Gut Akkermansia muciniphila ameliorates non-alcoholic fatty liver disease by L-aspartate via interaction with liver

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Abstract

Background and Purpose The human gut bacterium Akkermansia muciniphila has been increasingly recognized for its therapeutic potential in treating metabolic disorders. However, its efficacy in preventing non-alcoholic fatty liver disease (NAFLD) and the mechanism involved in its well-known metabolic actions are unknown. The present study explored the therapeutic effect and novel mechanism of A. muciniphila in intervening NAFLD. Experimental Approach The anti-NAFLD activity of A. muciniphila was evaluated in an obese mouse model induced by high-fat and cholesterol (HFC) diets using three different interventions. The gut microbiota composition, beneficial metabolic effects in the gut-liver axis were explored. The level and beneficial metabolic effects of L-aspartate in vitro and in vivo were further determined. Key Results Mice treated with A. muciniphila efficiently reversed NAFLD in the liver, such as hepatic steatosis, inflammatory, and liver injury. These therapeutic effects persisted after long-term drug withdrawal and were slightly weakened in a germ-free mouse model. A. muciniphila treatment efficiently increased mitochondrial oxidation and bile acid metabolism in the gut-liver axis, ameliorated oxidative stress-induced cell apoptosis in the gut, leading to the reshaping of the gut microbiota composition. These metabolic improvements occurred with increased L-aspartate levels in the liver that transported from the gut. The administration of L-aspartate in vitro or in mice displayed the similar beneficial metabolic effects mentioned above. Conclusion and Implications The anti-NAFLD activity of A. muciniphila correlated with lipid oxidation and improved gut-liver interactions through regulating L-aspartate metabolism. A. muciniphila would be a potent agent for clinical intervention in NAFLD.

Experimental Approach

The anti-NAFLD activity of *A. muciniphila* was evaluated in an obese mouse model induced by high-fat and cholesterol (HFC) diets using three different interventions. The gut microbiota composition, beneficial metabolic effects in the gut-liver axis were explored. The level and beneficial metabolic effects of L-aspartate *in vitro*and *in vivo* were further determined.

Key Results

Mice treated with *A. muciniphila* efficiently reversed NAFLD in the liver, such as hepatic steatosis, inflammatory, and liver injury. These therapeutic effects persisted after long-term drug withdrawal and were slightly weakened in a germ-free mouse model. *A. muciniphila*treatment efficiently increased mitochondrial oxidation and bile acid metabolism in the gut-liver axis, ameliorated oxidative stress- induced cell apoptosis in the gut, leading to the reshaping of the gut microbiota composition. These metabolic improvements occurred with increased L-aspartate levels in the liver that transported from the gut. The administration of L-aspartate *in vitro* or in mice displayed the similar beneficial metabolic effects mentioned above.

Conclusion and Implications

The anti-NAFLD activity of *A. muciniphila* correlated with lipid oxidation and improved gut-liver interactions through regulating L-aspartate metabolism. *A. muciniphila* would be a potent agent for clinical intervention in NAFLD.

Abbreviations

ACC, acetyl-CoA carboxylase; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; ASBT, apical sodium -dependent bile salt transporter; AST, aspartate aminotransferase; Bcl-2, B-cell lymphoma-2; BSEP, bile salt export pump; CHOP, C/EBP-homologous protein; CPT-1β, carnitine palmitoyltransferase 1β· CYP7A1, cholesterol 7α-hydroxylase; CYP8B1, 7-alpha-hydroxycholest-4-en-3-one 12-alpha-hydroxylase; CYP27A1, sterol 27α-hydroxylase; DAG, diacylglycerol; FABP1, fatty acid binding protein 1; FAS, fatty acid synthase; Fas, factor associated suicide; FXR, farnesoid X receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GluT2, glucose transporter 2; KEGG, Kyoto Encyclopedia of Genes and Genomes; FATP4, fatty acid transport protein 4; FAT/CD36, fatty acid translocase/CD36; IBABP, ilealum bile acid -binding protein; IL-6, interleukin-6; LKB1, liver kinase B1; LPL, lipoprotein lipase; LXR, liver X receptor; MCP1, monocyte chemotactic protein-1; MRP2/3, multidrug resistance resistance-associated protein 2/3; NAFLD, nonalcohol fatty liver disease; NASH, non-alcoholic steatohepatitis; NPC1L1, Niemann-Pick type C1 -like intracellular cholesterol transporter 1; NTCP, Na^+/taurocholate co-transporting polypeptide; HFC, high-fat and cholesterol diet; OATP, organic anion transporting polypeptides; ORO, oil red O staining; OSTβ, organic solute transporter β· OTU, operational taxonomic units; PCoA, principal co-ordinates analysis; PCoA, principal coordinates analysis; PGC-1α, peroxisome proliferator-activated receptorγcoactivator-1α· TGF-β1, transforming growth factor-β1; TGR5, G-protein -coupled bile acid receptor 1; TNF-α, tumor necrosis factor-α, TG, triglyceride; UCP2, uncoupling protein 2.

What is already known

- *A. muciniphila* serves as a potential probiotic to treat obesity-related metabolic disorders.
- L-aspartate is an approved therapeutic agent for acute and chronic hepatitis treatment.

What this study adds

The therapeutic potency of *A. muciniphila* in treating NAFLD in mice through a novel mechanism.

What is the clinical significance

A. muciniphila provides a promising new treatment for NAFLD through its regulation on L-aspartate.

INTRODUCTION

Nonalcohol fatty liver disease (NAFLD) is defined as a wide spectrum of liver diseases, including simple steatosis (NAFL), non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis, and even hepatocellular carcinoma (HCC) (Byrne & Targher, 2015; Rinella & Sanyal, 2016). NAFLD has become a leading liver disease, with a prevalence of 22%–29% in adults worldwide, greater than 75% of whom are overweight and obese (Cole et al., 2018). However, to date, no approved pharmacological agents are available for NAFLD/NASH treatment.

NAFLD is a metabolic syndrome characterized by heavy lipid accumulation, and associated with oxidative stress, lobular inflammation, apoptosis, and a certain degree of fibrosis progressively in clinical hepatic pathologic findings (Friedman et al., 2018). Among these manifestations, hepatic steatosis serves as the first initiator (first hit), displaying aggressive injury to hepatocytes, and followed by multiple hits, including oxidative stress, mitochondrial dysfunction and degeneration, and apoptosis, which lead to fibrosis in the liver (Schuster et al., 2018). Our previous and recent studies have demonstrated that increasing hepatic lipid oxidation by pharmacologic treatment or gene editing is an efficacious approach to combat NAFLD/NASH by decreasing the lipid content and ameliorating progressive oxidation stress, lipotoxicity, inflammation and

fibrosis (Rao et al., 2019; Yang et al., 2014; Zheng et al., 2017), findings that agree with the recommendation of anti-NASH drug development (Diehl & Day, 2017).

The human gut microbiota is a virtual metabolic and endocrine organ that comprises approximately 10^{14} microbial cells inside the gut (Heintz-Buschart & Wilmes, 2018). These microorganisms are associated with the regulation of host metabolism, and immunity*via* their abundant metabolites, such as short-chain fatty acids (SCFAs) and amino acids (Adak & Khan, 2019; Festi et al, 2014). Recently, the gut microbiome has drawn extensive attention and has served as a therapeutic approach in combating NAFLD/NASH (Kirpich et al., 2015; Zhu et al., 2015). *Akkermansia muciniphila* , a well-known probiotic bacterium (Zhang et al., 2019), represents 1% to 5% of the microbial community in humans. Several studies have revealed that *A. muciniphila* is an efficacious pre-authorized agent to treat metabolic disorders clinically, such as obesity and diabetes (Depommier et al., 2019; Plovier et al., 2017; Everard et al., 2013). Recently, a few studies have shown that the efficacious treatment of NAFLD/NASH *in vivo* by diet or drugs results in the enrichment of*A. muciniphila* in the gut. Moreover, to date, the detailed mechanism underlying the therapeutic ability of *A. muciniphilavia* metabolic regulation is not fully understood.

Presently, we explored the therapeutic properties of *A. muciniphila* in treating NAFLD in an obese mice model induced by high-fat and high-cholesterol (HFC) diets using different interventions. Moreover, we revealed a novel mechanism underlying the metabolic regulation of *A. muciniphila* in treating NAFLD in mice. Our data revealed that treatment with *A. muciniphila* in obese mice efficiently ameliorated NAFLD. Mechanistically, mice treated with*A. muciniphila* efficiently increased lipid oxidation in the gut, ameliorated metabolic stress and inhibited the apoptosis of intestine cells, thus maintaining the integrity of the gut barrier and reshaping the gut microbiota composition. Moreover, *A. muciniphila*treatment improved bile acid metabolism between gut-liver axis interactions. Interestingly, these beneficial metabolic effects were occurred with increased L-aspartate levels that transported from the gut. Moreover, gavage of L-aspartate in mice directly increased energy expenditure and improved bile acid metabolism between gut-liver axis interactions.

METHODS

2.1 Materials

L-aspartate(Sigma, O7125, China), Hochest 33342 probe (Thermo, China), Nile red probe (Sigma, N3013, China), Mito-tracker (Thermo, M7512, China), TG assay kit (Jiancheng Bio Cat# A110-2, Jiangsu, China) and DAG assay kit (Uscn Life Science Inc Cat# CEC038Ge, Shanghai, China), L-aspartate ELISA kit (SinoBestBio, YX-12010M, China), FAS antibody (Cell Signaling Technology Cat# 3180, RRID:AB_2100796),*ACC*antibody (Cell Signaling Technology Cat# 3662, RRID:AB_2219400),*IL-6*antibody (Cell Signaling Technology Cat# 12912, RRID:AB_2798059),*TNΦ-α*antibody (Affinity Biosciences Cat# AF7014, RRID:AB_2835319), p*LKB1*antibody (Santa Cruz Biotechnology Cat# sc-271924, RRID:AB_10610759), PGC-1α antibody (Abcam Cat# ab191838, RRID:AB_2721267), CPT-1β antibody (Affinity Biosciences Cat# DF3904, RRID:AB_2836257), mitochondrial complex antibody, CD68 antibody (Abcam# ab955, Guangzhou, China; RRID: AB_307338). CHOP antibody (Affinity Biosciences Cat# DF6025, RRID:AB_2838000), and E-cadherin antibody (Affinity Biosciences Cat# AF0131, RRID:AB_2833315), TRIzol reagent (Invitrogen, Cat# 15596018, Guangzhou, China), cDNA synthesis kit (Takara, Cat# 6210B, Dalian, China). Details of the primer sequences used in the study are shown in Supporting Information.

2.2 *Akkermansia muciniphila* culture

Akkermansia muciniphila strain ATCC BAA-835 was cultured in modified brain-heart infusion (BHI) liquid medium at 37 under anaerobic conditions. The following were added to the standard BHI (37 g/L, BD) base ingredients: mucin (2 g/L, Sigma) and*L-cysteine*(5 mg/L, Sigma) (10% H2, 10% CO_2, 80% N_2).

2.3 Animal experiments

All animal care and experimental procedures were approved by the Sun Yat-sen University Committee on Animal Ethics for the Use of Laboratory Animals in accordance with the Animal Welfare Legislation of

China. Every effort was made to minimize the use of the animals and their discomfort. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al, 2010; McGrath et al, 2015) and with the recommendations made by the British Journal of Pharmacology. Male C57BL/6 mice (IMSR Cat# JAX:000664, RRID:IMSR_JAX:000664) aged 7–8 weeks (18–20 g) bred at the Laboratory Animal Centre of Sun Yat-sen University (Guangzhou, China) were used for the study. The mice were housed under specific pathogen free and reared in line with standardized methods at 22 ± 1°C on a 12-hr light/dark cycle with free access to food and water.

After 1 week of acclimatization to the environment of this study, the mice were fed with a 60% high fat and 1.2%*cholesterol*(HFC) diet (ResearchDiet, USA) ad libitum for up to 17 weeks, and HFC fed mice were randomly divided into two subgroups at the beginning of week 11 to receive phosphate buffered solution (PBS, control group) or*A. muciniphila* treatment for 6 weeks. The *A. muciniphila*were dissolved in oxygen-free PBS and orally each other day with a final concentration of $1x10^8$ CFU per mL. Suspension solution (200 μL) was given every other day. The control subgroup mice were administered the same volume of vehicle.

For the germ-free mice model, HFC mice were orally fed with Antibiotics mixture (Abx) for 1 week prior of week 11 and then received the treatment of Abx or *A. muciniphila* alone or together for another 6 weeks. The Abx was dissolved in drink water. Mouse body weight were monitored daily.

2.4 Plasma biochemistry analysis

At the end of the study, mice were fasted for 8 h and anaesthetized by an *i.p.* injection of 80 $mg{\cdot}kg^{-1}$ ketamine and 10 $mg{*}kg^{-1}$ xylazine. When the mice were fully anaesthetized, the eyeball was removed to collect blood samples in a tube containing 1 mM EDTA for the measurement of hepatic relevant enzymes, such as alkaine phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

2.5 Determination of hepatic triglyceride (TG) and diacyglycerol (DAG) levels

Liver TG and DAG were extracted and determined as described previously (Rao et al., 2019). Briefly, the liver tissue was weighted, homogenized and lysis. The supernatant was collected and extracted with equal volumes of chloroform/methanol. The chloroform phase was removed to a new tube and dried and was then re-suspended in isopropyl alcohol as a total lipid extract sample. The quantities of total TG and DAG in the livers were then assayed according to the manufacturers' protocols.

2.6 Histological examination

The interested tissues were weighed and subjected to oil-red O staining or fixed in 4% formaldehyde solution, then embedded in paraffin after dehydration in a graded ethanol series (70–100%). Embedded samples were sectioned (4 μm thick) with a rotary microtome (Leica, Germany) and subject to haematoxylin and eosin (H & E). The liver macrophages were assessed by immunohistochemistry labelled with CD68 and IL-6. To determination of the metabolism, apoptosis and the integrity of gut barrier in gut, the level of protein peroxisome proliferator-activated receptorγcoactivator-1α (PGC-1α), C/EBP-homologous protein (CHOP), and E-cadherin were examined. Representative images were captured and quantification analysis was performed in 10 randomly selected fields per sample in a blinded manner as our previously reported (Rao et al., 2019).

2.7 16S rRNA sequencing amplicon

The bacterial DNA was extracted from fecal samples with a QIAamp DNA stool Mini Kit (Qiagen, Germany) and PCR amplification was conducted with barcoded specific bacterial primers targeting the variable region 3–4 (V3–V4) of the 16S rRNA gene: forward primer 5'-ACTCCTACGGGAGGCAGCA-3' and reverse primer 5'-GGACTACHVGGGTWTCTAAT-3'. Construction of sequencing libraries and paired-end sequencing was performed on an Illumina MiSeq platform at Biomarker Technologies Co, Ltd. (Beijing, China) according to standard protocols. Raw sequences were merged and quality filtered, and duplicates were removed by FLASH, Trimmomatic, and UCHIME, respectively. The resulting sequences were then aligned against the Greengenes database of 16S rRNA gene sequences. Reads were then performed using quantitative insights

into microbial ecology (QIIME) analysis. Raw sequences were deposited in the Sequence Read Archive database (http://www. ncbinlm.nih.gov/sra), with the accession numbers ranging from SAMN16252123 to SAMN16252158.

2.8 Total RNA extraction and real time quantative-PCR

Total RNA from cells or tissues was isolated using the TRIzol method. The first- strand cDNA was synthesized with a cDNA synthesis kit. Quantitative real-time PCR was carried out using 2 × RealStar Green Fast Mixture with ROX (GenStar, Cat# A301, Guangzhou, China). The results were analysed on an ABI StepOnePlus real-time PCR system (Applied Biosystems, USA, RRID: SCR_015805) using the 2^{-Ct}method. Primers were synthesized by Generay Biotech (Guangzhou, China), and primers sequences were listed in **Table S1** . Actin was used as a loading control, and relative mRNA levels were normalized to actin.

2.9 Immunoblotting and immunofluorescence

Immunoblotting and immunostaining were performed as previously described (Rao et al., 2019). Briefly, cells or tissues were lysed and total protein were extracted and quantified. Proteins were subjected to SDS-PAGE and transferred to PVDF membrane, and blocked with 5% bovine serum albumin (BSA) - Tris buffered saline Tween for 30 min at room temperature, then membrane were incubated with primary antibodies overnight and followed secondary antibody incubation, protein bands were visualized with an ECL kit (Millipore). The uncropped data of immunoblotting were uploaded as a supplemented file. For Immunostaining, cells were fixed and blocked, then incubated with primary antibodies at 4 °C overnight. After washing, cells were co-stained with fluorescent labeled secondary antibody and 2 μg/mL DAPI (for nucleus) at 37 °C for 1 h. Fluorescence images were acquired using confocal microscope (Zeiss, Germany) with a 60× UPlanApoN oil immersion lens (NA 1.40).

2.10 Hepatic metabolomics analysis

The metabolites in livers were extracted with 50% methanol Buffer. 20 μL of sample was extracted with 120 μL of precooled 50% methanol, vortexed for 1 min, and incubated at room temperature for 10 min; the extraction mixture was then stored overnight at -20 °C. After centrifugation at 4,000 g for 20 min, the supernatants were transferred into new 96-well plates for LC-MS analysis. In addition, pooled QC samples were also prepared by combining 10 μL of each extraction mixture. These samples were then subjected to LC-MS system followed machine orders. A high-resolution tandem mass spectrometer TripleTOF5600 plus (SCIEX, UK) was used to detect metabolites eluted from the ACQUITY UPLC BEH Amide column (100mm×2.1 mm, 1.7 μm, Waters, UK). The Q-TOF was operated in both positive and negative ion modes. The curtain gas was set 30 PSI, Ion source gas1 was set 60 PSI, Ion source gas2 was set 60 PSI, and an interface heater temperature was 650 °C. For positive ion mode, the ionspray voltage floating were set at -4500V, respectively. The mass spectrometry data were acquired in IDA mode. The TOF mass range was from 60 to 1200 Da. The survey scans were acquired in 150 millisecond and as many as 12 product ion scans were collected if exceeding a threshold of 100 counts per second (counts/s) and with a 1+ charge-state. Total cycle time was fixed to 0.56 s. Four time bins were summed for each scan at a pulser frequency value of 11 kHz through monitoring of the 40 GHz multichannel TDC detector with four-anode/channel detection. Dynamic exclusion was set for 4 s. During the acquisition, the mass accuracy was calibrated every 20 samples. Furthermore, in order to evaluate the stability of the LC-MS during the whole acquisition, a quality control sample (Pool of all samples) was acquired after every 10 samples.

2.11 Mitochondrial copy number quantification

Quantification of mitochondrial DNA (mtDNA) copy numbers was achieved by PCR. Briefly, DNA was extracted from cells or Liver tissue using a DNeasy Blood and Tissue kit (Tiagen Biotech, China). The copy numbers of nuclear DNA (nDNA) and mtDNA were assessed by PCR targeted toward the cytochrome C gene (for mtDNA) and 18S rRNA (for nDNA).

2.12 L-aspartate level determination

The levels of L-aspartate in liver, feces and plasma were measured by an L-aspartate ELISA kits according to the manufacturer's instructions. The feces and liver samples were dissolved and sonicated in saline buffer and centrifuged at 1,000 g for 10 min at 4°C to remove debris. Briefly, 20 μL samples, saline, and standard was added to the ELISA plate and incubated at 37 °C for 1 h. Wells were washed four times with elution buffer and then incubated with biotin-labelled antibodies at 37 degC in the dark for another 30 min followed by reaction with the corresponding substrate. The colour produced was proportional to the concentration of L-aspartate was measured at 450 nm and quantified against the standard curve from the known amount of the cytokine.

2.13 Metabolic flux assay

Cellular metabolic rates were measured using a XF96 Analyzer (Seahorse Bioscience, USA). L-02 cells were treated with vehicle or L-aspartate for 24 h, the ECAR and OCR were determined by a Seahorse Bioscience XF96 Extracellular Flux Analyzer (Seahorse Bioscience, USA), with 2 μM*oligomycin*, 1.5 μM FCCP and 1 μM antimycin A/rotenone injected during fixed time intervals. The OCR were normalized by protein level in each well. Mitochondrial respiration of BAT isolated from mice was determined using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience, USA) using 5 μg mitochondrial protein in a buffer containing 50 mM KCl, 4 mM KH_2PO_4, 5 mM HEPES, and 1 mM EGTA, 4% BSA, 10 mM Pyruvate, 5 mM Malate, 1 mM GDP. Mitochondria were isolated using a mitochondria isolation kit (Thermo, China), plated and centrifuged at 2,000 g for 20 min to promote adherence to the cell culture microplate. One millimole of*ADP*, 4 mM Oligomycin, 6 mM FCCP, and 2 mM each of Antimycin A/Rotenone were added during fixed time intervals.

2.14 Statistical analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018). Cell experiment included 5 independent experiment for each data set and 8 to 10 mice was included in each group of animal study, the results were presented as the mean ± SEM unless stated otherwise. Data are expressed as the mean ± SEM. Differences between two groups were analysed by Student's t-test using Graphpad Prism (Graphpad Software Inc, California, USA, RRID:SCR_002798). Statistical analysis for multiple groups was performed by one-way ANOVA followed by Tukey's HSD post hoc tests. A P value of [?].05 was considered statistically significant.

2.15 Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018; Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017a; Alexander et al., 2017b).

3. RESULTS

3.1 *A. muciniphila* attenuates NAFLD in obese mice

We evaluated the therapeutic effect of *A. muciniphila* in treating NAFLD in an obese mouse model induced by high-fat and high-cholesterol (HFC) diet feeding (**Figure 1A**). Compared with the HFC group mice, mice treated with *A. muciniphila* efficiently increased the abundance of *A. muciniphila* in the gut by 20 fold compared with that in HFC mice by quantitative polymerase chain reaction (qPCR) (**Figure 1B**). This activity led to decreased mouse body weight, as indicated by an approximately 20.8% body weight reduction in*A. muciniphila* -treated HFC mice after 6 weeks of treatment (**Figure 1C**). Intriguingly,*A. muciniphila* treatment efficiently attenuated hepatic steatosis in HFC mice, indicated by decreased hepatic TG (**Figure 1D**). Diacylglycerol (DAG) is a representative intermediate of lipid metabolism that induces activation of oxidative stress and displays toxicity to hepatocytes, leading to apoptosis and liver injury *in vivo* (Samuel & Shulman, 2018). Notably, treatment with *A. muciniphila* in obese mice markedly decreased the DAG levels in the liver (**Figure 1E**). The plasma levels of liver-specific enzymes revealed that *A. muciniphila*significantly ameliorated liver injury in HFC mice, as indicated by decreased levels of aspartate aminotransferase (AST),

alanine aminotransferase (ALT) and alkaline phosphatase (ALP) (**Figure 1F**). Additionally, pathologic examination further confirmed the therapeutic potency of *A. muciniphila* in ameliorating all the phenotypes of NAFLD in mice, namely hepatocyte injury and ballooning (indicated by HE staining), hepatic steatosis (indicated by ORO staining), and inflammation (indicated by IL-6 and CD68 examination) (**Figure 1G**). Gene expression analysis revealed that *A. muciniphila* markedly suppressed the expression of genes related to steatosis and inflammation at the transcriptional level, such as tumuor necrosis factor a (*TNF-a*), interleukin-6 (*IL-6*), monocyte chemotactic protein 1 (*MCP-1*), fatty acid synthase (*FAS*), and acetyl CoA carboxylase (*ACC*) (**Figure 1H**).

To explore whether these anti-NAFLD effects of *A. muciniphila*were drug-treatment dependent, the HFC mice were treated with *A. muciniphila* orally for 6 weeks, followed by the withdrawal of *A. muciniphila* for another 4 weeks (**Figure 1I**).Interestingly, *A. muciniphila* withdrawal caused efficient persistence of its anti-NAFLD activities, as indicated by significant reductions in the mouse body weight (**Figure 1J**), hepatic TG levels (**Figure 1K**), and plasma levels of AST, ALT, and ALP (**Figure 1L**) in the post-drug withdrawal group. Pathologic examination further confirmed the conclusion that *A. muciniphila*withdrawal caused sustained anti-NAFLD activity in mice (**Figure 1M**).

We further determined the anti-NAFLD properties of *A. muciniphila*in germ-free mice by treating the HFC mice with an antibiotic mixture (Abx) orally (**Figure S1A**). Treatment with Abx in mice efficiently decreased the abundance of *A. muciniphila* and its colonization in the gut (**Figure S1B**). This activity led to exacerbated liver injury and NAFLD manifestation in mice, as indicated by increased liver weight, heavy hepatic TG accumulation, and liver injury in Abx-treated HFC mice (**Figure S1C-H**). Additionally, there were still significant NAFLD-attenuating effects in the Abx and*A. muciniphila* co-treatment group compared with that in the HFC mice or Abx-treated HFC mice. Gene expression analysis further confirmed that the ability of *A. muciniphila* in downregulating the genes expression levels of *FAS* , *ACC* , *TNF-a* , *IL-6* , and *MCP1* (**Figure S1H**). The above data demonstrate that treatment with *A. muciniphila* efficiently alleviates NAFLD in mice.

3.2 Gavage of *A. muciniphila* reshapes the gut microbiota composition in obese mice

The data of 16S rRNA amplicon sequencing from the faeces of HFC or*A. muciniphila*- treated HFC mice were subjected to principal co-ordinates analysis (PCoA) and two distinguished clusters corresponding to the HFC mice and *A. muciniphila* -treated HFC mice revealed substantial differences in the microbial community structure between the groups (**Figure 2A**). Gavage of *A. muciniphila*in the mice increased the richness and diversity of gut microbiota by α-diversity analysis [indicated by observed operational taxonomic units (OTUs) and Shannon index] (**Figure 2B**) and improved the microbial community structure, including reducing the relative abundance of *Desulfovibrionacceae* , *Blautia* , *Lachnospiraceae* , and increasing the relative abundance of *A. muciniphila* ,*Faecalibaculum* , and *Lactobacillus* (**Figure 2C-D**). LEfSe [(linear discriminant analysis (LDA) score (log10) = 4] was employed to characterize the different distributions of the microbiota between the HFC control and *A. muciniphila* -treated HFC mice. Notably, *A. muciniphila* was identified as one of the distinguished microbiota components by LEfSe analysis ($p = 0.0096$; **Figure 2E**).

3.4 *A. muciniphila* activates lipid oxidation in the gut-liver axis and maintains gut barrier integrity

Studies have reported that treatment with *A. muciniphila*increases lipid oxidation and activates the AMP-dependent protein kinase (*AMPK)*pathway, which are the foundations underlying its therapeutic properties in obesity (Plovier et al., 2017; Everard et al., 2013). In the present study, mice treated with *A. muciniphila* showed increased hepatic mitochondrial copy numbers (**Figure 3A**) and activated a network of genes involved in lipid transportation [fatty acid transport protein 4 (*FATP4)* , fatty acid translocase/cluster of differentiation 36 (*FAT/CD36)*] and oxidation [lipoprotein lipase (*LPL*), peroxisome proliferator-activated receptor γ coactiva-tor-1a (*PGC-1a*), uncoupling protein 2 (*UCP2*), carnitine palmitoyltransterase-1β (*ΨIT-1β*), and liver X receptor (*LXR)*] in the liver of HFC mice (**Figure 3B**). Immunoblotting assays revealed

that treatment with *A. muciniphila* in obese mice activated the liver kinase B1 (LKB1)-AMPK axis, and increased the expression of energy metabolic regulators (PGC-1a and CPT-1β) and mitochondrial complexes (I, II, IV, and V) in the liver (**Figure 3C**).

Consistent with the beneficial metabolic profile in the liver, mice treated with *A. muciniphila* also activated a network of genes involved in glucose and lipid transportation (indicated by *FATP4* and *GluT2*) and oxidation (indicated by *ΠΓ-1a*) in the ileum and colon, respectively (**Figure 3D, Figure S2A**). Immunohistochemistry analysis of PGC-1a in the gut further confirmed the metabolic stimulation effects of *A. muciniphila* (**Figure 3E, Figure S2B**). These improved metabolic effects led to the reduction of the lipid levels (indicated by oil red O staining) and attenuated oxidation stress-induced cell apoptosis in the gut [indicated by C/EBP-homologous protein (CHOP) protein examination] (**Figure 3F, Figure S2C**), thus improving the integrity of the gut barrier, as indicated by examination of the marker for cell tight connection (E-cadherin) and HE staining (**Figure 3G** , **Figure S2D**).

3.5 *A. muciniphila* attenuates bile acid metabolism dysfunction in the gut-liver axis in obese mice

In vivo , bile acid is mainly synthesized in liver, transported to the tissues of the gut and gallbladder, and then refluxed to the liver. Additionally, its transportation is tightly regulated by a network of genes, including bile acid synthesis related genes [e.g., cholesterol 7α-hydroxylase (*CYP7A1*), 7-alpha-hydroxycholest-4-en-3-one 12-alpha-hydroxylase (*CYP8B1*), and sterol 27α-hydroxylase (*CYP27A1*)], bile acid transportation related genes in the liver [e.g., G-protein coupled bile acid receptor 1 (*TGR5*), bile salt export pump (*BSEP*), multidrug resistance associated protein 2/3 (*MRP2/3*)], bile acid salt reflux regulatory genes [apical sodium-dependent bile salt transporter (*ASBT*), ileal bile acid-binding protein (*IBABP*), organic solute transporter β (*OΣTβ*), Na^+/taurocholate co-transporting polypeptide (*NTCP*), and organic anion co-transporting polypeptides (*OATP*)] (Jia et al., 2018) (**Figure 4A**). Studies have reported that clinical obese and NAFLD populations often demonstrate abnormal levels of bile acid metabolism, such as high levels of bile acid metabolites in the gut and plasma (Rosso et al., 2018; Yu et al., 2018). Moreover, high levels of bile acids in the gut alter the composition in the gut and are harmful to gut microbiota survival, leading to the development of chronic metabolic disorders (Marra et al., 2018; Arab et al., 2017).

We then explored the modulating effect of *A. muciniphila* on bile acid metabolism in the gut-liver axis. As shown in **Figure 4B** , mice treated with *A. muciniphila* activated a network of genes involved in bile acid synthesis and transportation in the liver. Additionally, gavage of *A. muciniphila* in HFC mice increased the expression levels of bile acid reflux regulatory genes in the gut and liver, such as *ASBT* , *IBABP* , *OΣTβ* , *NTCP* and *OATP* (**Figure 4B -C**). Furthermore, treatment with *A. muciniphila* in mice inhibited cholesterol absorption [indicated by the decreased level of the cholesterol transporter Niemann-Pick type C1-like intracellular cholesterol transporter 1 (*NPC1L1*)] and bile acid synthesis related genes in the ileum (**Figure 4C**), demonstrating that *A. muciniphila* alters the bile acid composition in the gut and attenuates bile acid metabolism dysfunction in the gut-liver axis in mice. This finding explained its gut microbiota-resharping effect. Correspondingly, HFC mice treated with *A. muciniphila* decreased the plasma and hepatic cholesterol levels (**Figure 4D**), suggesting that *A. muciniphila* is beneficial for bile acid metabolism in the gut-liver axis.

Additionally, mice treated with *A. muciniphila* showed increased levels of *mucin 2* , *mucin 5ac* , *mucin 5b* , *mucin 12* and *mucin 20* in the colon (**Figure 4E**), genes that encode mucoproteins that are key substrates for *A. muciniphila* growth and colonization in the gut.

3.6 *A. muciniphila* increases the hepatic L-aspartate level by facilitating L-aspartate transportation from the gut

To reveal the possible mode of action underlying the anti-NAFLD activities of *A. muciniphila* , we determined its metabolic profile in the liver by metabolomics analysis. Principal coordinates analysis (PCA) revealed four distinguished clusters, corresponding to the HFC, HFC+*A. muciniphila* , HFC+Abx, and HFC+Abx+*A. muciniphila* mouse group (**Figure 5A**), suggesting substantial differences in the chemical

components among these groups. Compared with HFC mice, mice treated with *A. muciniphila* displayed a different metabolism profile, as indicated by the detection of 247 metabolites with significant differences (86 downregulated and 161 upregulated) (**Figure 5B, left panel**). The identified metabolites were assigned to categories according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. In total, 98 metabolites were classified into the top 20 KEGG second-level pathways (**Figure S3A**). Additionally, a more significantly differentially metabolites were detected in the livers of the combination group of *A. muciniphila*and Abx compared with those in HFC mice treated with Abx alone. In total, 628 significant differentially metabolites were detected (105 downregulated and 523 upregulated) (**Figure 5B, right panel**). These altered metabolites were annotated to be correlated with hepatic metabolism, such as lipolysis, fat digestion and absorption, insulin resistance, and bile acid biosynthesis (**Figure S3B**). Next, we performed metabolic trend analysis among these groups. Intriguingly, several interesting metabolites were identified, such as aspartic acid, corticosterone, acylcarnitine, and trans-retinoic acid, *etc* , and were assigned to categories according to the KEGG database (**Figure 5C**). Among them, L-aspartate was identified by occupying a higher significance level (˜2.18-fold) and a lower p value ($p = 0.0000114$) than that in HFC control mice (**Table S2**), a finding that was correlated with most of the annotated top 20 KEGG pathways, such as metabolic, pathway, pyrimidine metabolism, glycerolipid metabolism, alanine, aspartate, and glutamate metabolism, arginine and proline metabolism, and nitrogen metabolism. Quantification of hepatic L-aspartate level revealed that *A. muciniphila* treatment significantly increased the hepatic L-aspartate levels while reduced hepatic L-aspartate levels were detected in Abx-treated HFC mice (**Figure 5D**). Additionally, Abx treatment partly abrogated the stimulation effect of *A. muciniphila* in increasing the hepatic L-aspartate levels compared with *A. muciniphila* treatment alone. Furthermore, the hepatic L-aspartate levels was negatively correlated with the hepatic TG content (**Figure 5D**), indicating that L-aspartate might be a potential agent for NAFLD treatment, a finding that was consistent with the scenario that L-aspartate might be an efficacious agent in treating NAFLD (Canbay & Sowa, 2019).

We further determined the levels of L-aspartate in plasma and faeces.*A. muciniphila* treatment efficiently increased the L-aspartate levels in plasma and faeces while Abx treatment also increased the L-aspartate level in faces but decreased the level of L-aspartate in the plasma and liver (**Figure 5D-E**). These results demonstrated that the gut microbiota (e.g., *A. muciniphila*) might play important roles in the production and transportation of L-aspartate. Consistently, gene expression of the L-aspartate transporter (*Slc1a1 or Slc1a2*) in the ileum or liver revealed that mice treated with *A. muciniphila* increased the expression levels of *Slc1a1* or*Slc1a2* while Abx partially abolished the stimulation effect of*A. muciniphila* in increasing the gene expression of the L-aspartate transporter (**Figure 5F-G**).

3.7 L-aspartate directly activates lipid oxidation and the LKB1-AMPK axis

We exposed L-aspartate to hepatocytes (L-02 cell) in the presence of oleatic acid induction, a well-known reported cell model that mimics hepatic steatosis *in vitro* and found that the addition of L-aspartate efficiently decreased the cellular lipid levels, as indicated by the TG assay and Nile red staining (**Figure 6A-B**). This reduction occurred with increased expression levels of metabolic energy regulators (PGC-1α) and mitochondrial complexes and activation of the LKB1-AMPK axis (**Figure 6C**), leading to inhibition of oxidative stress, as indicated by a decrease in the reactive oxygen species (ROS) levels (**Figure 6D-E**). This beneficial metabolic effect was also replicated in intestinal cells (**Figure S4**). These novel findings agreed well with the latest report revealing the metabolic role of L-aspartate in activating the LKB1-AMPK axis in HCT-116 cells (Deng et al., 2020).

To further unravel the direct metabolic role of L-aspartate *in vivo* , mice orally treated with L-aspartate efficiently activated the gene expression of the L-aspartate transporter at the transcriptional level and increased L-aspartate levels in the liver (**Figure S5A-B**). Additionally, L-aspartate treatment activated a network of genes correlated with hepatic energy oxidation, such as *ΠΓ˜-1α* ,*UCP2* , and *˜ΠT-1β* (**Figure S5C**). Immunohistochemistry analysis of PGC-1α and UCP2 in the liver further confirmed the direct metabolic beneficial effect of L-aspartate in liver (**Figure S5D**). These metabolic improvements resulted in a slight reduction in the endogenous hepatic TG level without liver injury (**Figure S5E**).

We also explored the modulating effect of L-aspartate on bile acid metabolism in the gut-liver axis. Interestingly, L-aspartate treatment markedly activated a network of genes involved in bile acid synthesis and transportation in the gut-liver axis (**Figure S5F-G**). These data further confirmed the direct metabolic effects of L-aspartate in regulating energy metabolism, such as lipid oxidation and bile acid metabolism.

4. DISCUSSION

The present study investigated the therapeutic effects of the probiotic *A. muciniphila* on NAFLD and its underlying molecular mode in a mouse model. Our study showed that obese mice treated with *A. muciniphila* eliminated hepatic steatosis, inflammation, and liver injury. These therapeutic effects largely fulfilled the recommended criteria essential for the treatment of NAFLD (Diehl & Day, 2017). Interestingly, these anti-NAFLD actions of *A. muciniphila* in mice also persisted after the withdrawal of *A. muciniphila* treatment or the administration of *A. muciniphila* after antibiotics treatment. Notably, these therapeutic effects occurred with increases in the L-aspartate levels in the gut and liver, which direct stimulated lipid oxidation in the gut-liver axis, thus inhibiting oxidative stress, maintaining the integrity of the barrier. Furthermore, *A. muciniphila* or L-aspartate treatment activated bile acid metabolism in the gut-liver axis and improved gut-liver interactions. These metabolic effects were beneficial to reshape gut microbiota composition after *A. muciniphila* treatment.

The development of NAFLD/NASH is involved in multiple hits (Trépo & Valenti, 2020). Among them, hepatic steatosis is recognized as the initial factor, characterized by heavy lipid accumulation originated from increased *de novo* lipogenesis and/or excessive flux of exogenous FFAs (Feldstein et al., 2003). Hepatic lipogenesis is regulated by a series of lipogenic enzymes, including ACC and FAS, while FFA flux is regulated by its transporters, including FAT/CD36 and FATP4 (Bechmann et al., 2012). Our results showed that *A. muciniphila*treatment significantly downregulated the expression of lipogenic markers and increased the levels of FAT/CD36 and FATP4 in the liver. Furthermore, ectopic accumulation of lipids in the liver induced progressive oxidative stress, leading to mitochondrial dysfunction and degeneration (Rector et al., 2010). Additionally, the activation of oxidative stress caused hepatocyte apoptosis in the liver (Chen et al., 2020). Recent studies have confirmed that decreased lipid levels by promoting lipid oxidation is an efficacious approach in treating NAFLD (Rao, et al., 2019; Fan et al., 2020). Consistently, we found that *A. muciniphila* significantly increased the mitochondrial copy number, enhanced the lipid flux and oxidation in the liver, thus inhibiting hepatic steatosis and its accompanying oxidative stress and protecting the liver from steatosis induced by chronic HFC diet feeding. These results indicate that the mode of *A. muciniphila* in attenuating the first hit of NAFLD in mice is attributed to the inhibition of lipogenesis and increased oxidation of fluxed lipids.

A. muciniphila , an intestinal bacterium isolated from human faeces in 2004, has been demonstrated to have marked potential to treat metabolic disorders, such as obesity and diabetes (Deng et al., 2020). However, its therapeutic efficacies in curing NAFLD/NASH have been less documented. Presently, we revealed that mice treated with *A. muciniphila* showed significantly ameliorated NAFLD, which provides a new experimental clue to expanding the application of *A. muciniphila* in treating metabolic disorders in the clinic.

Although the beneficial metabolic activity of *A. muciniphilain vivo* has been well-documented, the detailed molecular mechanism remains elusive. Our study revealed that mice treated with *A. muciniphila* increased the L-aspartate levels in plasma and liver. Furthermore, *A. muciniphila* treatment increased the expression level of the L-aspartate transporter in the ileum and liver, suggesting that *A. muciniphila* plays an important role in regulating L-aspartate transportation. This novel finding was quite unfamiliar with the traditional theory of L-aspartate being mainly produced in the liver (Nuzzo et al., 2020; Leng et al., 2014). Moreover, a negative correlation was observed between the L-aspartate level and the hepatic TG level, suggesting that *A. muciniphila* ameliorated NAFLD in mice may be closely related to the increased levels of L-aspartate.

L-aspartate is a non-essential amino acid and involved in treatment for acute and chronic liver hepatitis and cirrhosis treatment in the clinic by regulating nitrogen metabolism, nucleic acid synthesis and the Kreb's cycle (Hou et al., 2017; Leng et al., 2014). Recently, a study reported that L-aspartate supplementation

ameliorated diabetic kidney disease in mice (Ichikawa et al., 2020). Moreover, Jiang et al revealed that L-aspartate directly interacted with phosphorylated LKB1 and activated the LKB1-AMPK axis in cells (Deng et al., 2020). A recent study speculated that L-aspartate could be an efficacious therapeutic agent in treating NAFLD/NASH (Canbay & Sowa, 2019). Presently, we examined the direct beneficial metabolic role of L-aspartate *in vitro* and*in vivo* and found that L-aspartate addition directly increased lipid oxidation and activated the LKB1-AMPK axis, and has been proven to be a beneficial tool in combating NAFLD (Seo et al., 2015).

Dysfunction of the gut-liver axis is closely correlated with the development of NAFLD (Albillos et al., 2020). Among activities in the gut-liver axis, bile acid metabolism dysfunction plays pivotal roles in reshaping the gut microbiota composition and regulating the homeostasis of glucose and lipids in the liver (Molinaro et al., 2018). Studies have revealed that the plasma of NAFLD or NASH populations is often accompanied by abnormal bile acid level, which are positively related to the degree of NAFLD (Rosso et al., 2018; Yu et al., 2018). Moreover, high-fat and cholesterol diet feeding alters the composition of bile acids in the gut, causing imbalances in the intestinal flora and aggravating bile acid metabolism disorders (Zeng et al., 2020). By contrast, enhanced bile acid metabolism is beneficial to ameliorate NAFLD (Shapiro et al., 2018; Wang et al., 2018). Our study revealed that both *A. muciniphila* and L-aspartate treatment markedly increased bile acid metabolism and transport in the gut-liver axis. These ameliorated effects are beneficial to remodel the gut microenvironment and promote the colonization of *A. muciniphila* . Additionally, recent studies have demonstrated that the activation of oxidative stress and induction of cell apoptosis in the gut are harmful to the gut barrier, leading to the development of certain disease, such as inflammatory bowel disease (IBD), obesity and NAFLD. Consistently*A. muciniphila* treatment efficiently ameliorated oxidative stress-induced cell apoptosis in the gut, preserved cellular tight junctions and maintained the integrity of the gut barrier. These data further confirmed the therapeutic effects of *A. muciniphila* in treating metabolic disorders.

The present study has a few limitations. (i) We did not directly explore the therapeutic effect of L-aspartate in treating NAFLD in a mouse model of NAFLD. Based on its beneficial metabolic role, we believe that L-aspartate is likely efficacious in treating NAFLD *in vivo* . (ii) We did not provide the another clues to prove where the increased L-aspartate originated form. This need a further study to explore its anti-NAFLD/NASH actions and origin using relevant animal model.

In summary, our present study, for the first time, showed that *A. muciniphila* is a potential therapeutic agent for NAFLD treatment through a novel mechanism by increasing the L-aspartate levels in the liver transported from the gut. Treatment with L-aspartate in cells and in mice largely replicated the beneficial metabolic effects of *A. muciniphila* in mice, including increased lipid oxidation, activation of the LKB1-AMPK axis and improved bile acid metabolism in the gut-liver axis, leading to the amelioration of lipid accumulation-induced metabolic disorders in the tissues of the gut and liver (**Figure 7/Graphic abstract**). Our findings suggest that *A. muciniphila*attenuates NAFLD in mice by improving the gut-liver interactions*via* regulating L-aspartate metabolism.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

This study was designed by Y. Rao., Y. J., Lu, and Z. - S. Huang. Y. Rao., Z. Q., Kuang., C. Li.,S. Y., Guo., Y. - H. Xu., S.-Y. G., Y. - T. Hu., B. - B. Song, and Z., Jiang, Z. H., Ge., X. Y., Liu., C. D., Li performed biologic evaluation, animal study and mechanism study. Y. Rao., Z. - S. Huang. and Y. J., Lu, provided reagents, materials and analysis tools. Data was analyzed and interpreted by Y. Rao., Z. Q., Kuang. The manuscript was written by Y. Rao., Z. Q., Kuang., Y. J., Lu, and Z. - S. Huang., with input from all authors.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigor of preclinical research as stated in the BJP guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Figure legends

Figure 1 . *A. muciniphila* attenuates obese NAFLD induced by HFC diet feeding. (A-H) Eight-week-old male C57BL/6 mice were fed with an HFC diet for 11 weeks to induce obese NAFLD. They were then randomly divided into two subgroups to treat with PBS as a control (HFC group) or *A. muciniphila* in PBS (HFC+*A. muciniphila*) orally every other day for 6 weeks. (A) Schematic diagram of *A. muciniphila* treatment. (B) Abundance of *A. muciniphila* in faeces. (C) Mouse body weight at week 17. (D-E) Hepatic triglyceride (TG) and diacylglyceride (DAG) levels. (F) Plasma levels of AST, ALT and ALP. (G) Pathologic examination of the liver by haematoxylin eosin (H&E) staining, oil red O staining (ORO), and immunofluorescence examination of IL-6 and CD68. Representative images were captured. Scale bar, 200 μm. (H) Expression of mRNA markers for steatosis and inflammation markers. The levels of genes in the HFC control group were set as 1, and the relative fold increases were determined by comparison with the HFC control group. N = 5-8 mice / group. * $p < 0.05$, compared with HFC control group mice. (I-M) Sustainability of the anti-NAFLD efficacy of *A. muciniphila* after the cessation of its administration. (I) Schematic diagram of the experimental design. After 6 weeks of administration, *A. muciniphila* was withdrawn from the HFC-induced obese NAFLD mice followed by a period of 4 weeks of washout to evaluate of the sustainability of the anti-NAFLD efficacy. (J) Mouse body weight. (K) TG level in the liver. (L) Plasma levels of AST, ALT and ALP. (M) Pathologic examination of the liver by HE staining, oil red O staining, and immunohistochemistry assays of IL-6. Representative images were captured. Scale bar, 200 μm. N = 5 mice / group. * $p< 0.05$, compared with HFC control mice.

Figure 2 . *A. muciniphila* reshapes the gut microbiota composition in obese NAFLD mice. HFC-induced obese NAFLD mice (11 weeks of feeding) were administered PBS as the control or *A. muciniphila* (6 weeks of treatment). The faeces were collected for the 16S rDNA amplicon sequencing assay. (A) PCoA analysis. (B) Gut microbiota species (indicated by operational taxonomic units (OTUs)) and diversity determination (indicated by the Shannon index). (C-D) Taxonomic analysis of gut microbiota at the phylum (C) and genus (D) levels. (E) The LDA score shows a significant bacterial difference between HFC and HFC+*A. muciniphila* mice. Each sample included 3 replicates with 5 mice in one replicate. * $p <0.05$, compared with HFC control mice.

Figure 3 . *A. muciniphila* increases markers related to lipid metabolism in liver and gut. HFC induced obese NAFLD (11 weeks of feeding) were administered with saline as the control or A. *muciniphila* (6 weeks of treatment) to assess liver and ileum parameters: A-C for parameters in the liver and D-F for parameters in the ileum. E-G, representative images of the ileum (Scale bar, for 100 μm). (A) Hepatic mitochondrial copy number determination. (B) Gene expression related to lipid uptake and oxidation in the liver of mice. (C) Protein levels of metabolic regulators mitochondrial complexes, and the LKB1-AMPK

axis in the liver of mice. The protein levels were quantified and normalized to the loading control actin (D) Gene expression of lipid uptake and oxidation in the ileum of mice. The gene level or protein level in the HFC group was set as 1, and the relative fold increases were determined by comparison with the HFC control group. (E) Immunohistochemistry analysis of PGC-1α in the ileum of mice. The brown dot indicates the examined protein. Representative images were captured. Scale bar, 100 μm. (F) TG level quantification (indicated by oil red O staining) and oxidative stress-induced cell apoptosis determination (indicated by CHOP examination) in the ileum. Representative images were captured. Scale bar, 100 μm. (G) Immunohistochemistry analysis of E-cadherin and HE examination in ileum of HFC mice. The brown dot indicates the target protein. Representative images were captured. Scale bar, 100 μm. N = 5-8 mice/group. * $p < 0.05$, compared with HFC control mice.

Figure 4 . *A. muciniphila* promotes the metabolism of bile acid in the liver and gut. HFC-induced obese NAFLD (11 weeks of feeding) were administered saline as the control or A. *muciniphila* (6 weeks of treatment) to assess the liver and ileum parameters. (A) Schematic diagram of bile acid shuttling between the liver and gut. (B) Expression of mRNAs involved in the regulation of the synthesis and transport of bile acids in the liver. (C) Expression of mRNAs involved in the regulation of the synthesis and transport of bile acids in the ileum (D) Plasma level of cholesterol in plasma or the liver. (E) mRNA levels of mucoprotein-related genes in the colon of HFC mice. The level of genes in the HFC control group were set as 1, and the relative fold increases were determined by comparison with the HFC control group. N = 5-8 mice/group. * $p < 0.05$, compared with the HFC control group mice.

Figure 5 . *A. muciniphila* increases the hepatic L-aspartate level in the gut. HFC-induced obese NAFLD (11 weeks of feeding) were administered saline (HFC), *A. muciniphila* , antibiotics (Abx) or a combination of Abx plus *A. muciniphila* (Abx+*A. muciniphila*) for (6 weeks of treatment) to assess liver metabolomics and the indicated assays. (A) PCA analysis. (B) Identification of significant differentially metabolites in *A. muciniphila*- or Abx+*A. muciniphila*- treated HFC mice compared with HFC or Abx-treated HFC mice by LC-MS analysis. Significant differentially metabolites were identified based on the criteria of fold [?] 2 and a Q value equal to or higher than 0.05. The dots represent differentially metabolites. N = 5 mice/group. (C) Annotation of the identified significant metabolites in the HFC, HFC+*A. muciniphila* , HFC+Abx, and HFC+Abx+ *A. muciniphila* groups by metabolomics trend analysis. (D) Comparative analysis of the hepatic L-aspartate and TG levels. (E) L-aspartate level in the faeces and plasma of mice. N = 5 mice/group. (F) Gene expression of *Slc1a1* in the ileum of mice. (G) Gene expression of*Slc1a2* in the liver of mice. The level of mRNA in the HFC control group was set as 1, and relative fold increases were determined by comparison with the HFC control group. N = 5-8 mice/group. * $p<0.05$, compared with HFC control mice. # $p<0.05$, compared with antibiotics (Abx)-treated HFC mice;$^{\$}$ $p<0.05$, compared with *A. muciniphila* -treated HFC mice.

Figure 6 . L-aspartate reduces lipid and ROS in hepatocytes along with increases in LKB1-AMPK activity and mitochondrial oxidative capacity. L-02 cells induced with oleatic acid (0.75 mM) were treated with L-aspartate (10, 40 μM) for 24 h, and then cells were harvested for the indicated analysis. (A) TG level in cell lysates. (B) Nile red staining using a Nile red probe at a final concentration of 5 μM. (C) Key proteins in the LKB1-AMPK axis and mitochondrial oxidation. The protein levels were quantified against the loading control GAPDH. The protein levels in control cells were set as 1, and the relative fold increases were determined by comparison with the control group. (D-E) Quantification of the ROS levels by flowcytometry or confocal microscopy using the DCFH-DA probe at a final concentration of 10 μM. Representative images were captured. Scale bar, 100 μm. The data shown are individual values with means ± SEM, N = 5 independent experiments. *$p < 0.05$, compared with control group cells.

Figure 7 . Proposed mechanism by which *A. muciniphila*ameliorates NAFLD in HFC diet-induced obese mice. *A. muciniphila*treatment increases L-aspartate levels in the gut-liver axis, which activates the LKB1-AMPK axis and increases lipid oxidation and bile acid metabolism in the gut-liver axis, leading to decreased lipid accumulation and oxidation stress-induced cell apoptosis. These metabolic improvements result in maintaining the integrity of the gut barrier and reshaping the gut microbiota composition for NAFLD

treatment in mice.