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RESEARCH ARTICLE

# *Akkermansia muciniphila* alleviates high-fat-diet-related metabolic-associated fatty liver disease by modulating gut microbiota and bile acids

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## Abstract

It has been reported that *Akkermansia muciniphila* improves host metabolism and reduces inflammation; however, its potential effects on bile acid metabolism and metabolic patterns in metabolic-associated fatty liver disease (MAFLD) are unknown. In this study, we have analysed C57BL/6 mice under three feeding conditions: (i) a low-fat diet group (LP), (ii) a high-fat diet group (HP) and (iii) a high-fat diet group supplemented with *A. muciniphila* (HA). The results found that *A. muciniphila* administration relieved weight gain, hepatic steatosis and liver injury induced by the high-fat diet. *A. muciniphila* altered the gut microbiota with a decrease in *Alistipes*, *Lactobacilli*, *Tyzzerella*, *Butyricimonas* and *Blautia*, and an enrichment of *Ruminiclostridium*, *Osclibacter*, *Allobaculum*, *Anaeroplasma* and *Rikenella*. The gut microbiota changes correlated significantly with bile acids. Meanwhile, *A. muciniphila* also improved glucose tolerance, gut barriers and adipokines dysbiosis. *Akkermansia muciniphila* regulated the intestinal FXR-FGF15 axis and reshaped the construction of bile acids, with reduced secondary bile acids in the caecum and liver, including DCA and LCA. These findings provide new insights into the relationships between probiotics, microflora and metabolic disorders, highlighting the potential role of *A. muciniphila* in the management of MAFLD.

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a chronic metabolic liver disease that includes various conditions such as simple steatosis, non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC). The prevalence of NAFLD is increasing globally, and it is now considered one of the primary causes of liver transplantation (Younossi et al., 2018). Recently, the term NAFLD has

been replaced by metabolic-associated fatty liver disease (MAFLD) to better reflect the pathophysiology and risk factors of the disease (Eslam et al., 2020). The development of MAFLD involves various pathogenic factors, including insulin resistance (IR), adipose tissue dysfunction, immune dysfunction and intestinal flora. The gut–liver circulation of blood and lymph carries a constant influx of intestine-derived products (e. g. antigens) into the liver, which plays a key role in triggering hepatic inflammation (Balmer et al., 2014; Barrow et al., 2021). These risk factors may work together to induce the onset and progression of MAFLD (Buzzetti et al., 2016). However, current medication for MAFLD/NASH is still limited, and the primary management of MAFLD remains lifestyle intervention and comorbidity control.

In recent years, the gut microbiota and bile acids have been found to play an important role in the development and progression of metabolic-associated fatty liver disease (MAFLD) and non-alcoholic steatohepatitis (NASH). Dysbiosis of the gut microbiota has been observed in obese patients with MAFLD, with increasing *Lactobacillus*, *Dorea*, *Robinsoniella* and *Roseburia* (Raman et al., 2013). The microbiota can affect the development of high-fat diet-induced MAFLD in mice under the same genetic backgrounds (Le Roy et al., 2013). The gut microbiota metabolizes primary bile acids (pBAs) to produce secondary bile acids (sBAs), and both are reabsorbed through the enteric epithelium. Dysbiosis of bile acids has been associated with MAFLD and increased synthesis of pBAs has been observed in patients with NASH (Xue et al., 2021). BAs can regulate host metabolism and immunity through the farnesoid X receptor (FXR), which is activated by bile acids (Pathak et al., 2018). The intestinal microbiota can modulate gut secretion of fibroblast growth factors (FGF15 in mice, FGF19 in humans) through FXR activation by BAs. FGF15/19 has been found to modulate host metabolism and bile acid synthesis in the liver (Betrapally et al., 2016). Probiotics have been found to reduce the absorption of bile acids and promote hepatic bile acid synthesis through the enterohepatic FXR-FGF15 axis (Degirolamo et al., 2014). Therefore, the use of probiotics to modulate the gut microbiota and improve liver diseases is a promising area of research.

*Akkermansia muciniphila* is the only isolated member of the phylum *Verrucomicrobia* and has been shown to be beneficial in metabolic and immune disorders by degrading mucin, which can be used as nutrients by enterocytes or other intestinal commensal microbes (Grander et al., 2018; Qin et al., 2012). Depletion of *A. muciniphila* has been linked to disrupted intestinal permeability and increased plasma lipopolysaccharide (LPS), causing systemic inflammation and MAFLD in mice (Shi et al., 2021). On the other hand, *A. muciniphila* supplementation has been shown to improve high-fat diet-related obesity by enhancing the gut barrier and improving dysglycaemia (Everard et al., 2013; Li et al., 2016; Plovier et al., 2017). The evidence suggests that *A. muciniphila* can be a promising candidate as a new probiotic (Derrien et al., 2017; Zhang et al., 2019). Our previous study has showed that short-term replenishment with high concentrations of *A. muciniphila* can reshape the gut microbiota and influence the mouse response to concanavalin A injection (Wu et al., 2017). However, the beneficial effect of *A. muciniphila* on MAFLD is limited. This study aims to investigate the specific roles and regulatory mechanisms of *A. muciniphila* in MAFLD development from the perspective of the gut–liver axis.

## EXPERIMENTAL PROCEDURES

### Strain culture and animal treatments

*Akkermansia muciniphila* Muc$^T$ (ATCC BAA-835) was purchased from ATCC and cultured as previously described (Wu et al., 2017). Briefly, the live bacteria were collected after repeated centrifugation and washing with PBS. The final concentration of live *A. muciniphila* in sterile PBS reached $1.5 * 10^{10}$ CFU/mL.

Six-week-old specific pathogen-free (SPF) C57BL/6 male mice of SLAC Laboratory, Shanghai were bred in a SPF environment. After 2 weeks of adaptive feeding, all the mice were divided into three groups randomly (LP, HP, and HA). Mice in the LP group (LFD + PBS) were fed a low-fat diet as a healthy control, while mice in the HP (HFD + PBS) and HA (HFD + *A. muciniphila*) groups were fed a high-fat diet (Research Diet D12492) for 21 weeks to construct MAFLD models. Mice in the HA group were treated with a daily oral gavage with 0.2 mL of live *A. muciniphilaus* suspension (approximately $1.5 * 10^9$ CFU total counts), while mice in the HP and LP groups were administered PBS instead. After 21 weeks, the mice were euthanized, and blood, liver, ileum, colon, and fat samples were collected and immediately stored at −80°C. All experiments were approved by the Local Committee of Animal Use & Protection and were performed in accordance with criteria of National Research Council's Guide for the Care and Use of Laboratory Animals.

### In vivo intestinal barrier permeability

FITC-labeled dextran (FD4) was weighed and fully dissolved in sterile PBS to prepare a 100 mg/mL FD4 stock solution. Before detection, mice were fasted for 4 h and given 60 mg/100 g FD4 by oral gavage; thereafter, mice were fasted for another 4 h. Venous blood was then acquired, and the fluorescence value in plasma was detected with a fluorescence microplate reader with 485 nm excitation wavelength and 528 nm receiving wavelength.

A standard curve was drawn, according to which the FD4 concentration in the samples was calculated.

## Intraperitoneal glucose tolerance (IPGTT)

Mice were fasted for 6 h to ensure baseline glucose levels. Whole blood glucose was measured from the tail vein with ACCU-CHEK Sensor Comfort Test Strips (Roche), and this value was taken as the 0-min blood glucose level. Then, a 20% glucose solution was injected intraperitoneally at a dose of 1 g/1 kg body weight. Blood glucose levels were measured at 15, 30, 60 and 120 min after injection. The blood glucose levels were plotted against time to draw a blood glucose curve. The area under the glucose tolerance test (GTT) curve was calculated.

## Histological analysis

Tissue samples were collected and immediately placed in 10% formalin fixing buffer. The fixed tissues were embedded in paraffin blocks. Sections of 4 μm-thick paraffin were produced and stained with haematoxylin and eosin. The NAFLD Activity Score (NAS) was applied to assess the severity of hepatic lesions according to different aspects including steatosis grade (0–3), lobular inflammation (0–3) and ballooning (0–2). Lipid accumulation was determined using Oil Red O staining. The colon mucus layer was measured by Alcian blue staining. For immunofluorescence, paraffin sections were stained with fluorescently labelled antibodies (occludin and tjp1) and scanned by a fluorescence confocal microscope (Zeiss).

## Liver function, systemic LBP and metabolic factor detection

Serum concentrations of alanine aminotransferase (ALT) and aspartate transaminase (AST) were detected using an automated analyser (SRL). The LPS-binding protein (LBP) in plasma was measured using an ELISA kit according to the manufacturer's instructions. The Bio-Plex Pro Mouse Diabetes eight-Plex Assay and Adiponectin Assay (Bio–Rad) were used to determine metabolic-related factors in serum, including insulin, ghrelin, glucagon-like peptide 1 (GLP-1), resistin, glucagon, gastric inhibitory peptide, leptin, plasminogen activator inhibitor-1 (PAI-1) and adiponectin.

## RNA isolation and real-time quantitative PCR detection

RNA was extracted and purified. and reverse-transcribed into cDNA using Prime Script™ RT Master Mix (TaKaRa Biotechnology). Real-time fluorescence quantitative PCR (RT–PCR) was performed using a 7500 real-time PCR system (Applied Biosystems). The reaction conditions were set as follows: a predenaturation step at 95°C for 30 s, followed by 40 cycles of two-step PCR denaturation (95°C for 5 s) and annealing extension (60°C for 34 s). The relative expression of target genes was calculated according to the $2^{-\Delta\Delta CT}$ method and normalized to b-actin as a reference (primers shown in Table S1).

## Bile acid profile analysis

Approximately 10 mg of caecum was weighed, and 10 mg of prechilled zirconium oxide beads and 15 μL of ultrapure water were added. Internal standards (LCA-d4, UDCA-d4, GCDCA-d4, TCA-d4, GCA-d4, CA-d4, GDCA-d4, DCA-d4, TCDCA-d9, and β-CA-d5) were used, and their concentrations were controlled to 150 nM at all calibration points. A prechilled acetonitrile and methanol mixture was prepared with a volume ratio of 80:20, and the above internal standards were added. Then, the mixture was added to the processed caecal solution. After centrifugation at 13,500 *g* and 4°C for 20 min, 50 μL of supernatant was carefully collected and gently mixed with 150 μL of acetonitrile/water solution (50:50, v/v) for later detection.

For liver samples, 10 mg of tissue was weighed and mixed with prechilled beads and ultrapure water. The samples were then homogenized with acetonitrile/methanol (8:2) as well as internal standards and centrifuged. The collected supernatant liquid was freeze dried. The lyophilized sample powder, dried calibrators and quality control samples (QCs) from BAP Ultra were reconstituted by acetonitrile/methanol (80/20, v/v) and ultrapure water. Finally, the centrifuged supernatant was collected for later detection.

A UPLC–MS/MS system (Waters Corp) was used for BA quantification following previous protocols (Xie et al., 2015). QuanMET software (version 1.0, MetaboProfile) was applied for further data processing. A standard curve was drawn to calculate the regression equation. The retention time in the ion chromatogram was compared to identify the specific components in the sample, and its corresponding concentration was calculated according to the signal value from this equation (Xie et al., 2015).

## Faecal 16S rDNA sequence and bioinformatics analysis

Faecal bacterial genomes were extracted. Universal primers with specific barcodes targeting the conserved V3-V4 regions of 16S rDNA were used (341F: 5′-CCTAYGGGRBGCASCAG-3′, 806R: 5′-GGACT

ACNNGGGTATCTAAT-3′). High-fidelity PCR of the genome was performed using Phusion® High-Fidelity PCR Master Mix with a GC Buffer Kit (New England Biolabs, Inc.).

A clone library was yielded by Ion Plus Fragment Library Kit and sequenced by Ion S5TM XL (Thermo Fisher Scientific Inc.). Single-ended reads (400 bp/600 bp) were acquired and processed to obtain clean reads. Sequences which have a similarity of higher than 97% were clustered into an operational taxonomic unit (OTU). Representative OTUs were extracted and annotated with Mothur based on the Silva database. a-diversity and b-diversity were calculated by QIIME software. Principal coordinate analysis (PCoA) based on UniFrac distance was conducted to compare the community distributions, and the significance was analysed by Anosim. The Wilcoxon rank sum test or *T* test was adopted for intergroup analysis. The community constitution at various taxonomic levels (phyla, families, genera, species) was analysed by MetaStat, and the *Q* value was obtained by correcting the *p* value.

## Statistical analysis

SPSS 20.0 (SPSS, Inc.) was used for data analysis, and a $p$ value <0.05 was considered statistically significant. An unpaired *t* test or Mann-Whitney U test was applied for two-group comparisons, while ANOVA or Kruskal-Wallis test was employed when three groups were involved according to data distribution. Figures were generated using Prism 6.0 software (GraphPad Software.).

# RESULTS

## *Akkermansia muciniphila* relieves weight gain, hepatic steatosis and liver injury

High-fat diet-induced MAFLD shares similar histologic and pathogenic characteristics with clinical MAFLD, featuring concomitant obesity and IR (Lau et al., 2017). Therefore, our study used a high-fat diet to construct a mouse MAFLD model. We found that mice fed with HFD (group HP) gained weight more rapidly than mice fed with a low-fat diet (LFD; group LP), and *A. muciniphila* remarkably inhibited HFD-induced weight gain (Figure 1A). This significance remained until the end of the experiment (group HA vs. HP, 35.95 ± 4.88 vs. 43.92 ± 5.26 g, $p < 0.001$, at week 21, Figure 1B). Compared to group LP, the liver volume of group HP increased with extensive lipid deposition in all lobes, and this was improved by *A. muciniphila* treatment. (Figure 1C,D). HFD induced the accumulation of lipid droplets in hepatocytes, as seen in Oil Red O staining, and these intracellular lipid vacuoles were lessened in *A. muciniphila*-treated mice (Figure 1D). HE staining showed disordered liver lobules with ballooning degeneration, the characteristic Mallory body, and scattered inflammatory infiltration. The histological NAS score was found to be much higher in HFD-fed mice than in the LP group and was decreased in *A. muciniphila*-treated mice (Figure 1E). In addition, intrahepatic triglycerides (TGs) significantly increased in the model group (HP), accompanied by elevated ALT and AST. *Akkermansia muciniphila* intervention markedly reduced the concentration of intrahepatic TGs, as well as serum ALT and AST (Figure 1F,G).

## *Akkermansia muciniphila* modulates the dysbiosis of the gut microbiota induced by HFD

To explore the alteration of the gut microbiota, we assessed faecal microbial communities using 16S rDNA sequencing analysis. PCoA clustering was employed to discriminate the construction with both unweighted and weighted UniFrac distances (Figure 2A). In contrast to the LP group, MAFLD mice presented a distinct construction ($p < 0.01$, ANOSIM multivariate data analysis). However, compared to the HP group, *A. muciniphila*-treated mice showed a disparate cluster of commensal microbes based on both an unweighted and weighted PCoA plot ($p < 0.01$, ANOSIM multivariate data analysis).

To elaborate specific changes in taxonomic compositions, microbial abundance at different taxonomic ranks was analysed. In the LP group, the phylum *Bacteroidetes* was found to be the dominant population, followed by *Firmicutes*, *Proteobacteria*, and *Actinobacteria* (Figure S1), which was consistent with previous findings. However, in the HP group, there was a significant decrease in *Bacteroides* and *Actinobacteria*, with a corresponding increase in *Firmicutes*, indicating a shift in the composition of the gut microbiota towards an unhealthy state (Figure S2, adjusted $q < 0.05$, compared to group LP). Interestingly, the administration of *A. muciniphila* did not significantly reverse the alteration at the phylum level. However, the relative abundance of the top 35 genera revealed a disparate distribution in the HA group compared to the HP group (Figure S3). Several genera, such as *Alistipes*, *Lactobacilli*, *Tyzzerella*, *Butyricimonas* and *Blautia*, were greatly reduced in the HA group compared to the HP group, whereas other genera, such as *Ruminiclostridium*, *Osclibacter*, *Allobaculum*, *Anaeroplasma* and *Rikenella*, were enriched in the HA group (Figure 2B, $p < 0.05$, by MetaStat). These findings suggested that *A. muciniphila* treatment had a selective effect on specific genera of gut microbiota, rather than a broad effect on the overall composition of the gut microbiota.

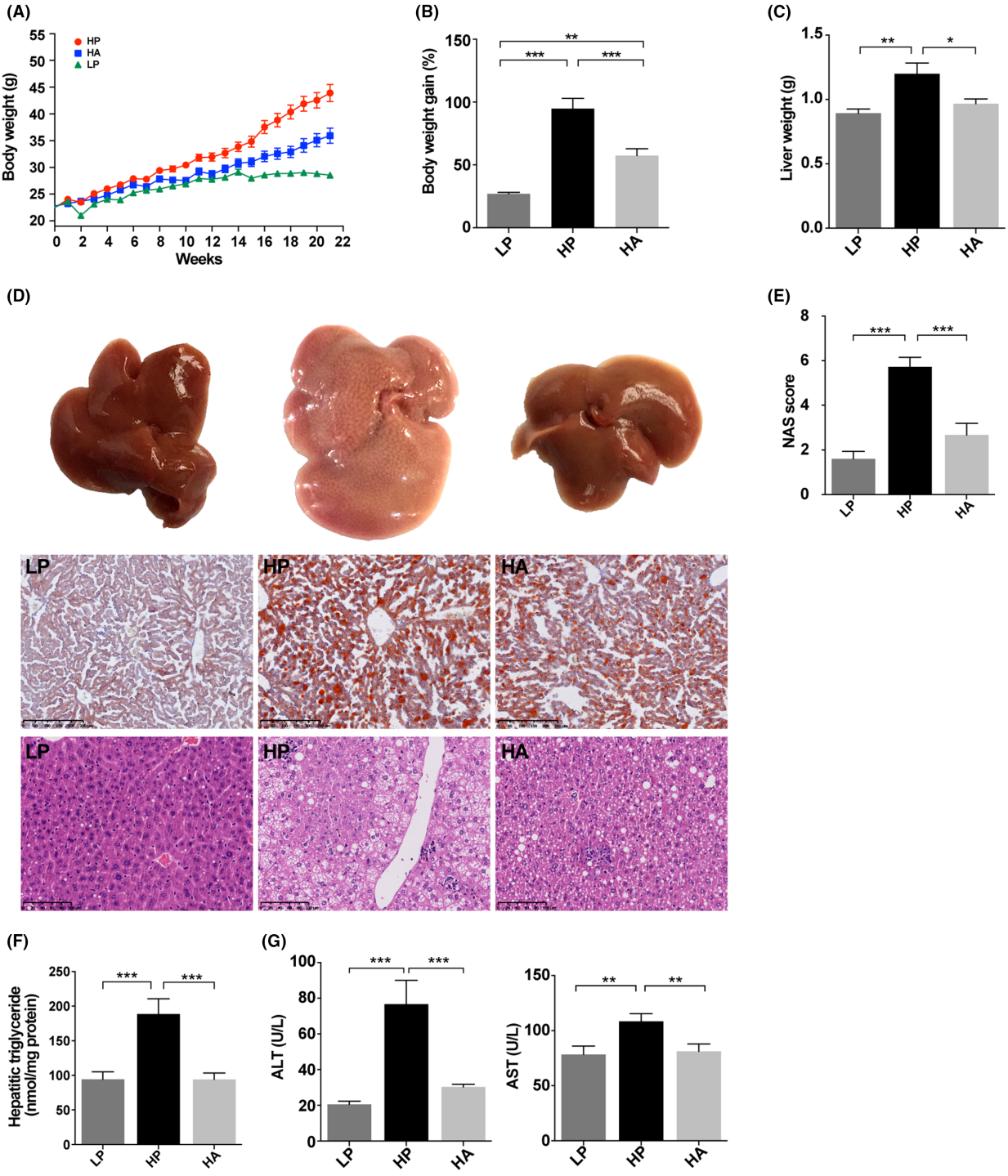


FIGURE 1 *Akkermansia muciniphila* relieves high-fat diet-induced weight growth, hepatic steatosis and liver injury. (A) body weight curves during experiments: mice were randomly distributed to three groups ($n = 10–12$ per group, red circle represents the model group fed with HFD (HP), blue square represents *A. muciniphila*-treated group (HA), and green triangle represents the control group (LP); (B) body weight and (C) liver weight at the end of experiments; (D) macroscopic view of liver tissue, HE staining and Oil-red O staining; (E) NAS scores of liver histopathology; (F) concentration of hepatic triglyceride; (G) serum ALT and AST. Statistical test: ANOVA; Significant post hoc comparisons: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; Data are shown as means ± SEM.

## *Akkermansia muciniphila*-modified gut microbiota is related to bile acid profiles

Primary BAs are modified and transformed into secondary BAs by gut commensal microbes, such as *Clostridia* and *Bacteroidetes species*. Thus, the concentration of BAs was quantified and the correlation between intestinal flora and caecum bile acids was analysed. Various genera were significantly correlated with cecal bile acids (Figure 3). *Allobaculum* was

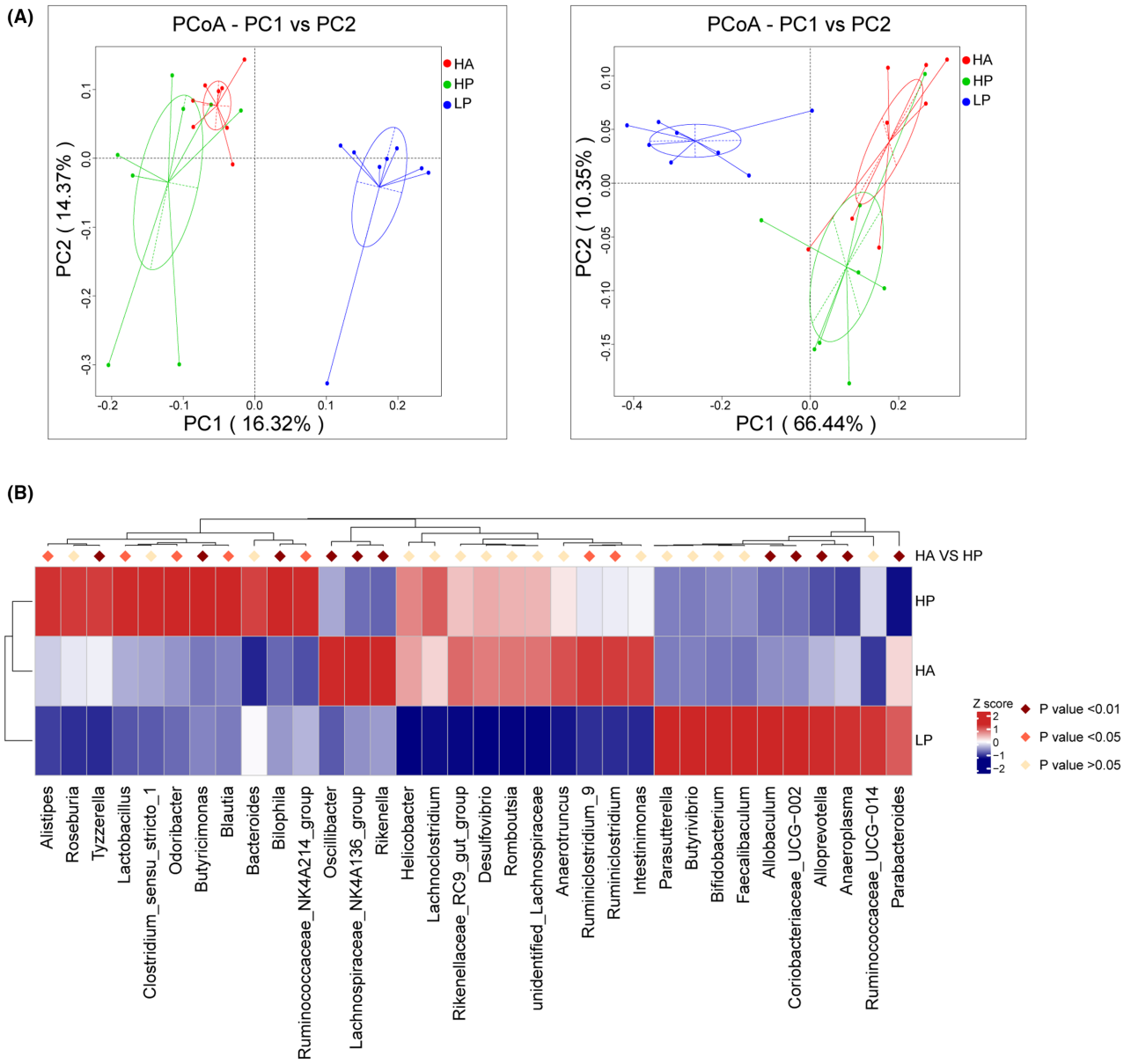


**FIGURE 2** *Akkermansia muciniphil*a modulates the dysbiosis of gut microbiota induced by high-fat diet. (A) Principal co-ordinates analysis (PCoA) among three groups based on un-weighted (left) and weighted (right) UniFrac distances, each point represents a sample (blue, group LP; green, group HP; red, group HA); (B) heat map of genus-level taxa among three group, each line depicts one group, between-group (HP and HA) significance was determined using MetaStat, orange diamond indicating $p < 0.05$, brown diamond indicating $p < 0.01$, white diamond indicating $p > 0.05$.

negatively related to several bile acids, including CDCA, α-MCA, LCA, DCA, UDCA and TDCA (Figure 3), which were enriched in the HP group. The abundance of *Allobaculum* was reduced in the HP group but elevated following *A. muciniphila* supplementation (Figure S3). DCA was negatively correlated with *Anaeroplasma* ($R = -0.48$) and positively correlated with *Tyzzerella* ($R = 0.57$). *Anaeroplasma* was enriched in the HA group, while *Tyzzerella* was enriched in the HP group. LCA was positively related to several genera, including *Alistipes*, *Lactobacillus*, *Tyzzerella*, *Lachnoclostridium*, *Clostridium_sensu_stricto_1* and *Roseburia*, most of which were increased in the HP group. These findings suggested that changes in the gut microbiota resulting from *A. muciniphila* intervention were associated with alterations in the structure of bile acid profiles.

## *Akkermansia muciniphila* inhibits adipocyte hypertrophy and improves dysbiosis of adipokines

Adiposity and altered profiles of adipokines (including leptin, adiponectin and resistin) are considered to be involved in the pathogenesis of MAFLD. Thus, we measured the enlargement of white adipose tissue (WAT)

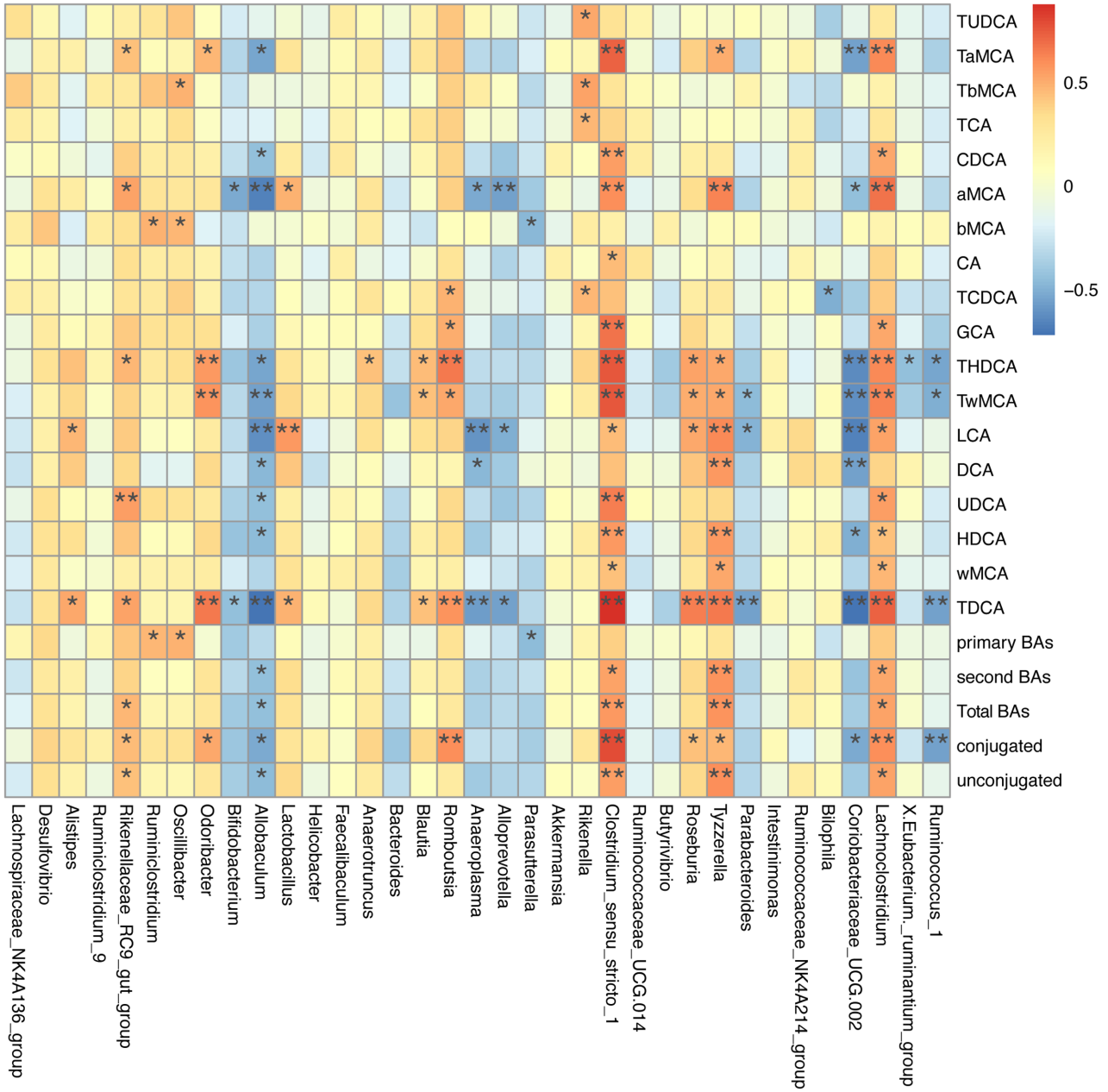


**FIGURE 3** Correlation heatmap of gut microbiota and cecum bile acids. Spearman's rho non-parametric correlation was adopted, significant relationships with $*p<0.05$ and $**p<0.01$.

and WAT-secreted adipokines in serum (Figure 4). Consistent with previous findings, *A. muciniphila* treatment significantly inhibited the development of WAT in total, subcutaneous (sWAT), mesenteric (mWAT), and epididymal tissues (eWAT) in mice fed a HFD (Figure 4A-C). The size of eWAT tissue (Figure 4C) as well as adipocytes (Figure 4D) in group HA was smaller than that in group HP. The hyperplasia of WAT was associated with dysbiosis of adipokines, as the serum concentration of insulin, leptin, and resistin in the HP group greatly raised, and the concentration of adiponectin was obviously decreased compared with those in the LP group. *Akkermansia muciniphila* significantly eliminated insulin, leptin and resistin (Figure 4E, $p<0.05$), whereas adiponectin was comparable between group HP and HA. In addition, *A. muciniphila* obviously improved glucose tolerance, with a smaller AUC of the IPGTT at week 21 (Figure 4F). These findings suggested that *A. muciniphila* might have therapeutic potential for MAFLD by improving adipocyte metabolism and related adipokines.

## *Akkermansia muciniphila* strengthens intestinal barriers

MAFLD patients exhibit increased permeability of intestinal barriers, which can lead to translocation of

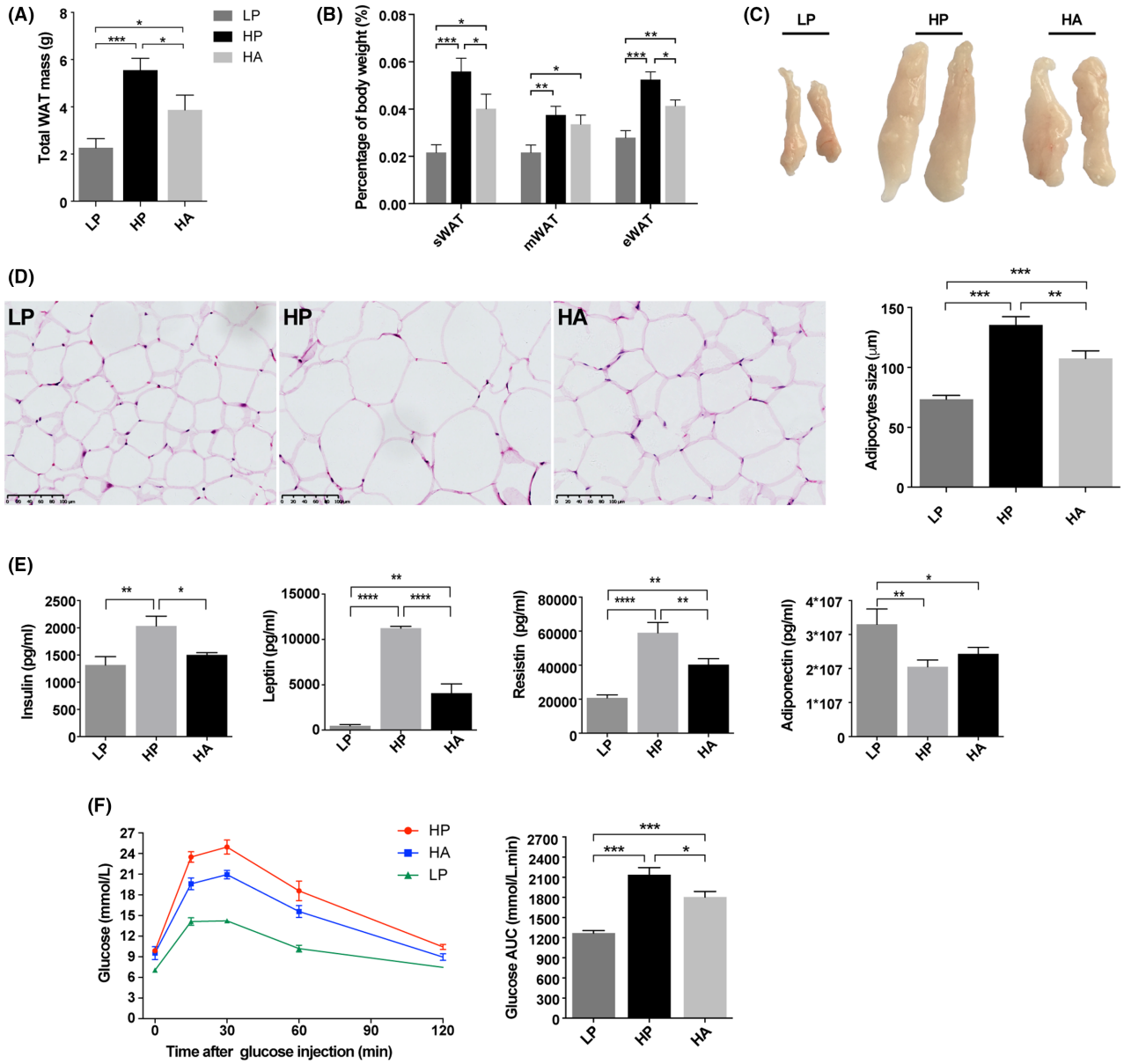


**FIGURE 4** *Akkermansia muciniphila* improves adipocyte hypertrophy, dysbosis of adipokines, and glucose tolerance. (A) total WAT mass; (B) mass percentage of subcutaneous, mesenteric, and epididymal WAT; (C) epididymal WAT tissues; (D) HE staining of epididymal WAT and diameter of adipocytes; (E) serum concentration of adipokines; (F) intraperitoneal pyruvate tolerance test and mean area under the curve (AUC) measured between 0 and 120 min after glucose injection. Statistical test: ANOVA; Significant post hoc comparisons: *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$; Data are shown as means ± SEM.

commensal metabolites, bacterial components and bacteria themselves, resulting in systemic inflammation (Cani et al., 2007). The mucus layer and tight junctions play a role in modulating gut permeability. Compared to the LP group, the blue mucus layer shown by alcian blue staining was thinner in the HP group and was thickened by *A. muciniphila* (Figure 5A). Moreover, the expression of tight junction-related structures (OCCLUDIN and TJP1) was stronger and more continuous in the LP and HA groups compared to the HP group (Figure 5B,C). The mRNA expression of *occludin* and *tjp1* was also suppressed in the HP group compared to the LP group (Figure 5D,E, $p<0.05$), but was upregulated by the *A. muciniphila* intervention ($p<0.01$). *I*n vivo intestinal permeability was measured by assessing the systemic concentration of FD4 after intragastric gavage. The results showed that after 4 h of FD4 gavage to fasting mice, the plasma FD4 concentration was significantly higher in group HP than in group LP, indicating increased intestinal permeability in group HP. However, this elevation was greatly attenuated by *A. muciniphila* supplementation (Figure 5F). We observed that HFD-fed mice had increased concentrations of LBP in plasma, whereas *A. muciniphila* supplementation resulted in a lower trend of LBP, although this trend did not reach statistical significance (Figure 5G, $p=0.08$).

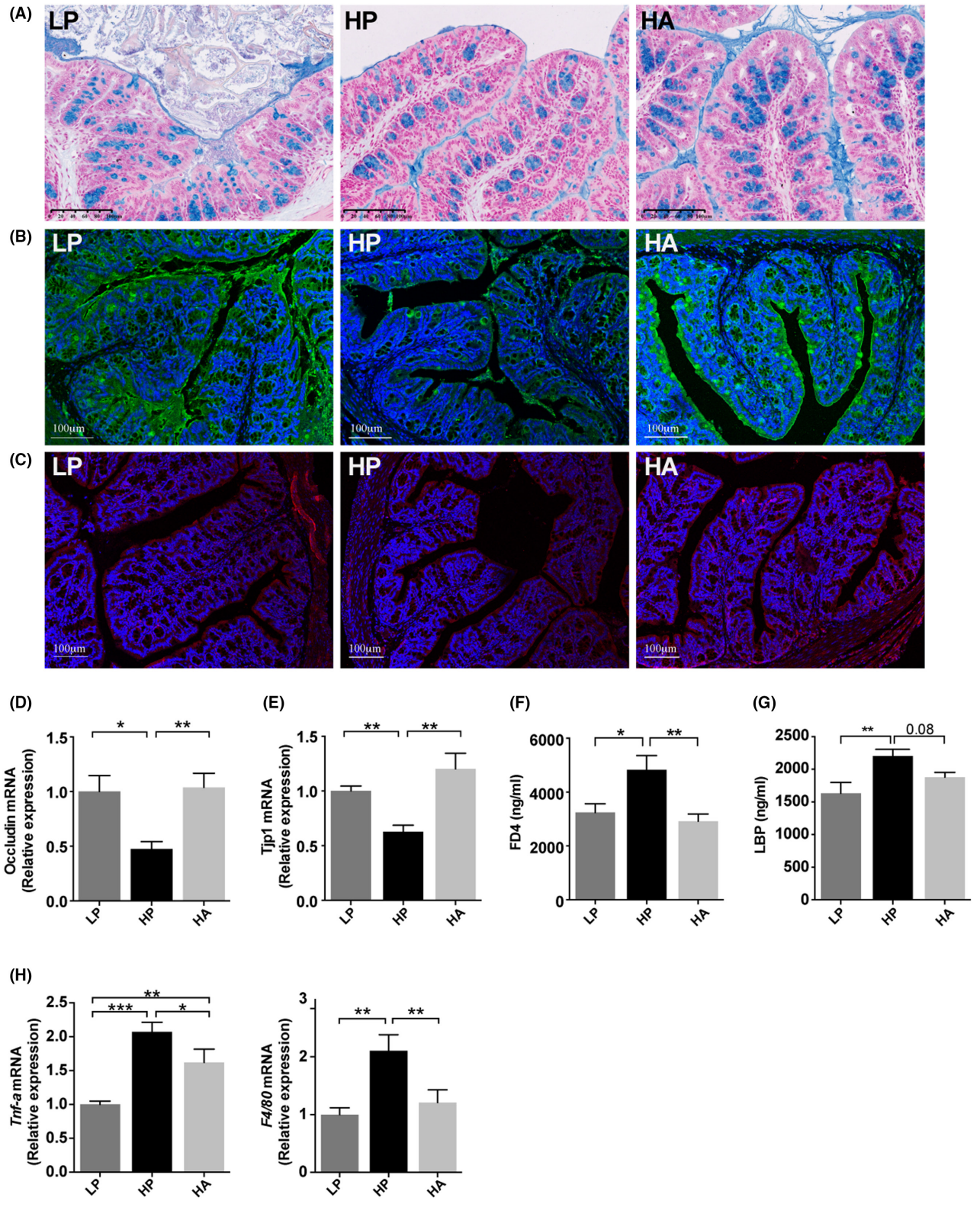


**FIGURE 5** *Akkermansia muciniphila* strengthens intestinal barriers. (A) representative alcian blue images of mucus layer, scale bar, 100 mm; (B) and (C) representative immunofluorescence staining of OCCLUDIN (green) and TJP1 (red) in colon, scale bar, 100 mm; (D) and (E) mRNA expression of occludin and Tjp1 in colon; (F) and (G) plasma concentration of FD4 and LBP, ng/mL; (H) mRNA expression of tnf-$\alpha$ and f4/80 in liver. Statistical test: ANOVA; Significant post hoc comparisons: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Data are shown as means ± SEM.

Leaky gut can lead to the onset of systemic inflammation and MAFLD progression. TNF-α and macrophage infiltration are closely related to liver inflammation and insulin resistance (Sell et al., 2012). Our study found that *tnf-α* and *f4/80* were significantly elevated in the HP group compared to their levels in the LP group, but were significantly decreased under the administration of *A. muciniphila* (Figure 5H, $p < 0.05$). The above results suggested that intestinal barrier permeability increased during MAFLD modelling, while *A. muciniphila* improved intestinal barrier function, reduced endotoxin translocation, and relieved intrahepatic inflammation.

## *Akkermansia muciniphila* upregulates FGF15 and inhibits hepatic bile acid synthesis

The FXR-FGF15 axis is a regulatory pathway that plays an important role in the regulation of host metabolism and is considered a therapeutic target for obesity and MAFLD (Arab et al., 2017). We proposed that the reshaped microbiota might, in turn, affect the activation of BA receptors and BA enterohepatic circulation. We found that the mRNA expression of *fxr* and *shp* in the ileum was significantly inhibited in the HP group ($p < 0.05$), while the mRNA expression of *fxr* and *shp* was significantly increased in the HA group. Correspondingly, the *fgf15* mRNA concentration in the ileum was diminished in the HP group ($p < 0.05$, compared to the LP group) and significantly reversed by *A. muciniphila* supplementation (Figure 6A). FGF15 can activate the hepatic tyrosine kinase receptor FGFR4 through portal venous reflux, thus inhibiting the expression of CYP7A1, an important rate-limiting enzyme for bile acid synthesis. In addition, BAs can directly bind and activate hepatic FXR receptors, thereby inhibiting the expression of CYP7A1 through the transcription factor SHP (Li et al., 2013). We found that the *fgfr4* mRNA level in group HA greatly increased compared to the HP and LP groups (Figure 6B, $p < 0.05$), while the intrahepatic *fxr* and *shp* mRNA levels were comparable among the three groups. Furthermore, it was observed that HFD feeding significantly increased hepatic mRNA level of *cyp7a1* (HP vs. LP, $p < 0.001$, Figure 6B), but *A. muciniphila* supplementation apparently inhibited the overexpression of *cyp7a1* ($p < 0.01$).

As shown in Figure 6C, MAFLD mice presented a distinctively higher concentration of total BAs in the caecum (HP vs. LP, $p < 0.001$), which was mainly attributed to increased sBAs (HP vs. LP, $p < 0.001$). A similar change was observed with an elevated concentration of total BAs in hepatic tissue from the HP group, in contrast to that of the LP group. In addition to sBAs, intrahepatic pBAs were higher in group HP (HP vs. LP, $p < 0.01$). *Akkermansia muciniphila* greatly reduced the concentration of total and secondary BAs both in the caecum and liver (HA vs. HP, $p < 0.05$), as well as decreasing hepatic pBAs. The above results suggested that *A. muciniphila* could promote gut-derived *fgf15*, which might in turn suppress bile acid synthesis by inhibiting the expression of hepatic *cyp7a1*.

## *Akkermansia muciniphila* regulates the construction of intestinal BAs and reduces secondary BAs

To further explore the detailed alteration of BA enterohepatic circulation, the composition of 18 major bile acids in the caecum and liver among the three groups was analysed. As seen in Figure 7A mice from the LP group had a unique hepatic BA profile, with TCA and β-MCA being the major components. In contrast, MAFLD mice from the HP group showed an altered hepatic BA profile, with elevated TCDCA, LCA and TDCA, and decreased GCA, ω-MCA and Tω-MCA. *Akkermansia muciniphila* partially restored the disturbed hepatic BA profile by reducing TCDCA, LCA and TDCA as well as restoring ω-MCA and Tω-MCA (Figure 7A). In the caecum, unconjugated secondary BAs were the main proportion of BAs in control mice fed LFD, with ω-MCA as the predominant BA, followed by DCA, β-MCA, HDCA, LCA, and α-MCA. HFD diets restructured the constitution of BAs. The percentage of DCA was markedly elevated after HFD feeding (43.63 ± 4.35% vs. 32.39 ± 12.52%, HP vs. LP, $p < 0.05$). In addition, LCA and TDCA were higher in the HP group. However, *A. muciniphila* significantly reduced the increased DCA and LCA levels and showed a decreasing trend in TDCA levels ($p = 0.08$, Figure 7B).

# DISCUSSION

Several studies have investigated the beneficial roles of *A. muciniphila* in metabolic diseases and the associated mechanisms. This study adopted an HFD-induced MAFLD model because its pathogenesis is similar to the clinical course of MAFLD. We observed that *A. muciniphila* treatment inhibited weight gain and reduced lipid deposition in hepatocytes. Interestingly, we found that *A. muciniphila*-treated mice had a differentiated gut flora and bile acid profiles, in addition to enhanced intestinal barrier function and improved systemic inflammation. We proposed that the mechanisms be related to the regulation of the FXR-FGF15 axis in the intestine and liver and the improvement of liver lipid and bile acid metabolism.

Increased intestinal barrier permeability is related to the metabolic dysfunction of visceral adipose tissue (Lam et al., 2012). Gut-derived endotoxin can trigger fat inflammation, which is considered to be the main driver of systemic inflammation in metabolic

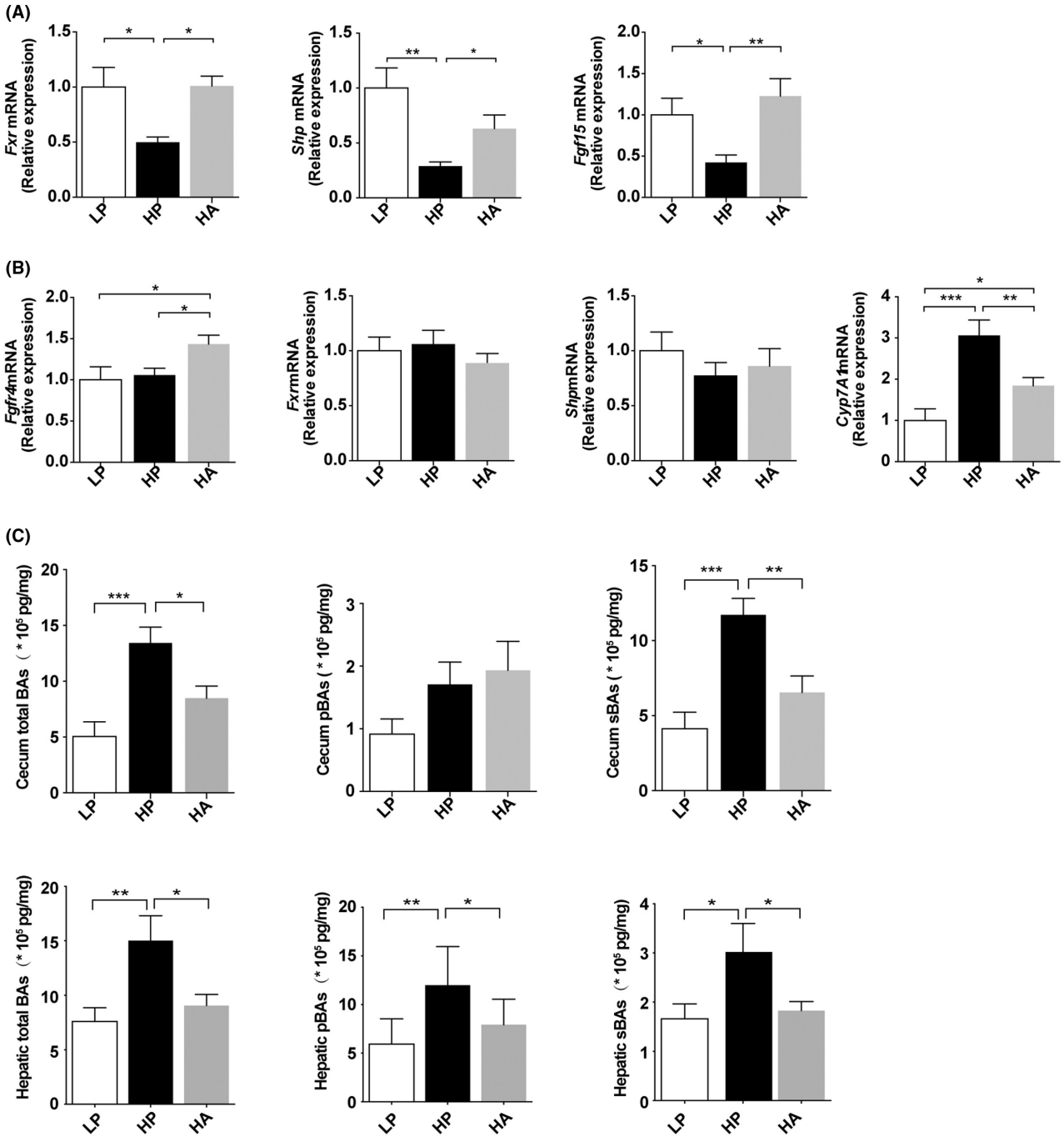


**FIGURE 6** *Akkermansia muciniphila* up-regulates FGF15 and inhibits hepatic bile acid synthesis. (A) mRNA expression of fxr, shp, and fgf15 in ileum; (B) mRNA expression of fgfr4, fxr, shp, and cyp7a1 in liver; (C) concentration of total, primary, secondary BAs in cecum and liver. Statistical test: ANOVA; Significant post hoc comparisons: $*p<0.05$; $**p<0.01$; $***p<0.001$; Data are shown as means ± SEM.

syndrome by releasing adipokines (Schaffler & Scholmerich, 2010). Adipose tissue can synthesize and release proinflammatory cytokines and adipokines, including leptin, adiponectin, and resistin; and these factors make up one of the driving mechanisms of IR (Brunt, 2004). Adipokines can promote the occurrence of simple fatty liver and are related to the progression of MAFLD to NASH or even liver cirrhosis (Polyzos et al., 2009). As it is known, WAT is kept in a normal range under physiological conditions, maintaining its secreted cytokines and adipokines in equilibrium. However, hypertrophy of WAT can result in overproduction of leptin and resistin, leading to sustained insulin resistance and hepatic steatosis. Moreover, leptin and resistin can promote inflammation and liver fibrosis (Polyzos et al., 2015, 2016). Adiponectin is significantly reduced when simple fatty liver progresses to NASH (Polyzos et al., 2010). Our study found that *A. muciniphila* inhibited WAT hypertrophy in MAFLD mice and improved adipokine

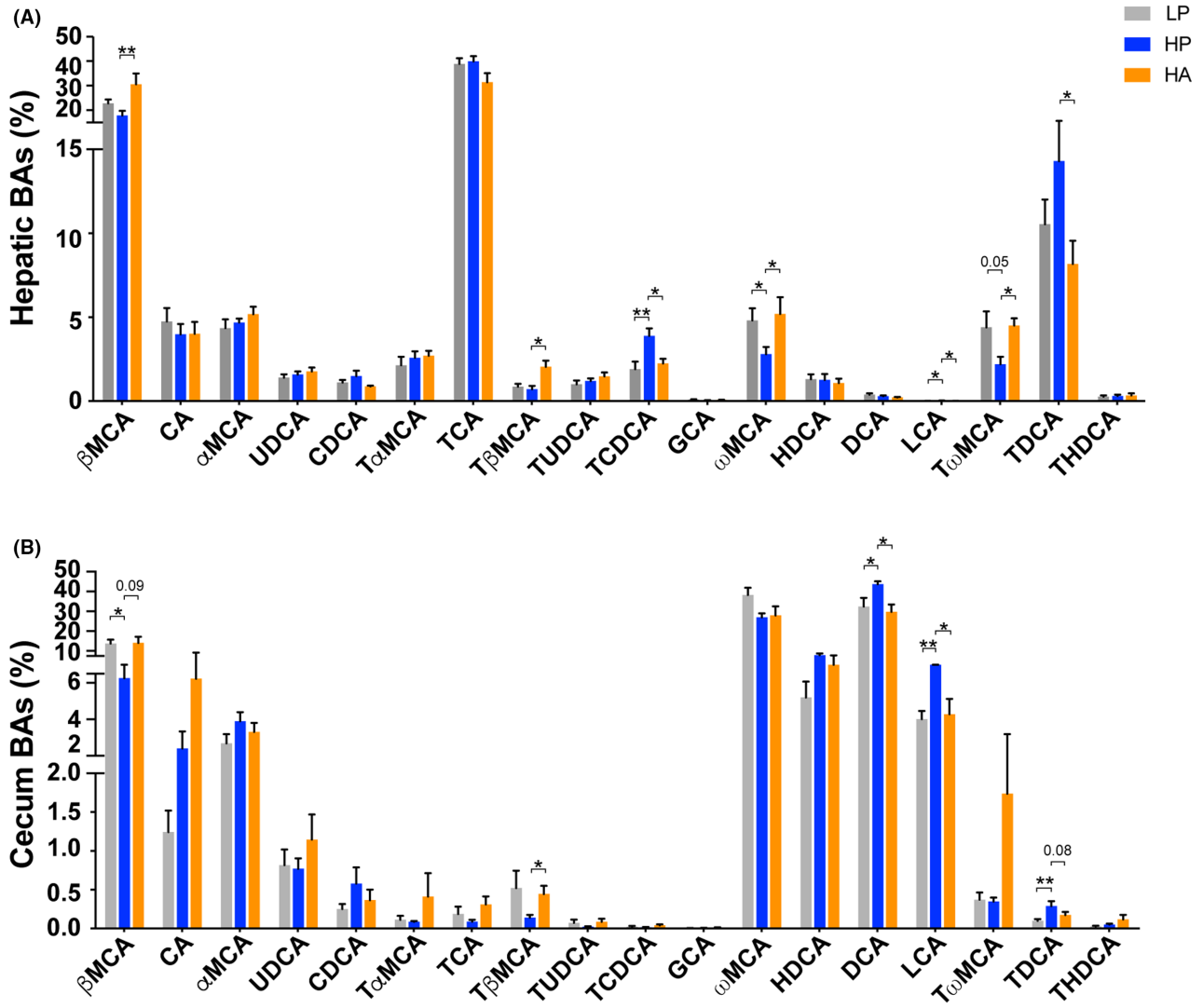


**FIGURE 7** *Akkermansia muciniphila* regulates the construction of intestinal BAs and reduces several secondary BAs. (A) relative percentage of 18 major bile acids in liver; (B) relative percentage of 18 major bile acids in cecum. Statistical test: ANOVA or Kruskal-Wallis test according to data distribution; Significant post hoc comparisons: $*p<0.05$; $**p<0.01$; Data are shown as means ± SEM.

disorders. Schneeberger et al. found that the abundance of *A. muciniphila* progressively decrease with HFD feeding and is inversely correlated with WAT inflammation and serum concentrations of leptin and insulin (Schneeberger et al., 2015). Li et al. also found that administration of *A. muciniphila* can reduce metabolic inflammation in *Apoe*−/− mice, thereby improving atherosclerosis (Li et al., 2016). Amuc_1100, a membrane protein derived from *A. muciniphila*, has been found to be able to activate the AC3/PKA/HSL pathway, which can lead to the activation of lipolysis and browning (Zheng et al., 2023). Thus, *A. muciniphila* has a favourable effect by inhibiting the hypertrophy of adipose tissue and regulating the balance of adipokines.

As the receptor of bile acids, FXR is expressed by intestinal epithelial cells. When FXR is activated, it induces the secretion of downstream FGF15, which plays a role in regulating bile acid synthesis and metabolism. Several bile acids, including CDCA, TCA, DCA, LCA and CA, can activate FXR in vitro. However, in the presence of CDCA, which is present under physiological conditions, DCA and LCA appear to be antagonists of the FXR receptor (Parks et al., 1999; Yu et al., 2002). It suggests that the balance of different bile acids be important in regulating FXR signalling and bile acid metabolism. In addition, DCA and LCA are cytotoxic, and DCA is associated with the development of colon cancer (Bernstein et al., 2011). Jiao et al. (2018) found that MAFLD patients present elevated primary and secondary bile acids, among which the proportion of DCA is increased and CDCA is decreased. Additionally, hepatic CYP7A1 expression is enhanced. In mice fed a high-fat diet, there is a significant change in the profile of intestinal bile acids, while total BAs, primary BAs, and secondary BAs, including CA, GCA, TDCA, DCA, and LCA, are greatly increased (Zheng et al., 2017). Consistently,

our study also observed a differential intestinal BA profile in HFD-fed mice with enrichment of secondary BAs, especially DCA and LCA.

Our study revealed that *A. muciniphila* did not significantly influence hepatic *fxr* expression in HFD-fed mice, while intestinal *fgf15* was increased and hepatic *fgfr4* was upregulated. FGFR4 downregulates the expression of CYP7A1, thereby reducing hepatic synthesis of bile acid (Li et al., 2014). Previous research showed that *fgf15*−/− mice fed HFD for 24 weeks present more imbalanced bile acid profiles, more severe insulin resistance and higher serum triglycerides than HFD-fed WT mice (Schumacher et al., 2017). It is recognized that intestine-secreted FGF15 also plays a pivotal role in the regulation of energy metabolism and liver cell regeneration (Potthoff et al., 2011). *Fgf15*−/− mice show overexpression of *cd36*, which acts as a main receptor of hepatocytes for the uptake of free fatty acids (Schumacher et al., 2017). Therefore, it is speculated that *A. muciniphila* prevents HFD-induced MAFLD through decreased intrahepatic bile acid synthesis mediated by *fgf15*.

Primary bile acids synthesized in the liver are secreted into the gastrointestinal tract, where they are metabolized and modified by gut microbiota to be converted to secondary bile acids. Thus, different structures of intestinal flora will influence the modification of bile acids. A high-calorie diet disrupts the structure of the gut microbiota, leading to the overgrowth of opportunistic and/or proinflammatory bacteria (Kong et al., 2019). Shen et al. sequenced gut microbial construction of MAFLD patients, observing an enrichment of *Proteobacteria*, *Enterobacteriaceae*, and *Streptococcaceae*; moreover, the increased *Escherichia coli* and *Blautia* are correlated with the progression of MAFLD to NASH and liver fibrosis (Shen et al., 2017). *Alistipes* is a common resident of the digestive tract, and its abundance has been reported to be positively associated with the severity of alcoholic liver injury (Llopis et al., 2016). *Alistipes* can produce succinic acid (Liu et al., 2016), which is significantly increased in feces, serum, and liver tissue from MAFLD patients (Loomba et al., 2017; Schofield et al., 2017). Succinic acid can activate the GPR91 receptor of hepatic stellate cells and thus promote the progression of MAFLD (Li et al., 2015). *Oscillibacter* has been found to be associated with obesity (Xu et al., 2017), and its abundance is negatively correlated with intestinal barrier function (Lam et al., 2012). *Roseburia* and *Blautia* can produce short-chain fatty acids (SCFAs), such as acetic acid, propionic acid, and butyric acid (Canfora et al., 2019). SCFAs are known to be related to the regulation of energy balance. However, the role of SCFAs in MAFLD remains controversial. Excessive production of SCFAs in the intestinal tract can be absorbed and converted into lipids or glucose by the liver. Moreover, gluconeogenesis is thought to be associated with the development of MAFLD. Thus, the enrichment of *Blautia* and its effect on MAFLD still needs further study. It is known that bile acids have a direct impact on microbial populations. The abundance of *Bilophila* and *Blautia* immediately increase after 1 day of intervention with a high-fat diet, which may be related to their ability to tolerate bile salts (Zheng et al., 2017). In addition, we found that *Tyzzerella* was enriched in the MAFLD group. *Tyzzerella* can form spores and is associated with coronary heart disease (Kelly et al., 2016). Bile salt hydrolase (BSH)-containing bacteria is a prerequisite for conjugated-BA hydrolysation. Some bacteria of the genus *Lactobacillus*, such as *Lactobacillus plantarum*, possess BSH enzymatic activity (Kumar et al., 2012). In this study, we found that the relative abundance of *Lactobacillus* was significantly enriched in the HP group and highly associated with TDCA and LCA. *Akkermansia muciniphila* administration reduced HFD-induced enrichment of *Lactobacillus*, which may be one of the reasons for the decrease in secondary bile acids. Recent research found that *A. muciniphila* increases mitochondrial oxidation and bile acid metabolism in the gut–liver axis, leading to the reshaping of gut microbiota (Rao et al., 2021). Our results suggested that *A. muciniphila* improved bile acid metabolism by altering the structure and function of the gut microbiota. This may include promoting the growth of beneficial bacteria that are involved in bile acid metabolism or inhibiting the growth of harmful bacteria that disrupt bile acid homeostasis. It has been reported that outer membrane vesicles released from *A. muciniphila* is able to restore the dysbiosis of gut microbiota by selectively promoting the proliferation of beneficial bacteria (Wang et al., 2023). And *A. muciniphila* can modulate the immune system and influence the microbiota-host interaction through the production of immunomodulatory lipids (Zhang et al., 2023). Besides, Li et al. (2022) also found that *A. muciniphila* can suppress nonalcoholic steatohepatitis associated tumorigenesis by regulating CXCR6+ natural killer T cells. These evidence suggests an important role of *A. muciniphila* in the regulation of gut microbiota and the gut-liver axis.

Nevertheless, there are some limitations in this study. The exact mechanisms by which *A. muciniphila* regulates the intestinal FXR-FGF15 axis and the gut microbiota are not fully understood. Further research is warranted to identify the specific molecular mechanisms and to compare its effects with those of other gut microbes.

## CONCLUSION

Overall, our results reveal a potential mechanism underlying the beneficial effects of *A. muciniphila* in MAFLD. *Akkermansia muciniphila* can improve bile acid metabolism by regulating the intestinal FXR-FGF15 axis

and altering the structure and function of the gut microbiota. This study provides evidence to support the use of *A. muciniphila* as a potential probiotic remedy for MAFLD in the future.

## AUTHOR CONTRIBUTIONS

**Wenrui Wu:** Conceptualization (equal); methodology (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Wang Kaicen:** Conceptualization (equal); data curation (equal); methodology (equal); validation (equal); visualization (equal). **Xiaoyuan Bian:** Conceptualization (equal); data curation (equal); methodology (equal); validation (equal); visualization (equal). **Liya Yang:** Conceptualization (equal); data curation (equal); methodology (equal); validation (equal); visualization (equal). **Shi Ding:** Conceptualization (equal); data curation (equal); methodology (equal); validation (equal); visualization (equal). **Yating Li:** Conceptualization (equal); data curation (equal); methodology (equal); validation (equal); visualization (equal). **Shengjie Li:** Conceptualization (equal); data curation (equal); methodology (equal); validation (equal); visualization (equal). **Aoxiang Zhuge:** Data curation (equal); methodology (equal); validation (equal); visualization (equal). **Lanjuan Li:** Conceptualization (equal); methodology (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

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## CONFLICT OF INTEREST STATEMENT

None.

## DATA AVAILABILITY STATEMENT

Sequence data are available at https://doi.org/10.6084/m9.figshare.19624749.v1. All data are available from the corresponding author upon reasonable request.

## ETHICS STATEMENT

All experiments were approved by the Local Committee of Animal Use & Protection and were performed in accordance with criteria of "Guide for the Care and Use of Laboratory Animals".

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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