CLHFD mice

Sterol regulatory element binding protein 1c (SREBP-1c) is a major transcription factor regulating fatty acid synthesis and can directly bind to the promoter region of the fatty acid synthase (FAS) gene to initiate its transcription^[21]. FAS is a key enzyme in fatty acid synthesis, and enhanced expression of which directly leads to an increase in fatty acid synthesis in the liver. As shown in the Figure 2E, the HF group could increase the gene expression of SREBP-1c and FAS, while the expression of SREBP-1c in the LHF group was lower than that in the HF group, and higher than that in the LF group and the LHAP group. The gene expression of FAS in the LHF group was not different from that in the LF group and the HF group, but higher than that in the LHAP group. Peroxisome proliferator-activated γ receptor (PPAR γ) is a key transcriptional regulator of adipocyte differentiation and lipid metabolism. When ligand (fatty acid/lipid derivatives from high-fat diet) binds to PPAR γ , the conformation of PPAR γ is changed, and its ability to bind to peroxisome proliferator-responsive elements (PPREs) on DNA is strengthened, which activates the transcription of its target genes^[22]. The expression of PPAR γ was higher in the HF group than in the LF group, and there was no difference between the LHF and LHAP groups and the LF group. Stearoyl coenzyme A desaturase 1 (SCD1) is a central regulator of fuel metabolism and controls adipogenesis^[23]. SCD1 gene expression was lower in the HF group than in the LF group. The LHAP group increased the reduction of SCD1 gene transcription level in the LHF group. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) plays an important role in metabolic reprogramming in response to dietary availability by coordinating the expression of multiple genes involved in glucose and fatty acid metabolism^[24]. The HF group had a lower gene expression of PGC-1 α than the LF group. The LHF group had no difference in gene expression of PGC-1 α from the LF and HF groups. The relative gene expression levels of SREBP-1c, PPAR γ , FAS and SCD1 in the LHAP group were the same

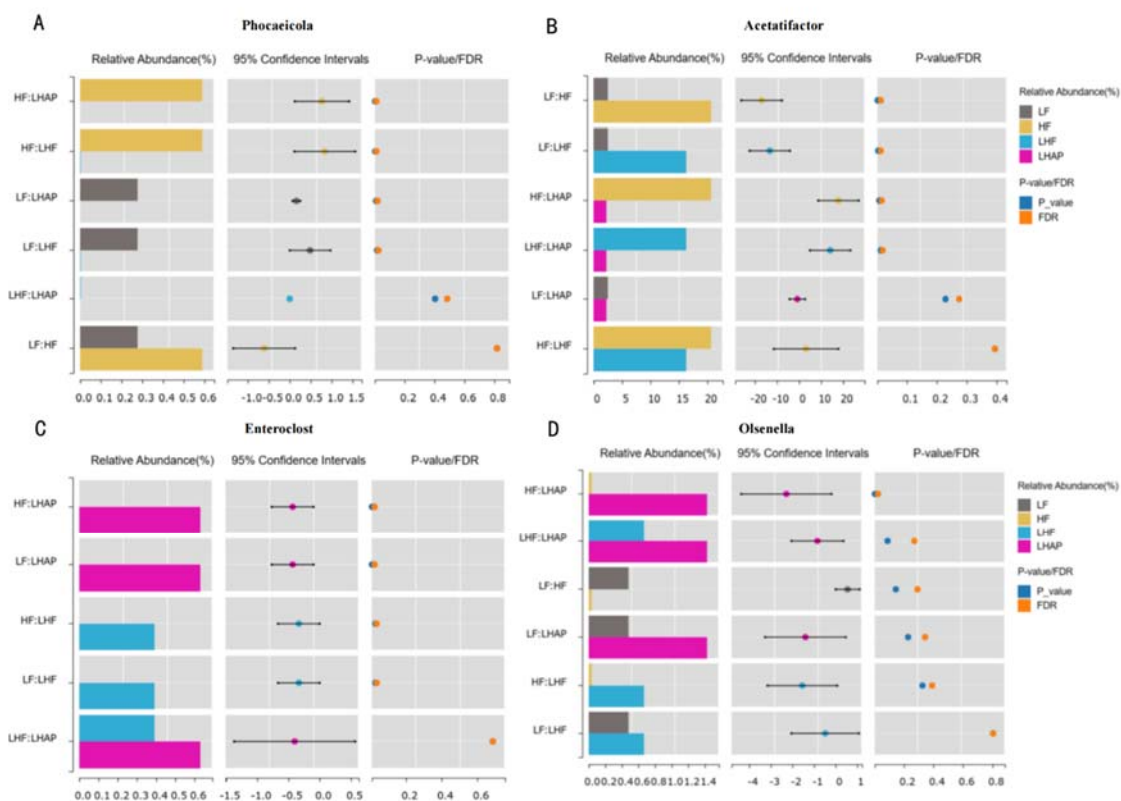
as those in the LF group, while the relative gene expression level of PGC-1 α in the LHAP group was higher than that in the LHF group.

Cholesterol 7 α -hydroxylase (CYP7A1) is the rate-limiting enzyme in the classical pathway of BA synthesis, which is mainly regulated by hepatic cholesterol content, BA feedback regulation, and hormone (e.g., insulin, glucagon) [25]. The gene expression of CYP7A1 in the HF group was lower than that in the LF group and LHF group, and that in the LHAP group was higher than that in the LHF group (Figure 2E). Inflammatory factors produced during a HFD process, such as LPS, can suppress the expression of CYP7A1. The downregulation of CYP7A1 results in decreased conversion of cholesterol into BAs, thereby causing cholesterol accumulation and upregulating the expression of SREBP-1c, FAS, and SCD1. This subsequently enhances the synthesis of fatty acids and TG [26]. Furthermore, the reduction in BA levels weakens intestinal Farnesoid X receptor (FXR) signaling, which impairs the secretion of intestinal hormones, such as fibroblast growth factor 19 (FGF19) [27]. This cascade further diminishes hepatic fatty acid oxidation capacity, ultimately leading to TG accumulation. AP reduced the concentration of fecal LPS in CLHFD mice, upregulated the gene expression of CYP7A1, and prevented the accumulation of TG in the liver.

3.4. AP regulated gut microbiota disorder in CLHFD mice

To investigate the species composition of each sample, OTUs were clustered and species annotated for all samples and plotted as a Wayne diagram, and the results were shown in Figure S1A, which showed that there were 93 OTUs common to the LF, HF, LHF and LHAP groups, 94 OTUs specific to the HF group, 138 OTUs specific to the LHF group, and 60 OTUs specific to the LHAP group. In order to visualise the differences in microbial community structure between the samples, PCA analysis was performed to reflect the differences between the samples (Figure S1B). The results showed that the LF and HF groups can be separated better, and the LHF, LHAP and LF groups are also separated, indicating that not only HF can significantly change the gut microbiota, but also CLHFD and AP have an effect on the gut microbiota. The LEfSe analysis (LDA Score > 3.5, $P < 0.05$) in Figure S1C successfully identified four groups (LF, HF, LHF, LHAP groups) with significantly different biomarkers of gut microbiota at the genus level. The results showed that with LDA Score > 3.5, the characteristic enriched genera in LF group were mainly *g_Sporofaciens* (LDA = 4.62), *g_Faecalibaculum* (LDA = 4.57), *g_Christensenella* (LDA = 3.51), *g_Prevotellamassilia* (LDA=3.51). And the HF group selectively enriched *g_Lawsonibacter* (LDA = 4.51), *g_Lactobacillus* (LDA = 4.17), *g_Limosilactobacillus* (LDA = 3.58), and *g_Paludihabitans* (LDA = 3.55). Moreover, *g_Adlercreutzia* (LDA = 3.67) was the most significant marker in the LHF group, while the LHAP group was characterised by the enrichment of *g_Duncaniella* (LDA = 4.95) and *g_Olsenella* (LDA = 4.11).

A Wilcoxon rank-sum test was conducted on the marker bacteria based on the genus-level relative abundance distribution across the four groups (Figure S1D). The results indicated that CLHFD significantly decreased the relative abundance of *Phocaeicola* (Figure 3A), with the LF group exhibiting significantly higher levels compared to the LHF group. *Phocaeicola* is positively correlated with unconjugated BAs^[28]. CLHFD effectively increased the relative abundance of *Acetatifactor*, with the LHF group exhibiting significantly lower levels compared to the LF group (Figure 3B). Notably, AP supplementation reduced the elevated relative abundance of *Acetatifactor* in CLHFD. *Acetatifactor* is a genus of bacteria related to the experiments on NAFLD induced by HFD and closely related to BAs metabolism disorders, which positively correlated with the weakened ability of fatty acid metabolism^[29, 30]. Additionally, CLHFD significantly increased the relative abundance of *Enterocloster* (Figure 3C), which was more abundant in the LHF group compared to the LF group. Furthermore, the relative abundance of *Duncaniella* in the LHAP group was significantly higher than in the LHF group (Figure 3E). *Duncaniella* may improve BAs turnover and lipid metabolism^[31]. To visualize the impact of AP on the microbial community, box plots were used to represent the relative abundances of *Bacteroides* (Figure 3F) and *Helicobacter* (Figure 3G). These visualizations confirmed that AP significantly enhanced the abundance of both *Bacteroides* and *Helicobacter* in CLHFD mice. *Bacteroides*, a dominant commensal genus residing in mammalian intestinal ecosystems, can produce BSH to promote the metabolism of BAs and has particular therapeutic potential in the treatment of intestinal diseases and systemic metabolic disorders^[32, 33].



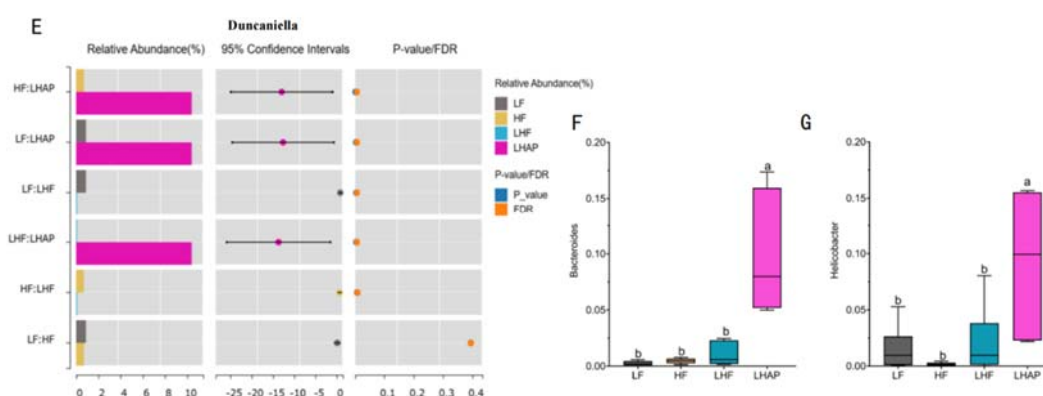


Figure 3. AP regulated gut microbiota disorder in CLHFD mice. Differential abundance analysis included Wilcoxon rank-sum test results for species differences between the two groups: *Phocaeicola* (A), *Acetatifactor* (B), *Enterocloster* (C), *Olsenella* (D) and *Dunaliella* (E), as well as boxplots for *Bacteroides* (F) and *Helicobacter* (G). For the Wilcoxon rank-sum test, histograms represent the mean relative abundance of species within each group, log2-transformed ratios indicated the fold change in mean relative abundances between groups, and p-values and false discovery rate (FDR) values were provided. A species was considered significantly different if both the p-value and FDR are below 0.05 ($P < 0.05$). Significant differences in the scatter plot were denoted as different letters (a and b) among the groups ($P < 0.05$).

3.5. AP influenced the fluctuations of gut microbiota in CLHFD mice during the dietary intervention process

To satisfy our curiosity about the time-dependent dynamic changes of the gut microbiota during the transition from low-fat to high-fat diets and the regulatory role played by AP, we further investigated the dynamic changes in gut microbiota composition within the LHF and LHAP groups during the last cycle (days 25-30). Specifically, days 25-27 corresponded to the low-fat diet phase, while days 28-30 represented the high-fat diet phase. Figures 4A and 4B illustrated the temporal dynamics of genus-level relative abundance in the LHF and LHAP groups, respectively. Notably, the relative abundance of *Acetatifactor* (Figure 4C) was significantly greater in the LHF group compared to the LHAP group and remained elevated during the low-fat diet period. The relative abundance of *Ligilactobacillus* (Figure 4D) was higher in the LHF group than in the LHAP group on days 28 and 30, with a significant increase observed on day 28 compared to day 27. In contrast, the relative abundance of *Dunaliella* and *Olsenella* was significantly higher in the LHAP group than in the LHF group, although the abundance of *Dunaliella* decreased on day 28 compared to day 25 in the LHAP group. *Dunaliella* significantly increased in the inulin diet and has potential metabolic regulatory functions [34]. Additionally, the relative abundance of *Bacteroides* was markedly higher in the LHAP group, reaching up to 25%, but exhibited a reduction on day 29 compared to day 27, suggesting a decline in *Bacteroides* abundance under high-fat dietary conditions. Collectively, these findings indicated that gut microbiota composition is distinctly influenced by low-fat and high-fat dietary interventions. *Acetatifactor* was enriched in CLHFD and was related to the metabolism of BAs. [35]. Studies have shown that citrus pectin oligosaccharides can increase the abundance of *Bacteroides*, up-regulate CYP7A1, and regulate BAs metabolism [36]. *Bacteroides* contains BSH, which can convert primary conjugated BAs into free BAs, and then undergo 7 α -dehydroxylation to form secondary BAs [37]. The differences in the abundance of these

microbiota and the variations of the same species under low-fat and high-fat diets suggest that AP may positively regulate the production of BAs in CLHFD.

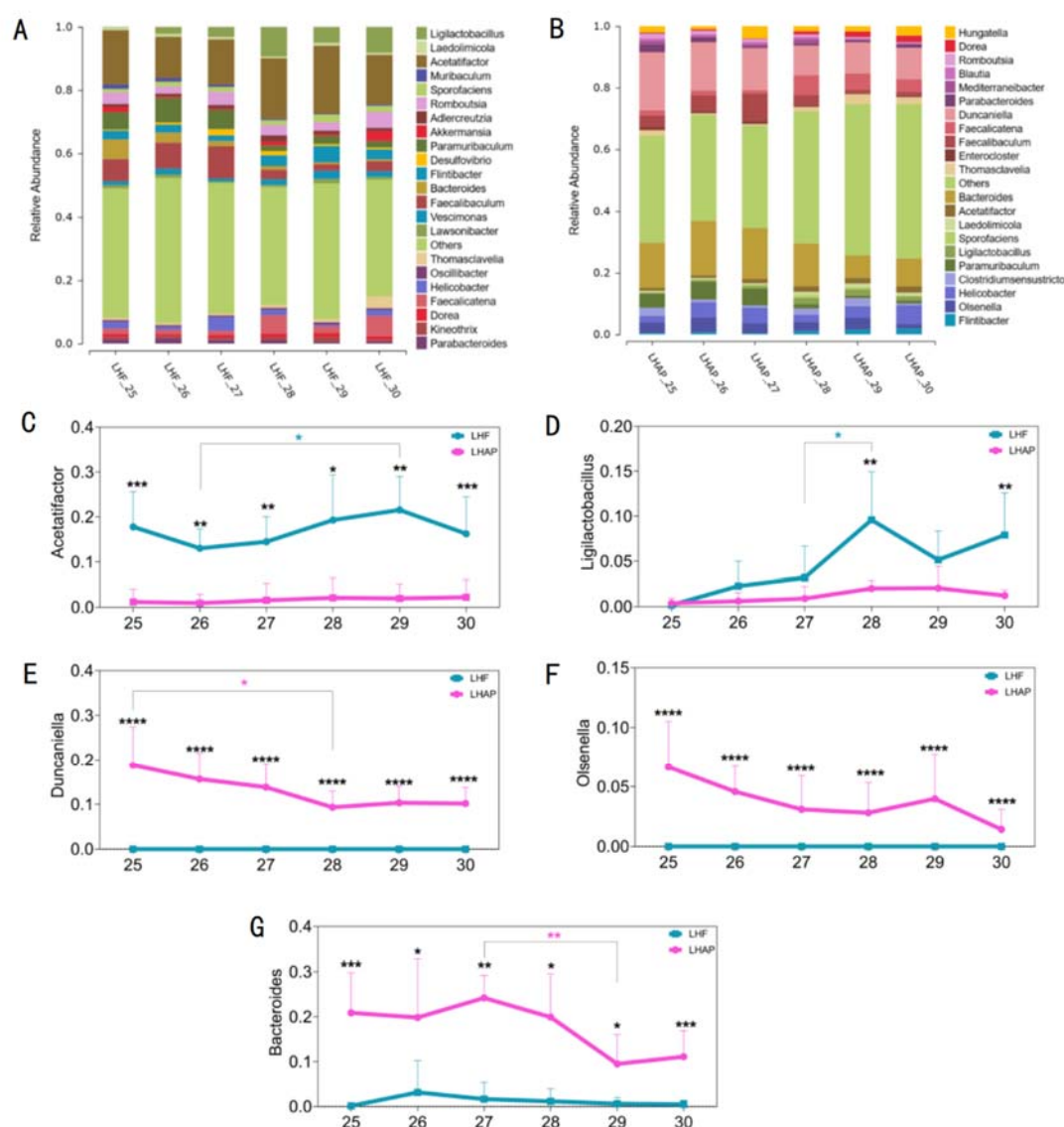


Figure 4. AP influenced the fluctuations of gut microbiota in CLHFD mice during the dietary intervention process. Relative abundance at the genus level of the effects of CLHFD and AP on gut microbiota at cycle 5 (day 25, day 26, day 27, day 28, day 29, day 30) (A-B), as well as the comparison of daily changes in relative abundance of *Acetatifactor* (C), *Ligilactobacillus* (D), *Duncaniella* (E), *Olsenella* (F), and *Bacteroides* (G). Significant differences in the curves were indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

3.6. AP played an optimizing role in BAs metabolism in CLHFD mice during dietary intervention

BAs are synthesized by hepatocytes from cholesterol (primary BAs) and subsequently conjugated with glycine or taurine to form water-soluble BA species. These conjugated BAs are secreted into the intestines via bile to facilitate lipid digestion and absorption. Fecal BAs primarily consist of secondary BAs that are not reabsorbed (the majority), along with minor amounts of unconjugated primary BAs and free BAs [38]. Given the significant roles of CLHFD and AP in regulating hepatic metabolic abnormalities, as evidenced by result 3.5, the present study further investigated fecal BA metabolism in the LHF and LHF+AP groups during the period from day 25 to day 30. Notably, the two groups exhibited distinct differences in BA composition,

irrespective of whether the BAs were primary, conjugated, or secondary. Specifically, the results demonstrated that for primary BAs, the concentration of CA (Fig. 5A) was significantly higher in the LHF group compared to the LHAP group on days 25 and 26. Additionally, the CA levels were elevated in the LHF group on day 29 relative to day 27. Conversely, the CDCA levels (Fig. 5B) were higher in the LHF group than in the LHAP group on day 25 but lower from day 28 to 30. Notably, the CDCA levels in the LHAP group were significantly increased on day 28 compared to day 27. Furthermore, the UDCA content (Fig. 5C) was consistently higher in the LHAP group than in the LHF group from days 26 to 30. The β MCA levels (Fig. 5C) were also elevated in the LHAP group compared to the LHF group on days 25, 27, and 28, with a significant increase observed on day 28 compared to day 27 within the LHAP group. For conjugated BAs, the levels of TCA (Figure 5F) were significantly lower in the LHAP group compared to the LHF group on days 25-30. Additionally, within both groups, TCA levels were higher on day 28 than on day 27. The levels of TCDCA (Figure 5G) were lower in the LHAP group than in the LHF group on days 29 and 30. For TUDCA (Figure 5H), the LHAP group exhibited lower levels than the LHF group specifically on day 25. Furthermore, TDCA levels (Figure 5I) were higher in the LHAP group than in the LHF group on days 25, 26, 28, 29, and 30, while the LHF group showed a decrease in TDCA levels compared to day 27. Lastly, TLCA levels (Figure 5J) were consistently lower in the LHAP group than in the LHF group from day 25 to day 30, with a notable increase in TLCA content observed on day 28 compared to day 27. For secondary BAs, the LHAP group exhibited significantly lower levels of DCA (Figure 5K) compared to the LHF group on days 25-30. Notably, both groups demonstrated elevated DCA levels on day 28 relative to day 27. Additionally, the LHAP group presented with lower levels of LCA (Figure 5M) in comparison to the LHF group on days 25, 28, 29, and 30. Furthermore, both groups showed increased LCA levels on day 28 compared to day 27.

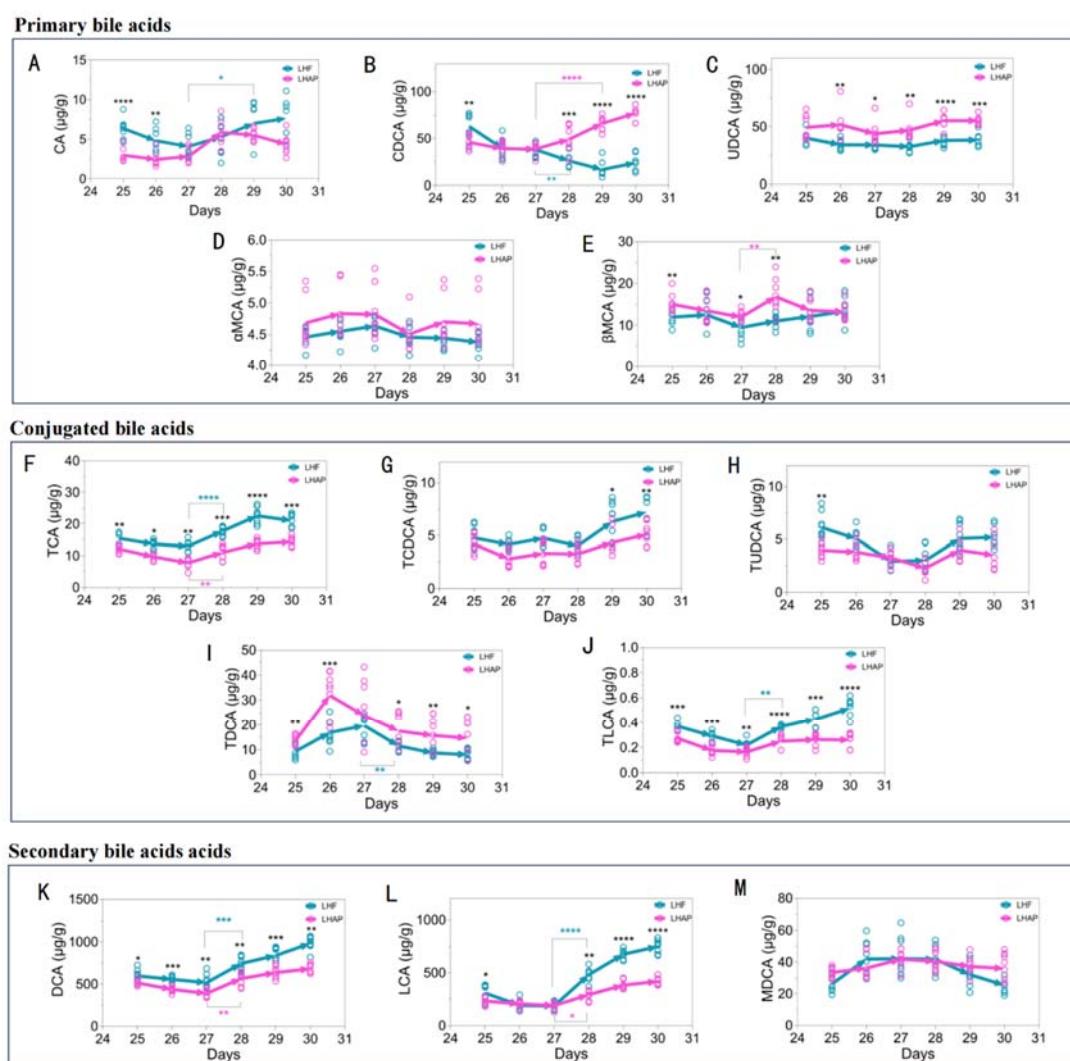


Figure 5. AP played an optimizing role in BAs metabolism in CLHFD mice during dietary intervention. Effects of CLHFD and AP on BAs metabolism at cycle 5 (day 25, day 26, day 27, day 28, day 29, day 30) in primary BAs (cholic acid (A), chenodeoxycholic acid (B), ursodeoxycholic acid (C), α -methylcholic acid (D), β -methylcholic acid (E)), conjugated BAs (taurine sodium salt hydrate (F), taurine chenodeoxycholic acid sodium salt (G), taurine deoxycholic acid (H), taurine deoxycholic acid (I), taurine lithocholic acid (J)), secondary BAs (deoxycholic acid (K), lithocholic acid (L), murine deoxycholic acid (M)) ($P < 0.05$). Significant differences were expressed as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$.

HFD significantly affects the metabolism of BAs. According to the results of this study, in the CLHFD model, after switching from a low-fat diet to a high-fat diet, the concentration of CA in primary BAs increased, while the concentration of CDCA decreased. The concentrations of TCA and TLCA in conjugated BAs increased, while TDCA decreased. This is similar to the situation of primary BAs in hepatitis, possibly due to the inhibition of BA synthesis in the liver by a HFD, such as the down-regulation of CYP7A1 expression, and the enhancement of intestinal reabsorption, thereby reducing the excretion of primary BAs in feces^[39]. The concentrations of secondary BAs (DCA and LCA) increased, which is similar to previous studies. A short-term high-fat diet rapidly affects the alteration of the gut microbiota, increasing the levels of DCA and LCA, thereby exacerbating colitis^[40]. On the other hand, AP could reduce the increase in secondary BAs (DCA and LCA) concentrations in CLHFD mice. High concentrations of DCA are cytotoxic and

pro-inflammatory^[41], whereas LCA directly contributes to intrahepatic cholestasis, causing metabolic disorders in the liver^[42]. In addition, AP might alleviate abnormal BA metabolism induced by CLHFD. For example, after switching to HFD, AP can promote the synthesis of primary BAs (CDCA, UDCA, β MCA), while decreasing the synthesis of bound BAs (TCA, TCDCA, TLCA), and decreasing the synthesis of secondary BAs (DCA and LCA), which could regulate cholesterol metabolism, maintain the homeostasis of BAs, and keep the host healthy. These findings suggested that AP has considerable potential in regulating BA metabolism.

4. Conclusion

In summary, during the CLHFD cycled from a low-fat diet to a high-fat diet and back to a low-fat diet, the gut microbiota demonstrated a "rapid response" to the short-term high-fat diet intervention. Combined with this rapid response, AP showed good bioactivity, which not only alleviated the CLHFD-induced abnormalities of hepatic lipid metabolism, but also regulated the maintenance of gut microbiota homeostasis. We observed the dynamic alterations of gut microbiota and BAs metabolism before and after changes in dietary structure. More importantly, we found that AP exerts a positive influence in BAs metabolism, which provides new insights for subsequent exploration of diet-gut microbiota-host cross-interactions.

Data availability statement

All data relevant to the study are included in the article.

Conflict of Interest

The authors declare that there is no conflict of interest.

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