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Dietary Intervention With *Lycium barbarum* Polysaccharide and Quinoa Ultrafine Powder Ameliorates Lipid Metabolism in Nonalcoholic Fatty Liver Disease by Regulating Gut Microbiota and Activating AMPK/SREBP-1C/SCD-1 Signaling

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ABSTRACT: Changes in intestinal microecology play a crucial role in the pathogenesis and progression of metabolic diseases. *Lycium barbarum* polysaccharide (LBP) and quinoa ultrafine powder diet is a promising sources of prebiotics. However, the potential synergistic effects of combining them as a microbiota-targeted dietary supplement to mitigate lipid accumulation in nonalcoholic fatty liver disease (NAFLD) remain unclear. Our study aims to provide evidence for the application food/nutrient synergy interventions in NAFLD management, and also to support the clarification of these mechanisms. Following 12 weeks of a high-fat diet (HFD)-inducing NAFLD and an 11-week intervention in rats, the combination of LBP and quinoa ultrafine powder significantly decreased hepatocyte lipid accumulation and improved lipid metabolism disorders compared with those using either LBP or quinoa ultrafine powder alone. The combination increased beneficial intestinal microbiota, such as *Lactobacillus acidophilus*, *Roseburia*, *Ruminococcus2*, and *Prevotella*, promoting the production of short-chain fatty acids, notably butyric acid, and then activated AMPK/SREBP-1C/SCD-1 signaling. Combining LBP with quinoa ultrafine powder is a promising microbiota-targeted dietary supplement for ameliorating lipid disorders in NAFLD.

Keywords: nonalcoholic fatty liver disease; lycium barbarum polysaccharide; quinoa; gut microbiota; short-chain fatty acid

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide with a current global incidence as high as 20%–33%[1]. NAFLD is characterized by the accumulation of liver fat that is not caused by alcohol consumption. It is a multisystem disease associated with extrahepatic conditions such as obesity, type II diabetes, and cardiovascular disease[1, 2]. These associations impose a significant clinical and economic burden, impacting multiple organ systems and health outcomes[1, 2]. Although various drug

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candidates have undergone clinical trials, there are currently no approved pharmacological treatments for NAFLD[3]. Thus, there is an urgent need to identify effective intervention strategies for the treatment of NAFLD.

Dietary interventions and modulation of intestinal microecology play pivotal roles in preventing and treating non-alcoholic fatty liver disease (NAFLD)[4, 5]. Nutrients such as protein, sugars, dietary fiber and polyphenols in the diet or dietary supplements can affect the gut microbiota[6]. Among them, functional oligosaccharides and dietary fiber in carbohydrates play an important role in the influence of the gut microbiota. These compounds can be fermented by the gut microbiota to promote short-chain fatty acids (SCFAs) production, change the gut environment, and boost the abundance of the beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*[6, 7]. SCFAs serve as one of the important metabolites produced by gut microbiota, including acetic acid, propionic acid and butyric acid, etc. SCFAs promote intestinal hormone secretion and inflammatory response, maintain the intestinal barrier, and affect the processes of glucose and lipid metabolism[8, 9]. It has been reported that SCFAs activate AMPK signaling pathways related to energy balance by binding to G protein-coupled receptors such as GPR41 and GPR43, promoting fatty acid oxidation, inhibiting lipid synthesis, and improving lipid accumulation in the liver[7, 10, 11]. In particular, propionic acid and butyric acid have been shown to inhibit liver lipid production by activating AMPK, thereby reducing liver fat deposition[12, 13]. Therefore, supplementation of intestinal prebiotics, increasing SCFA concentration and AMPK activity may be beneficial strategies and therapeutic targets for prevention and improvement of NAFLD.

Previous studies have identified quinoa and *Lycium barbarum* polysaccharide (LBP) as effective sources of prebiotics[14, 15]. LBP is a plant-based extraction from goji berries/wolfberry, and serves as a good source of prebiotics in clinical trials[15]. LBP increases the number of intestinal *Bifidobacterium*, *Lactobacillus*, *Enterobacterium* and *Enterococcus*[16]. It regulates glucose and lipid metabolism, anti-inflammatory, antioxidant functions[17, 18]. Quinoa is a pseudo-grain rich in protein, dietary fiber, minerals, and a variety of phytochemicals that can be used as a daily diet[19]. Studies have found that fermentation of these substances has a positive effect on the production of SCFAs[6], and reduces blood lipids by reducing the absorption of cholesterol and increasing the output of cholesterol[20], thereby controlling obesity and promoting intestinal health[21]. To maximize nutrient intake and health benefits, people often take a mixture of natural extracts and grains as a dietary supplement[20]. Therefore, LBP and quinoa ultrafine powder diet may be a beneficial strategy to supplement intestinal prebiotics and SCFAs. However, the effect and mechanism of LBP combined with quinoa ultrafine powder diet on the prevention and treatment of NAFLD have not been reported.

In this study, we investigated the effect of LBP and quinoa ultrafine powder diet on lipid accumulation in NAFLD rats. The KEGG function prediction analysis of gut microbiota showed that dietary intervention of the LBP and quinoa ultrafine powder diet could reduce lipid synthesis and glycerophospholipid synthesis. SCFAs may play an important role in this process[22]. SCFAs are absorbed by intestinal cells and transported to the liver through the portal vein. The liver is one of the major tissues involved in energy metabolism. SCFAs can

affect liver lipid metabolism through the AMPK-dependent pathway, and then improve NAFLD rats by reducing liver fat[23, 24]. Our data provide direct evidence that LBP and quinoa ultrafine powder diet ameliorate lipid accumulation by regulating gut microbiota, increasing SCFAs and then activating AMPK signaling.

2. Materials and Methods

2.1 Development of a Diet based on LBP and Quinoa Ultrafine Powder

2.1.1 Source and preparation of quinoa ultrafine powder and LBP

The preparation process of quinoa ultrafine powder included: (1) material selection, (2) impurity removal, (3) moistening wheat, (4) rinsing, (5) freezing, (6) drying, (7) stir-frying, (8) low-temperature vibration crushing using a Vibratory Ultrafine Mill, (9) sieving the ultrafine flour through a 300-mesh sieve. The stir-frying temperatures were controlled at 52℃.

LBP source: LBP was acquired from Ningxia Bairuiyuan Wolfberry Co., LTD., LBP content ≥50% (g/100 g).

2.1.2 Quinoa ultrafine powder nutrient composition analysis

The crude protein, fat, ash, and dietary fiber content were determined using the National Standard for Food Safety of China (GB standard) by the Kjeldahl method[25], Soxhlet extraction[26], Dry ash method[27], and Gravimetric method[28], respectively. The content of starch and resistant starch (RS) were determined by kits (A148-1-1, Nanjing Jiancheng Institute of Biological Engineering, Inc., Nanjing, China and DS-W-DF009-48, Addison Biotechnology Co., Ltd., Jiangsu, China).

2.1.3 Monosaccharide composition of LBP

Using pre-column derivatization high performance liquid chromatography (HPLC) method analyzed the monosaccharide components of LBP[29]. Mixed standard preparation as follows: 10 mg mannose, 10 mg rhamnose, 10 mg arabinose, 10 mg fucose, 10 mg galactose and 10 mg glucose were accurately weighed. Then the above solution (0.5 mL mannose, 0.5 mL rhamnose, 0.5 mL arabinose, 0.5 mL fucose, 0.5 mL galactose, 0.5 mL glucose) , water were added to a 10 mL volumetric flask mark for making a mixed standard solution.

2.1.4 Selection of the optimal ratio of quinoa ultrafine powder and LBP

2.1.4.1 Determination of quinoa ultrafine powder and LBP ratios

The optimized quinoa ultrafine powder was divided into three doses: 0.9 g, 1.8 g, and 2.7 g. Similarly, LBP was categorized into three doses: 30 mg, 50 mg, and 100 mg. The quinoa ultrafine powder and LBP were then combined separately according to these specified doses. A total of nine combination samples were explored in this study.

2.1.4.2 In vitro simulated gastrointestinal digestion

Based on the protocols outlined in the “INFOGEST static in vitro simulation of gastrointestinal food digestion” guidelines[30], the in vitro digestion test included oral, gastric, and intestinal digestion phases.

Simulation of oral digestion: 5 g of each combination sample was mixed with 5.5 mL of deionized water for 5 minutes until a homogeneous paste was obtained. 4 mL of SSF electrolyte solution, $CaCl_2$ solution (25

μL, 0.3 mol/L), and alpha-amylase (0.5 mL, 1500 U/mL) were then sequentially added. The resulting mixture was placed in the GI20 in vitro digestion instrument (NI Australia) and incubated at 37℃ while oscillating for 10 minutes at a frequency of 60~250 r/min to simulate oral digestion. These processes yielded the oral digestive fluid.

Simulation of gastric digestion: The oral digestive products were combined with 8 mL of SGF electrolyte solution, $CaCl_2$ solution (5 μL, 0.3 mol/L), and pepsin (1.6 mL, 25000 U/mL). The pH of the resulting mixture was then adjusted to 3.0 using 5 mol/L HCl solution. After thorough mixing, each combination sample was subjected to simulated gastric digestion in the GI20 instrument at 37℃ without light for 2 hours at a frequency of 60~250 r/min. These processes produced the gastric digestive fluid.

Simulation of intestinal digestion: After simulating the gastric digestion phase, 10 mL of SIF electrolyte solution, $CaCl_2$ solution (0.04 mL, 0.3 mol/L), trypsin (5 mL, 8 USP), and pig bile salt solution (3 mL, 10 mmol/L) were added to the digestive solution. The pH of the resulting mixture was adjusted to 7.0 using 5 mol/L NaOH solution. Once the mixing was completed, the sample underwent simulated intestinal digestion in the GI20 instrument at 37℃ without light for an additional 2 hours at a frequency of 60~250 r/min.

2.1.5 Culturing and growth conditions of Lactobacillus acidophilus

A 200 μL suspension of *Lactobacillus acidophilus* (purchased from BNCC) was inoculated onto the medium sequentially and then anaerobically incubated at 37℃ for 48 hours. Colony size and quantity were observed and recorded at 12 hours, 24 hours, 36 hours, and 48 hours according to the guidelines of the National Standard for Food Safety of China (GB4789.35-2016)[31]. The culture medium followed the formula of MRS agar medium: a ratio of 1 part digestive fluid to 9 parts MRS agar medium (v/v). The blank control group utilized MRS agar medium, with three petri dishes prepared for each medium.（Fig.1C）

2.2 Animal Experiment

During the experiments, all animal protocols were approved by the Experimental Animal Ethics Committee of Ningxia Medical University (IACUC-NYLAC-2022). All animals received humane care according to the criteria outlined in the NIH “Guide for the Care and Use of Laboratory Animals”.

2.2.1 Animal experiment design

Male Sprague-Dawley rats (180-220 g) were obtained from the Laboratory Animal Center of Ningxia Medical University (lot number SCXK (NING) 2021-0002) and housed under specific conditions (temperature: 20℃-25℃, humidity: 45%-55%, 12 hours light/dark cycle). After adaptation, they were randomly divided into two groups: the Normal group (NC group, n=6, fed a normal diet from Beijing Keao Xieli Feeds Co., Ltd.) and the Model group (n=24, fed a high-fat diet (HFD) containing 60 kcal% from fat, MD12033, Medicience Biomedicine Ltd., Co.) ad libitum.

After 12 weeks, rats in the Model group were randomly divided into four groups, with six rats in each group, according to the experimental design:

(1) Model group (MC group, n=6): rats were fed with HFD diet.

(2) Quinoa ultrafine powder group (QC group, n=6): rats were fed with HFD containing quinoa ultrafine powder.

(3) LBP group (LC group, n=6): rats were fed with HFD containing LBP.

(4) Quinoa ultrafine powder +LBP group (QL group, n=6): rats were fed with HFD containing both quinoa ultrafine powder and LBP (quinoa ultrafine combined with LBP diet, hereinafter referred to as QL).

All rats had free access to feed and water during the experiment. Their energy intake , weight, and length were measured on a weekly basis. After 11 weeks of intervention, rats underwent a 12 hour fast before being euthanized under ether anesthesia. Tissue samples from the liver, epididymis, and perirenal adipose tissue, and blood were collected. Serum was isolated by centrifuging the blood at 2000 × g and 4℃ for 10 minutes. Tissue samples were immediately frozen in liquid nitrogen and stored at -80℃ for subsequent analysis. The dietary compositions of the diets provided for each experimental group are summarized in Table 1.

Table 1 The formulation of high fat feed and intervention feed for rats

Parameters	MC group	QC group	LC group	QL group
Casein	25.84%	24.52%	25.84%	24.52%
L-Cystine	0.39%	0.39%	0.39%	0.39%
Maltodextrin	16.17%	15.45%	16.17%	14.75%
Cane sugar	8.89%	3.44%	8.64%	3.44%
Cellulose	6.46%	5.62%	6.46%	5.62%
Soybean oil	3.23%	2.56%	3.23%	3.01%
Lard	31.66%	31.66%	31.66%	31.66%
Pellet	7.36%	7.36%	7.36%	7.36%
Quinoa	0.00%	9.00%	0.00%	9.00%
LBP	0.00%	0.00%	0.25%	0.25%
Total	100.00%	100.00%	100.00%	100.00%

Note: Ingredient list of animal experimental feed. Different intervention feeds were prepared according to the energy supply ratio of equal calories.
Abbreviations: MC group = HFD group, QC group = quinoa ultrafine powder group, LC group = LBP group, QL group = quinoa ultrafine powder + LBP.

2.2.2 Histological analysis

Liver tissues were fixed in 10% neutral formalin and embedded in paraffin for histological analysis of pathological changes. Sections (4μm thick) were stained with hematoxylin and eosin (H&E) as well as Oil Red O (from Nanjing Jiancheng Institute of Bio Engineering, Inc., Nanjing, China), then observed under a light microscope (Olympus Medical Systems, Tokyo, Japan). The assessment of histological liver damage was carried out using the NAFLD Activity Score (NAS)[32].

2.2.3 Biochemical and ELISA analysis

The level of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), total cholesterol (TC), low density lipoprotein-Cholesterol (LDL-C), and high density lipoprotein-Cholesterol (HDL-C), liver TC, liver TG, liver free fatty acid (FFA) were examined using a specific assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Serum levels of LPS, leptin (LEP) and adiponectin (ADPN) were examined using the ELISA kit (Jiang lai Bioendo Technology, Co., Ltd., Shanghai, China). Liver tissue homogenate were prepared by

homogenizing 0.1 g of liver tissue with 0.9 mL phosphate-buffered saline with an ultrasonic tissue grinder, and the supernatant was used for further analysis.

2.2.4 Western blot

Using liver tissue of experimental rats performed western blot analysis. Western blot analysis performed as described in previous studies[33]. The target proteins included AMPK, pAMPK, SREBP-1C (sterol regulatory element binding protein-1c), FAS (fatty acid synthase), SCD-1 (stearoyl-CoA desaturase-1) and β-actin (beta actin). The primary antibodies used as follows: AMPK(1:1000, Cell Signaling Technology), pAMPK(1:1000, Cell Signaling Technology,T172), ACC (1:1000, Cell Signaling Technology), FAS (1:1000, Cell Signaling Technology), β-actin(1:1000, Cell Signaling Technology), SREBP-1C (1:1000, Affinity), SCD-1(1:8000, Proteintech), and secondary antibodies (1:1000, Affinity).

2.3 Gut microbiota analysis

The colon contents of rats from different groups were collected and stored at -80℃. Genomic DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) based on the manufacturer's instructions. The integrity of genomic DNA was assessed through agarose gel electrophoresis, while its concentration and purity were measured using the Nanodrop 2000 and Qubit 3.0 Spectrophotometer. Amplicon libraries covering the V3-V4 hypervariable regions of the bacterial 16S-rRNA gene were amplified using primers 341F: 5'-CCTACGGGNGGCWGCAG-3', and 806R: 5'-GACTACHVGGGTATCTAATCC-3'. The DNA was sequenced by the Illumina NovaSeq 6000 sequencer, and the microbiota composition was analyzed using QIIME 2.0. Sequences with ≥100% similarity were grouped into identical operational taxonomic units (OTUs). The unweighted UniFrac beta distance was employed in principal coordinate analysis (PCoA), and the unweighted pair group method with arithmetic mean (UPGMA) clustering was used to analyze the complex and multidimensional data. The results were visualized in master coordinates using PCoA. The bar plots of different classification levels were plotted with T package V3.4.1 and R package "ggplot2", respectively. The Wilcox test method was used to compare the significant differences between the two groups, and the Kruskal-Wallis test method was used to compare the three groups or more. A P-value < 0.05 was considered statistically significant.

2.4 Determination of SCFAs content in the colon

Colon samples (approximately 50 mg) were homogenized in 500 μL of water with 100 mg of glass beads for 1 minute, followed by a centrifugation at 4℃ for 10 min at 12,000 r/min. Subsequently, a mixture of 200 μL of supernatant and 20 μL of 375 μL/mL 4-methylvaleric acid solution as an internal standard was acidified with 100 μL of 15% phosphoric acid. The resulting solution was then extracted with 280 μL of ethyl ether under continuous stirring for 1 min. The mixture was then centrifuged at 4℃ for 10 min at 12,000 r/min. The resulting supernatant was transferred to a vial and analyzed using GC-MS. Identification of SCFAs was performed by comparing their mass spectra with retention times of six standard SCFAs (acetic, propionic, butyric, isobutyric, valeric, and isovaleric acid).

2.5 Statistical procedures

Data were presented as the mean ± standard error of the means (mean ± SEM). The statistical analysis was conducted using one-way analysis of variance (ANOVA), kruskal-wallis test, repeated measure ANOVA. Tukey test or Bonferroni post hoc test was used to assess the differences between individual groups. A *P*-value < 0.05 was considered statistically significant. All analyses were conducted using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) or SPSS version 24.0 (Chicago, IL, USA).

3. Results

3.1 The development of QL diet

3.1.1 Chemical composition of quinoa ultrafine powder

The chemical composition outcomes of quinoa ultrafine powder were presented as follows: (13.36 ± 0.12) g/100 g of protein, (9.34 ± 0.12) g/100 g of dietary fiber, (7.46 ± 0.16) g/100 g of fat, (43.88 ± 0.40) g/100 g of starch and (240 ± 0.01) mg/100 g of RS (Table 2).

Table 2 Chemical composition of quinoa ultrafine powder

Nutrient Composition	Content
Ash (g/100 g)	1.43 ± 0.02
Protein (g/100 g)	13.36 ± 0.12
Dietary fiber (g/100 g)	9.34 ± 0.12
Fat (g/100 g)	7.46 ± 0.16
Starch (g/100 g)	43.88 ± 0.40
RS (mg/100 g)	240 ± 0.01

Note: RS=Resistant starch.

3.1.2 Composition of LBP

The results of HPLC retention times of standard glucose and the chromatogram of LBP samples showed that 5 monosaccharide compositions in LBP can be determined, including mannose, rhamnose, glucose, galactose, and arabinose. (Figure 1A, 1B).

3.1.3 QL diet in vitro digestion promotes Lactobacillus acidophilus proliferation

To further investigate the potential of QL as a prebiotic source, we evaluated its effect on *Lactobacillus acidophilus* proliferation in vitro across nine different QL combination samples. Compared to MRS medium, the number of *Lactobacillus acidophilus* reduced to varying degrees in mediums containing LBP or quinoa ultrafine powder digestates, with statistically significant differences ($P < 0.01$). The digestates of all QL combination samples showed significant promotion effect on *Lactobacillus acidophilus* proliferation with the exception of the sample combining 100mg LBP with 2.7g quinoa ultrafine powder (all $P < 0.05$). The digestates with 50 mg LBP and 1.8 g quinoa ultrafine powder demonstrated the highest promotion effect on *Lactobacillus acidophilus* proliferation in vitro. When compared to other digestates, the digestates with 50 mg LBP and 1.8 g quinoa ultrafine powder also showed the highest proliferation rate at the 24th hour of the experiment (Fig. 1C).

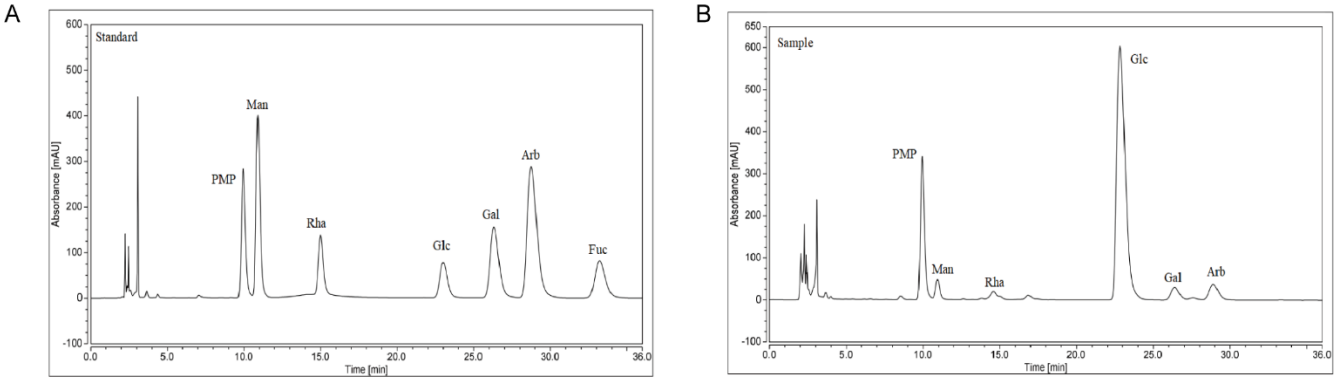
3.2 Effects of QL diet on lipid metabolism profiles in rats with NAFLD

3.2.1 Effects of QL diet on energy intake, weight, liver weight and visceral fat weight in NAFLD rats

To assess the impact of QL as a daily dietary source for NAFLD rats, animal experiments were conducted. At the end of the modeling period, the average food calorie intakes were (327.00 ± 6.85) kcal/week for the NC group, (294.83 ± 6.85) kcal/week for the MC group, (314.10 ± 11.91) kcal/week for the QC group, (295.75 ± 6.24) kcal/week for the LC group, and (315.02 ± 4.46) kcal/week for the QL group. We performed repeated measures ANOVA to assess changes in food intake over 11 consecutive weeks of weight measurements among different treatment groups of rats. The within-group analysis revealed a significant difference in food intake across weeks ($F_{(6, 20)}$ = 12.762, P < 0.001). In contrast, the interaction between time and group did not yield a significant difference ($F_{(6, 20)}$ = 0.869, P > 0.05). The between-group analysis further indicated no significant differences in total food intake among the groups ($F_{(4, 20)}$ = 1.653, P > 0.05). These findings suggest that while food intake changes significantly over time, the trends remain similar across treatment groups, with no statistically significant differences in overall food intake between groups (Fig.1E).

At the end of the modeling period, significant increases in body weight and Lee's index were observed in the MC, QC, LC, and QL groups compared to the NC group (all P < 0.05). Throughout the intervention, rats' body weights in all groups increased. At the end of the intervention, the Lee's index of rats in the MC group (40.17 ± 0.74) g/cm was significantly higher than that of the NC group (31.64 ± 0.47) g/cm, QC group (33.64 ± 0.94) g/cm, and QL group (32.82 ± 1.02) g/cm. No significant difference were observed between the LC group (39.43 ± 1.88) g/cm and the MC group. Among all intervention groups, QL intervention had the strongest effect (Fig.1F, 1G, 1J).

For the average liver index and visceral fat index, higher value were observed in the MC group when compared to other groups. There was significant differences observed between the MC group and the QC group (P < 0.05) and the QL group (P < 0.001) in liver index (Fig.1H, 1K). Furthermore, the average visceral fat index, higher value were observed in the MC group when compared to other groups, QL intervention resulted in more pronounced reductions in visceral fat index than LC intervention alone or QC intervention alone (Fig. 1I, 1L). LEE's index and other fat weight parameters related to lipid accumulation. These results suggest that QL reduced Lee's index, liver index, and visceral fat index in NAFLD rats, the QL intervention have a superior effect on fat reduction.



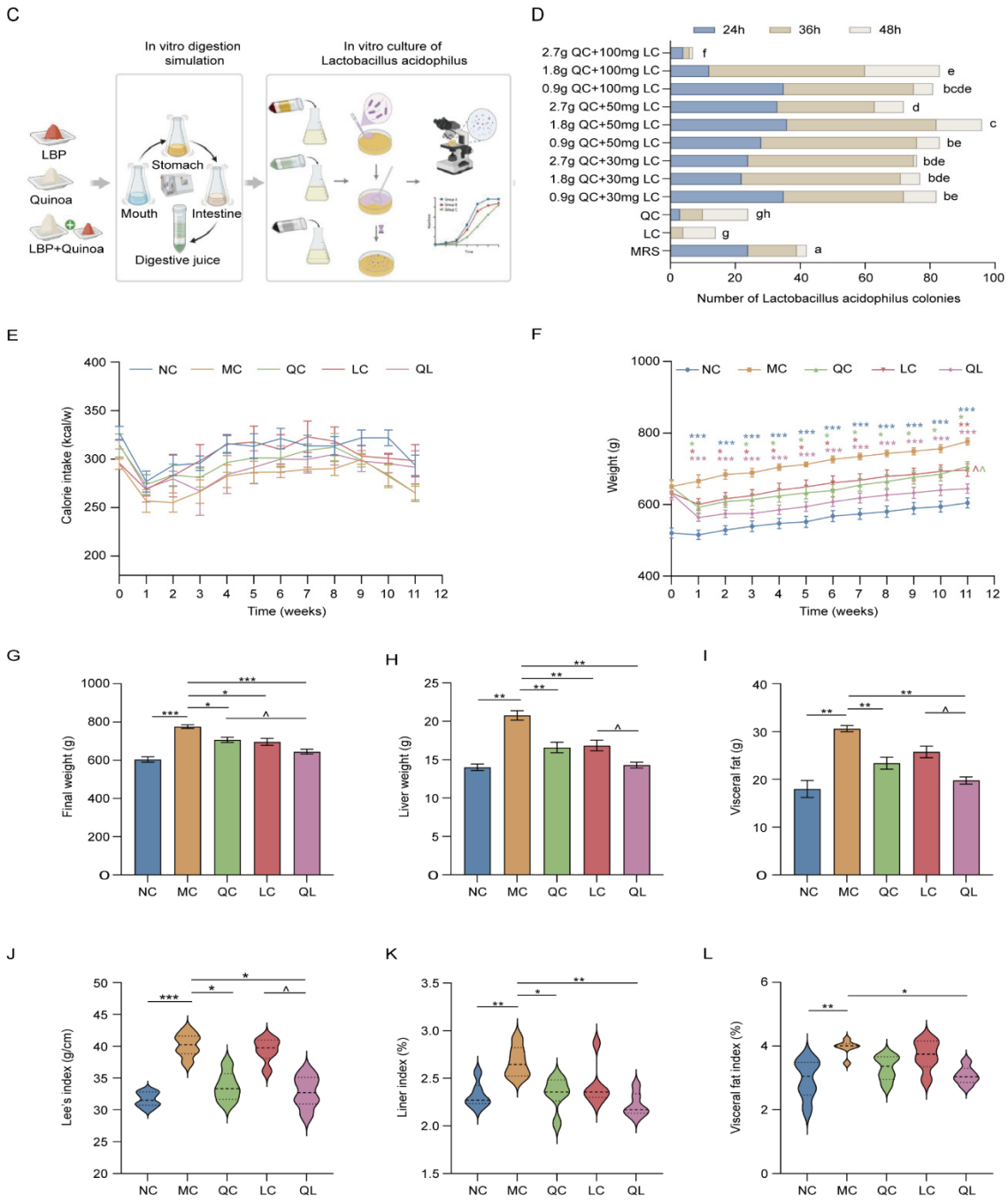


Figure 1. QL diet development and effect on weight of NAFLD rats. (A) Standard monosaccharides, (B) Monosaccharides released from LBP, (C) Experiment design of QL diet development, (D) Culturing of *Lactobacillus acidophilus*, values with different letters indicate significant differences($P < 0.05$), (E) Weekly calorie intake during intervention, (F) Weight change during intervention, (G) Final weight, (H) Liver weight, (I) Visceral fat, (J) Lee's index, (K) Liver index, (L) Visceral fat index .*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ^ $P < 0.05$,*compared to the MC group; ^compared with the QL group. Abbreviations: Man= mannose, Rha= rhamnose, Glc= glucose, Gal= galactose, Arb= arabinose, Fuc= fucose. MRS = MRS agar medium; QC= MRS agar medium containing quinoa ultrafine powder digestive fluid; LC= MRS agar medium containing LBP digestive fluid. NC=NC group, rats were fed a standard diet; MC=MC group, rats were fed HFD; QC =QC group, rats were fed the HFD supplemented with quinoa ultrafine powder; LC=LC group, rats were fed the HFD supplemented with LBP, QL group, rats were fed the HFD supplemented with both quinoa ultrafine powder and LBP.

3.2.2 Effect of QL diet on lipid biomarkers in NAFLD rats

Lipid biomarkers are valuable clinical parameters in assessing the progressive of NAFLD[34]. Measurements of the levels of blood and liver lipid indicators were included to enhance the validity and reliability of this study. For blood lipid indicators, significant higher levels of plasma TG, TC, LDL-C, AST,

and ALT, with a significant lower level of plasma HDL-C were observed in MC group when compared to those of NC group (all $P < 0.01$). After the 11-week intervention, significant lower levels of plasma TC (all $P < 0.05$), TG ($P_{QL} < 0.05$), AST (all $P < 0.01$), ALT ($P_{QL} < 0.01$), and LDL-C (all $P < 0.01$) were observed in the QC, LC and QL groups than those of the MC group, with the QL group showed the most significant reduction among the three intervention groups (Fig.2A, 2B, 2C, 2D, 2E). A significant higher level of plasma HDL-C were observed in the QC, LC and QL groups than those of MC group while the QL group showed the most significant improvement among the three intervention groups (Fig.2F). In terms of liver lipid indicators, significant higher concentration of liver TC (all $P < 0.01$), TG (all $P < 0.01$) and FFA (all $P < 0.001$) were observed in the MC group than those in the NC group (Fig.2G, 2H, 2I). After the 11-week intervention, intervention groups (QC group, LC group, QL group) showed significantly lower concentration of liver TC (all $P < 0.01$), TG (all $P < 0.01$), and FFA (all $P < 0.01$) than the MC group. The QL group had lower concentration of liver TG ($P < 0.01$) and FFA ($P < 0.001$) among all three intervention groups. QL intervention improved liver injury, decreased dyslipidemia, and improved liver function in rats with NAFLD.

3.2.3 Effect of QL diet on adipocytokines and inflammatory factors in NAFLD rats

For the adipocytokine profile, significantly higher level of serum leptin and significantly lower level of ADPN were observed in the MC group compared to the NC group (all $P < 0.01$). All three intervention groups showed lower levels of serum leptin (all $P < 0.01$) and higher levels of ADPN (all $P < 0.01$) compared to the MC group. Rats in the QL group demonstrated the most significant reduction in the level of serum leptin levels (Fig. 2J–K).

In terms of the expression levels of inflammatory factors, the serum IL-6, TNF-α, and LPS levels were significantly higher in the MC group compared with the NC group ($P < 0.001$). Significant reduction of serum IL-6, TNF-α, and LPS expression levels were observed in QC, LC, and QL groups ($P < 0.001$) than the MC group. Among all these intervention groups, the most significant reduction was observed in the QL group (Fig.2L–N).

3.2.4 Effect of QL diet on liver pathological changes in NAFLD rats

Histomorphological analysis was conducted to H&E staining and Oil red O staining of this study. The NC group showed normal liver structure with clear lobules and well-organized hepatocyte cords, without any ballooning degeneration. The MC group exhibited disorganized hepatocyte cords, narrowed or absent hepatic sinusoids, increased fatty vacuolar degeneration, and nuclei that were extruded and deformed. When compared to the staining outcomes of the MC group, the QC, LC, and QL groups showed significantly reduced liver lesions, characterized by only a small amount of fatty vacuolar degeneration. Among all the three intervention groups, the QL group showed the most pronounced effect on improve liver pathology (Fig.2O).

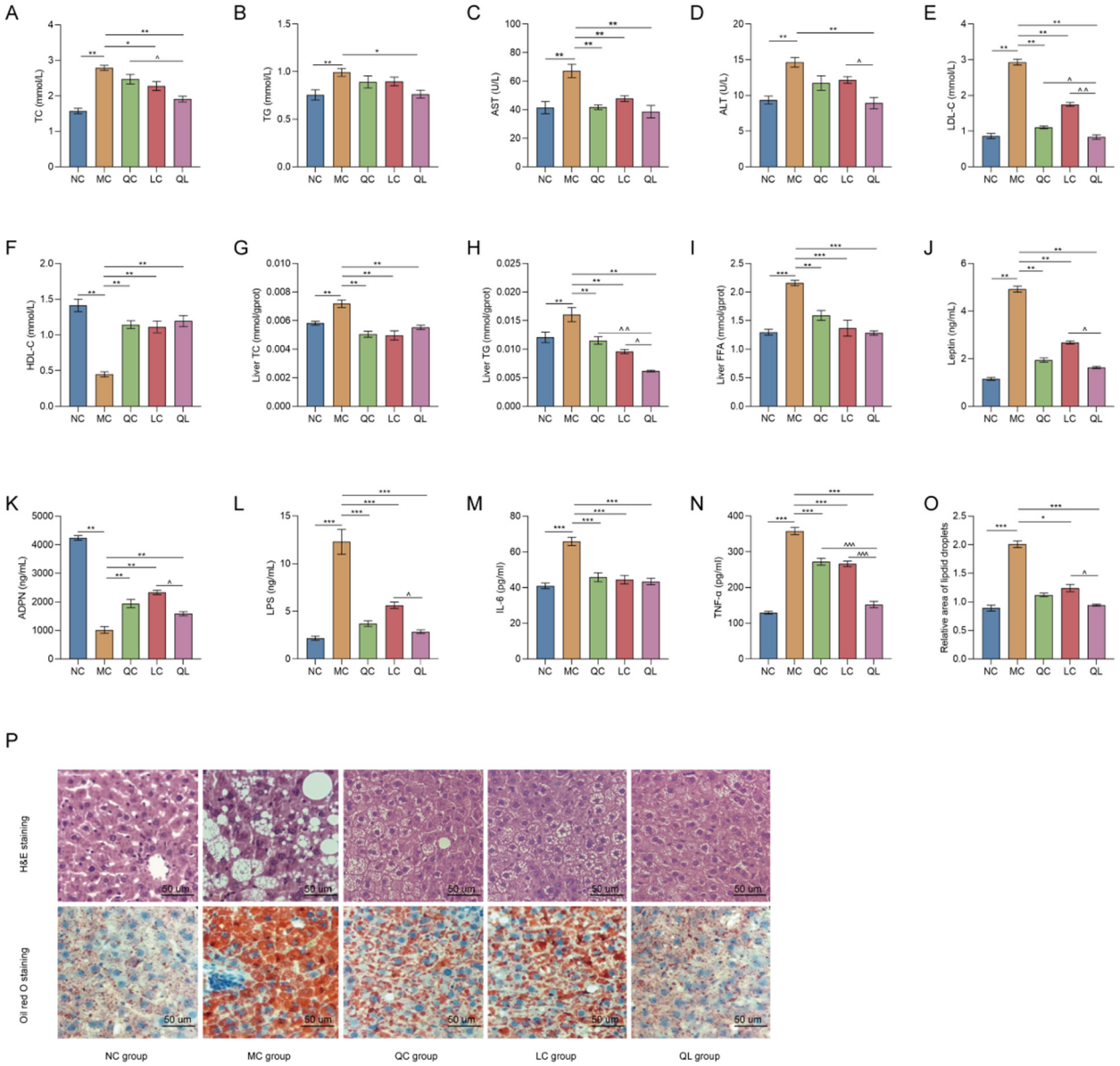


Figure 2. QL alleviates HFD diet-induced NAFLD. (A) Plasma TC, (B) Plasma TG, (C) Plasma AST, (D) Plasma ALT, (E) Plasma LDL-C, (F) Plasma HDL-C, (G) Liver TC, (H) Liver TG, (I) Liver FFA, (J) Serum leptin, (K) Serum ADPN, (L) Serum LPS, (M) Serum IL-6, (N) Serum TNF-α, (O) Oil red O score, (P) Representative images of liver sections stained with H&E and Oil red O (×200, scale bars 50 um). *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ^ $P < 0.05$, ^^ $P < 0.01$, ^^^ $P < 0.001$,*compared to the MC group; ^compared with the QL group. Abbreviations: NC=NC group, rats were fed a standard diet; MC=MC group, rats were fed HFD; QC =QC group, rats were fed the HFD supplemented with quinoa ultrafine powder; LC=LC group, rats were fed the HFD supplemented with LBP, QL group, rats were fed the HFD supplemented with both quinoa ultrafine powder and LBP.

3.4 The impact of QL diet on gut microbiota profiles

3.4.1 Gut microbiota Alpha diversity index and Beta diversity analysis

16S-rRNA analysis on colonic contents from all rats were conducted across five groups to elucidate how QL intervention alters the gut microbiota. The Alpha diversity of gut microbiota was assessed using observed species and chao1 index. Significantly lower number of observed species and level of chao1 index were observed in the MC group compared to NC group (all $P < 0.01$). Significantly increased number of observed species and level of chao1 index were observed in the QL group ($P < 0.05$) when compared to the MC group (Fig.3A, B).

PCoA was used to visualize the inter-group Beta diversity. The Beta diversity of samples from the MC group was clearly separated from the NC and QL groups, while QC and LC groups were not completely separated from the MC group and partially overlapped. Variations in gut microbiota composition in response to LC, QC, and QL interventions, while QL had a more pronounced effect than QC and LC interventions (Fig.3C).

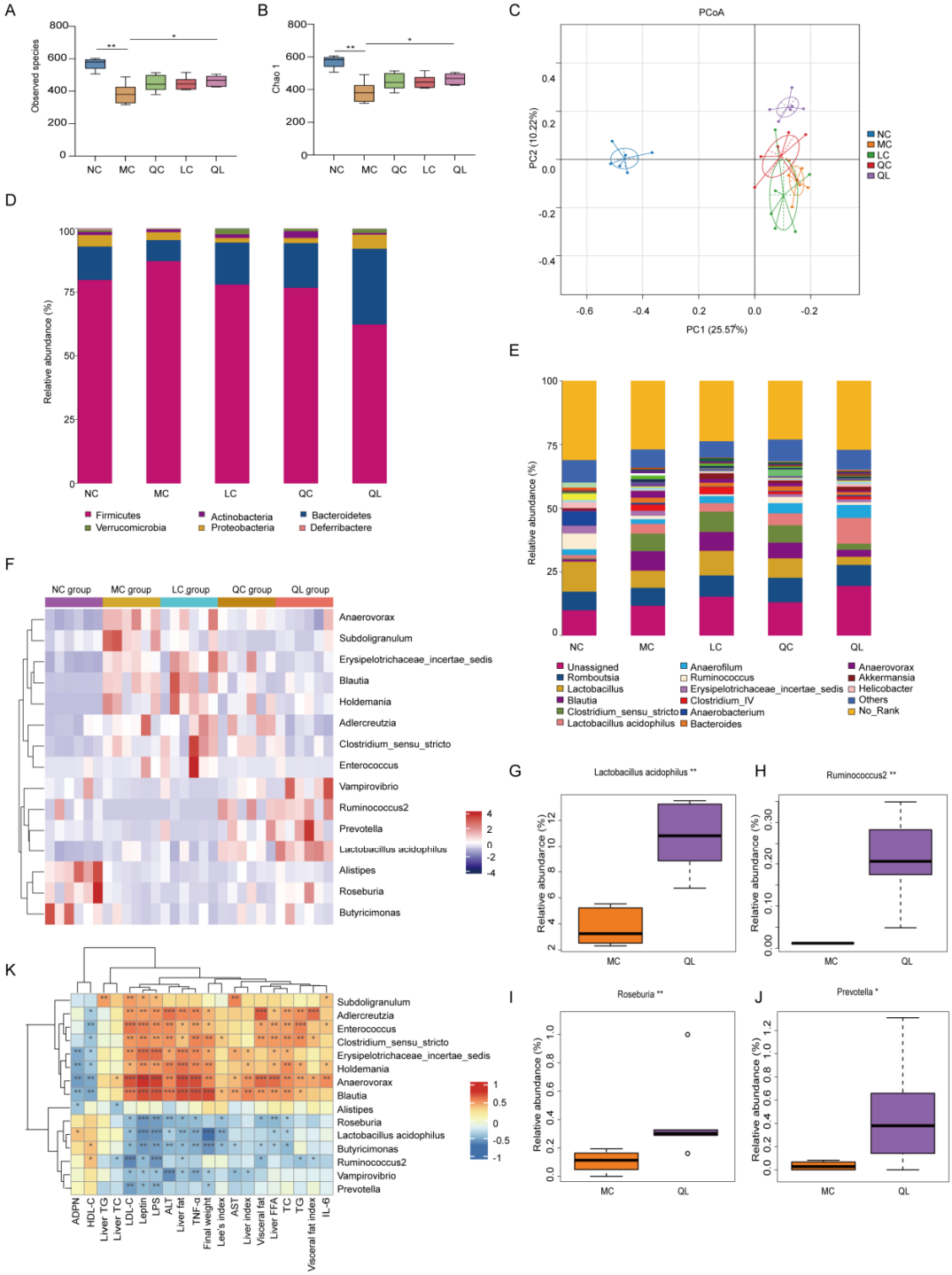


Figure 3. Effects of QL on the gut microbiota structure. (A) Observed species, (B) Chao1 index, (C) PCOA, (D) Bacterial profile at phylum level, (E) Bacterial profile at genus level, (F) Heat map of significant different bacteria among all groups (the top 15), (G-J) Significant different bacteria of QL group versus MC group, (K) Correlation between gut microbes and biochemical parameters. Values are presented as mean ± SEM, *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviations: NC=NC group, rats were fed a standard diet; MC=MC group, rats were fed HFD; QC =QC group, rats were fed the HFD supplemented with quinoa ultrafine powder; LC=LC group, rats were fed the HFD supplemented with LBP, QL group, rats were fed the HFD supplemented with both quinoa ultrafine powder and LBP.

3.4.2 Analysis of differences in gut microbiota

The gut microbial composition of rats in all groups was mainly dominated by *Bacteroidetes* and *Firmicutes*. At the phylum level, compared with the NC group, the MC group showed an increase in *Firmicutes* and a decrease in *Bacteroidetes*. All three interventions increased the abundance of *Bacteroidetes* and decreased the abundance of *Firmicutes*. The most significant increase in the abundance of *Bacteroidetes* was in the QL group among all intervention groups (Fig.3D).

At the genus level, using Kruskal-Wallis test identified the top 15 differentiated bacteria, including *Lactobacillus acidophilus*, *Ruminococcus2*, *Prevotella*, *Roseburia*, *Blauita*, *Vampirovibrio*, *Alistipes*, *Butyricimonas*, *Clostridium_sensu_stricto*, *Adlercreutzia*, *Erysipelotrichaceae_incertae_sedis*, *Anaerovorax*, *Enterococcus*, *Subdoligranulum*, *Holdemania* (Fig.3E, F).

Further comparison between the two groups was carried out with the Wilcox test. Compared to NC group, the abundance of *Clostridium_sensu_stricto*, *Anaerovorax*, *Holdemania*, *Lactobacillus acidophilus*, *Erysipelotrichaceae_incertae_sedis*, *Subdoligranulum*, *Adlercreutzia*, *Enterococcus, Blauita* were significantly increased (all $P < 0.05$), *Alistipes*, *Butyricimonas* were significantly decreased (all $P < 0.05$). *Roseburia*, *Prevotella*, *Vampirovibrio*, and *Ruminococcus2* tended to decrease in abundance but were not statistically different in MC group (Fig.3F).

Compared to MC group, the abundance of *Subdoligranulum* was significantly decreased (all $P < 0.05$) in LC group. Compared to MC group, the abundance of *Ruminococcus2*, *Prevotella* were significantly increased (all $P < 0.05$), *Erysipelotrichaceae_incertae_sedis*, *Anaerovorax*, *Enterococcus*, *Subdoligranulum* were significantly decreased (all $P < 0.05$). Compared to MC group, the abundance of *Lactobacillus acidophilus*、*Ruminococcus2*、*Roseburia*、*Prevotella*、*Vampirovibrio* were significantly increased (all $P < 0.05$), *Anaerovorax*、*Enterococcus*、*Subdoligranulum*、*Erysipelotrichaceae_incertae_sedis*、*Adlercreutzia* were significantly decreased (all $P < 0.05$). While *Alistipes*, *Butyricimonas* showed only an increasing trend in abundance, and *Clostridium_sensu_stricto*, *Holdemania* showed only a decreasing trend in abundance, none of which were statistically different (all $P > 0.05$) in QL group (Fig.3F-J).

3.4.3 Correlation analysis and functional prediction

Spearman correlations were used to link specific bacteria to NAFLD improvement with QL. Indicator included basal indicators, lipid metabolism, and inflammatory cytokines, correlating with gut microbiota (the top 15).

Lactobacillus acidophilus、*Ruminococcus2*、*Roseburia*、*Prevotella*、*Vampirovibrio* which were significantly increased in QL group, showed a negative correlation with LDL-C, Leptin, LPS, ALT, liver fat, TNF-α, final weight, Lee's index, visceral fat, and TC. However, there was no statistically significant difference in the negative correlation with IL-6 (Fig.3K).

KEGG analysis showed enhanced protein digestion and absorption, as well as other glycan degradation pathways, in the QL group compared to the MC group ($P < 0.05$). Conversely, fatty acid biosynthesis, glycolysis/gluconeogenesis, peptidoglycan biosynthesis, and glycerophospholipid metabolism were reduced

in the QL group ($P < 0.05$). Fatty acid biosynthesis and glycerophospholipid metabolism associated with disorders of lipid metabolism in NAFLD (Fig.4A).

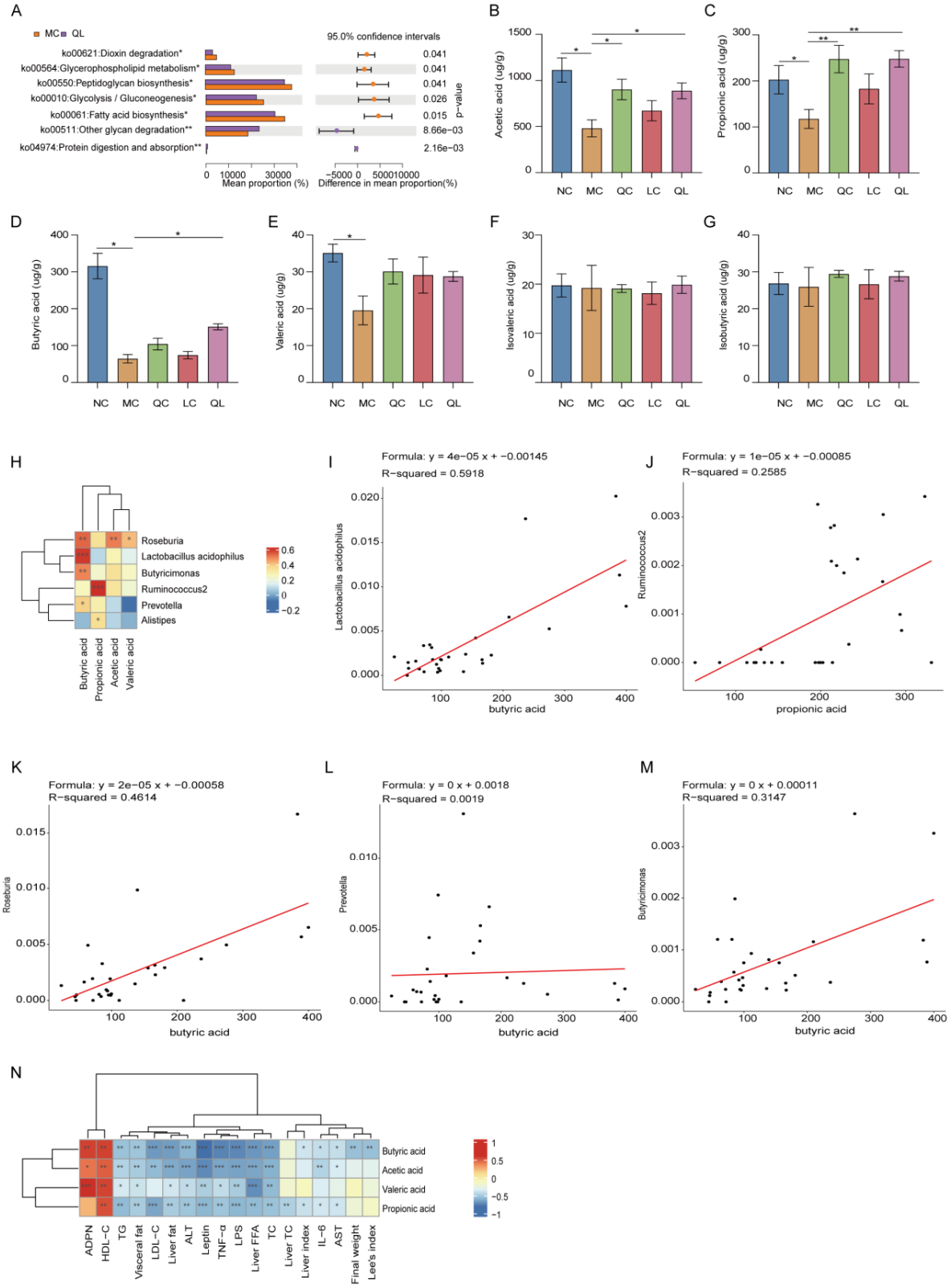


Figure 4. QL improve the content of SCFAs in NAFLD rats. (A) Functional difference analysis of gut microbiota, (B) Acetic acid, (C) Propionic acid, (D) Butyric acid, (E) Valeric acid, (F) Isovaleric acid, (G) Isobutyric acid, *$P < 0.05$, ** $P < 0.01$, *compared to the MC group. (H) Correlation between SCFAs and gut microbes, (I-M) Linear correlation between SCFAs and gut microbes, (N) Correlation between SCFAs and biochemical parameters. Abbreviations: NC=NC group, rats were fed a standard diet; MC=MC group, rats were fed HFD; QC =QC group, rats were fed the HFD supplemented with quinoa ultrafine powder; LC=LC group, rats were fed the HFD supplemented with LBP, QL group, rats were fed the HFD supplemented with both quinoa ultrafine powder and LBP.

3.5 QL diet regulation of SCFAs function

3.5.1 Effect of QL diet on SCFAs in the colon of NAFLD rats

SCFAs were found to influence the development and progression of the NAFLD. To further determine whether the QL diet had impacts on SCFAs metabolism in the colon, we compared the content of SCFAs in the colon. The concentrations of acetic acid, propionic acid, and butyric acid in the MC group lower than those in the NC, QC, LC, and QL groups. Among all intervention group, only the QL intervention significantly increased butyrate content compared to the MC group ($P < 0.05$) (Fig.4B-G).

3.5.2 Correlation analysis

The correlation between differential bacteria and SCFAs after QL intervention was analyzed. The results showed that *Ruminococcus2* was positively correlated with propionic acid (R^2=0.2585), *Roseburia* was positively correlated with butyric acid (R^2=0.4616), and *Lactobacillus acidophilus* was positively correlated with butyric acid (R^2=0.5918). The correlation between *Prevotella* and butyric acid was weak (R^2=0.0019), and *Bytricimonas* was positively correlated with butyric acid (R^2=0.3147). (Fig.4H-M).

In addition, acetic acid, propionic acid, and butyric acid showed a positive correlation with ADPN, HDL-C, and negative correlation with TG, visceral fat, LDL-C, liver fat, ALT, leptin, TNF-α, LPS, liver FFA, TC, liver TC, liver index, IL-6, AST. Only butyric acid was negatively correlated with final weight, Lee's index (Fig.4N).

The dominant bacteria in QL group were significantly positively correlated with butyric acid, while butyric acid was not only negatively correlated with blood lipid indexes, but also negatively correlated with weight loss indexes.

3.6 QL diet effect on the AMPK dependent pathway

All results suggest that QL diet may stimulate AMPK phosphorylation and improve liver lipid metabolism through AMPK dependent pathway in liver. Therefore, we further detected the protein expression of AMPK, pAMPK, SREBP-1C, FAS, SCD-1 in liver tissues. Compared with the NC group, pAMPK/AMPK ratio ($P < 0.01$) in the MC group was significantly decreased, while the pAMPK/AMPK ratio in the QC group, LC group and QL group were increased compared with the MC group, and the QL group was the most significantly increased ($P < 0.05$). In the lipid synthesis pathway, compared with the NC group, SREBP-1C ($P < 0.01$), FAS ($P < 0.01$), SCD-1 ($P < 0.01$) in the MC group were significantly increased. After intervention, SREBP-1C, FAS, SCD-1 were decreased compared with the MC group, and the QL group was the most significantly decreased (all $P < 0.05$). (Fig.5A-H).

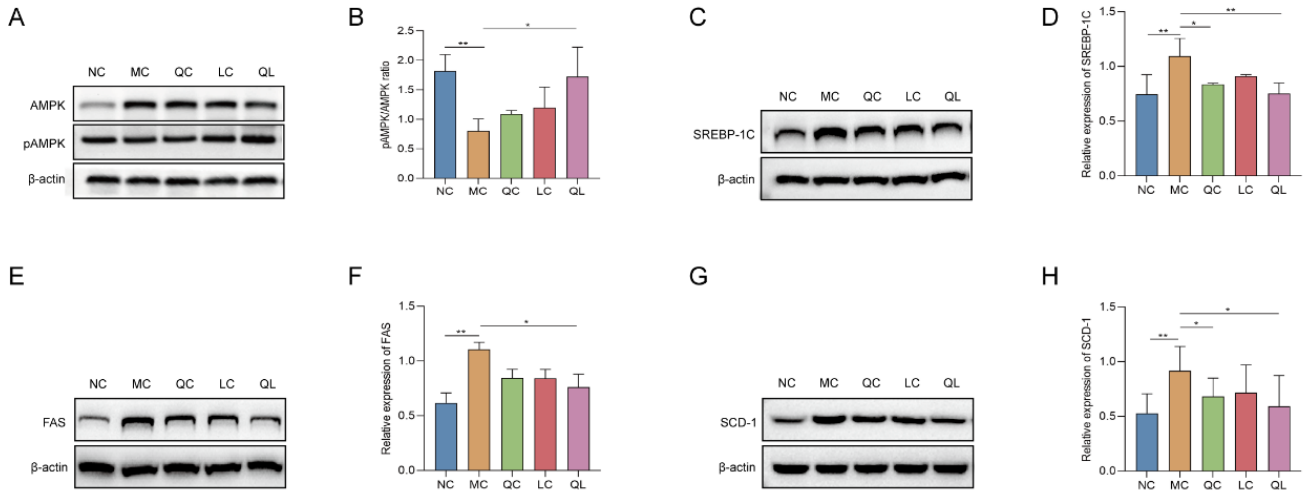


Figure 5. QL affects lipid synthesis through AMPK signaling pathway. (A) Protein expression of pAMPK/AMPK, (B) pAMPK/AMPK protein expression ratio analysis statistical graph, (C)Protein expression of SREBP-1C, (D) SREBP-1C protein expression ratio analysis statistical graph, (E) Protein expression of FAS, (F) FAS protein expression ratio analysis statistical graph, (G) Protein expression of SCD-1, (H) SCD-1 protein expression ratio analysis statistical graph. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ^ $P < 0.05$, ^^ $P < 0.01$, ^^^ $P < 0.001$,*compared to the MC group; ^compared with the QL group. Abbreviations: NC=NC group, rats were fed a standard diet; MC=MC group, rats were fed HFD; QC =QC group, rats were fed the HFD supplemented with quinoa ultrafine powder; LC=LC group, rats were fed the HFD supplemented with LBP, QL group, rats were fed the HFD supplemented with both quinoa ultrafine powder and LBP.

4. Discussion

Dietary patterns and compositions represent one of the most substantial factors for alterations in gut microbiota[35, 36]. In this study, HFD was used to induce a rat model of NAFLD, allowing investigation of the relationship between the gut microbiome and lipid metabolism by QL dietary intervention.

We found that the combination of LBP and quinoa ultrafine powder can serve as an excellent fermentation substrate for the gut to promote the proliferation of SCFAs. Based on this finding, we compared the effects of different doses and found that the combination of LBP (50 mg) and quinoa ultrafine powder (1.8 g) may be a promising treatment for NAFLD. SCFAs such as acetic, propionic, and butyric acid that produced by the fermentation of dietary fiber and RS contribute to reducing colon pH and supporting the prevention of colon cancer, diabetes and obesity[37, 38]. Natural RS is mainly found in grains, seeds, heated starches and starch-containing foods[39]. Quinoa is rich in protein, starch, and dietary fiber[40], which has been shown to benefit obesity and intestinal-related diseases by reducing blood glucose levels, blood lipid levels, and body weight[41]. In previous studies, *Lactobacillus acidophilus* was identified as a SCFA-producing bacterium[42]. The consumption of *Lactobacillus acidophilus* has been identified as a beneficial treatment option for high cholesterol[43]. Rapidly fermentable non-digestible carbohydrates produces lactic acid through *Lactobacillus acidophilus*[22]. Under normal conditions, some bacterial species, e.g., *Eubacterium hallii*, that can convert lactic acid into different SCFAs[44].

The severity of NAFLD is associated with dysbiosis and loss of metabolic function in commensal bacteria[45-47]. SCFAs are important gut microbe-related metabolites derived from the end-products of bacterial fermentation of indigestible dietary fiber in the gut[23, 48, 49]. Their levels in the gut depend on the availability of carbohydrate substrate, the degree of degradation, and the particular bacterial strain present[23,

[48, 49]. This study found a significant increase in the proportion of *Ruminococcus2* after the intervention in the quinoa ultrafine powder group and the combination of LBP and quinoa ultrafine powder groups. This finding is consistent with Abell et al,[50], who found that found that the richness of RS and dietary fiber in whole grains with their fermentation products can promote the growth of *Ruminococcus* and the production of propionate. Increasing levels of propionate in the human colon stimulate the secretion of peptide YY and glucagon-like peptide-1 from colonic cells. This process reduces weight gain, abdominal adipose tissue, liver cell lipid content, and improves insulin sensitivity[51].

In addition, we found that the combination of LBP and quinoa ultrafine powder diet significantly increased the abundance of *Roseburia*, *Lactobacillus acidophilus* in the gut. Butyric acid is the main production of *Roseburia*[50, 52] while lactic acid is the main production of *Lactobacillus acidophilus.* Lactic acid can be converted to butyrate by butyric acid-producing bacteria[53]. *Butyricimonas* is a kind of butyric acid-producing bacterium[54], the combination of LBP quinoa ultrafine powder intervention increased the abundance of the abundance of *Butyricimonas*. Therefore, it may the reason the combination of LBP and quinoa ultrafine powder group contained more butyric acid than the separate group after the intervention. In our study, KEGG functional prediction indicated that the combination of LBP and quinoa ultrafine powder group reduced lipid and glycerophospholipid synthesis. Correlation analysis with clinical indicators and SCFAs suggested that the combination of LBP and quinoa ultrafine powder intervention improves NAFLD lipid metabolism disorders by altering gut microbiota composition to enhance butyric acid production.

Disturbance of lipid metabolism significantly impact the occurrence and progression of NAFLD[55]. Overactivation of lipid synthesis is an important pathophysiological mechanism of liver steatosis[56]. Liver function and blood biochemical indexes are crucial physiological parameters for assessing NAFLD. Different diets had varying effects on improving lipid and liver function in NAFLD rats. The quinoa ultrafine powder intervention notably reduced Lee's index and liver index, consistent with weight loss effect of quinoa[57]. LBP intervention did not significantly affect weight loss in NAFLD. Combining quinoa ultrafine powder and LBP into the combination of LBP and quinoa ultrafine powder diet resulted in a more significant reduction in Lee's index, liver index, and visceral index, with a pronounced effect on improving NAFLD liver function and reducing lipid deposition. However, calorie intake had no significant differences among the groups, suggesting that the observed weight loss may be related to energy metabolism.

In vitro and in vivo experiment revealed a close link between dietary patterns and NAFLD development. The combination of LBP and quinoa ultrafine powder diet was found to be a potent fermentation substrate in the intestines, promoting SCFAs proliferation. Dietary supplementation with SCFAs prevents and reverses metabolic abnormalities induced by HFD in mice， several findings also validated the link between stimulated oxidative metabolism in the liver and increased AMPK activity[58]. Butyric acid is one of the important SCFAs, which has the potential to regulate lipid metabolism, modulate innate immunity and protect intestinal health[59]. Sodium butyric acid directly increases LKB1 phosphorylation in hepatocytes, leading to an elevation in the intracellular AMP/ATP ratio and the activation of AMPK and inhibits the cleavage activation

of SREBP-1C. SREBP-1C is the main regulator of lipid production[60]. SREBP-1C regulates key genes in fat production, such as FAS and SCD-1. FAS is a central enzyme in *de novo* lipogenesis, while SCD-1 is an enzyme involved in the production of unsaturated fatty acids. Downregulation of SREBP-1C reduces the expression of lipogenic proteins such as FAS[61], thereby reducing *de novo* lipid synthesis in hepatocytes and alleviating hepatocyte steatosis[56, 62]. This is consistent with our findings.

Nonetheless, while the combination of LBP and quinoa ultrafine powder diet has the preventive potential of against the progression of NAFLD, its efficacy remains somewhat limited, a long-term strategy (> 3 months) dietary intervention and further investigation through clinical trials is warranted.

5. Conclusions

In conclusion, our findings indicate that compared with LBP or quinoa ultrafine powder alone, consuming 50 mg of LBP with 1.8 g of quinoa ultrafine powder has a better effect on reducing weight and tissue fat, and improving lipid metabolism disorders caused by HFD. These positive effects are likely attributed to its regulatory impact on gut microbiota dysbiosis, including enhancing bacterial diversity and altering the relative abundances of *Lactobacillus acidophilus*, *Roseburia*, and *Ruminococcus2* at the genus level, promoting the production of SCFAs, especially butyric acid. The activation of AMPK/SREBP-1C/SCD-1 signaling by butyric acid may be the mechanism of improves liver lipid accumulation by QL diet. Therefore, LBP combined with quinoa ultrafine powder serves as a promising natural prebiotic dietary option for NAFLD prevention in our daily lives.

Conflicts of Interest

The authors declare no conflicts of interest.

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