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Distinct effects of highland barley and common buckwheat on gut microbiota and liver transcriptomics in high-fat diet-induced mice

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ABSTRACT: Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver condition, influenced by factors such as obesity, insulin resistance, and hyperlipidemia. While ancient grains like highland barley (HB) and common buckwheat (BW) have shown potential to mitigate these factors, direct comparisons of their effects on NAFLD remain unclear. The present study investigated the impact of HB and BW on HFD-induced NAFLD and the underlying mechanisms, including the influence of gut microbiota and fecal metabolic profile. HB supplementation for 12 weeks significantly reduced fat accumulation, serum triglycerides, insulin resistance, and hepatic steatosis visualized through H&E staining. BW supplementation also reduced these parameters, though less pronouncedly. Besides, HB and BW altered the gut microbiota composition, enriched the abundance of probiotics, while reducing harmful bacteria. Specifically, HB increased the abundance of *Ileibacterium* and *norank_f_Muribaculaceae*, while reducing that of *Mucispirillum* and *Helicobacter*. BW elevated levels of *Akkermansia* and *Lachnospiraceae_UCG-001*, and lowered those of *Mucispirillum* and *norank_f_Peptococcaceae*. In addition, HB supplementation greatly increased fecal levels of succinic acid and L-aspartic acid, whereas BW supplementation raised levels of 2,3-dihydroxybenzoic acid, p-salicylic acid, pyrocatechol, and gentisic acid. Moreover, liver transcriptomic analysis revealed that HB upregulated LEPR, AMPK α 1, and PGC-1 α , while BW enhanced PI3K, AKT, and BCL-2 expression. Overall, HB and BW alleviated HFD-induced NAFLD through distinct modulation of gut microbiota, fecal metabolites, and hepatic transcriptomic profiles.

Keywords: Highland barley; Common buckwheat; Non-alcoholic fatty liver disease; Gut microbiota; Fecal metabolites; Liver transcriptomics

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by pathologic fat accumulation in the liver and is regarded as the most common liver disease worldwide. Risk factors contributing to NAFLD development and progression include obesity, diabetes, and hypertriglyceridemia ^[1,2]. At present, no

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pharmacological therapies have been approved specifically for NAFLD. Instead, recommended management focuses on lifestyle modification, particularly dietary control and increased physical activity.

Studies on the relationship between NAFLD and gut microbiota have shown that harmful bacteria such as *Mucispirillum*, *Desulfovibrio*, *Anaerotruncus*, and *Desulfovibrionaceae* are sequentially enriched during the progression of NAFLD [3]. Conversely, several beneficial bacteria, including *Muribaculaceae*, *Akkermansia*, *Lactobacillus*, and *Prevotella*, are significantly reduced in NAFLD models compared to healthy controls [4–7].

Therefore, it has become possible to alleviate NAFLD by modulating the gut microbiota through dietary intervention. Whole-grain-rich diets, which provide more fiber and phenolic compounds, improve metabolic risk factors and may protect against NAFLD through their prebiotic effects on gut microbiota and metabolites. For example, dietary fiber can be fermented by certain intestinal microorganisms to generate organic acids such as succinate, acetate, propionate, and butyrate. These metabolites can regulate inflammatory responses, thereby suppressing the progression of NAFLD [8,9]. Also, the gut microbiota metabolizes complex dietary polyphenols into low-molecular-weight phenolic acids that are more bioavailable and biologically active and interact with host liver cells via the gut-liver axis [10].

With advances in precision nutrition, it has become clear that different whole grains confer distinct health benefits. In Sprague Dawley (SD) rats fed a high-fat diet, millet supplementation (50%, 4 weeks) reduced serum total cholesterol and modified gut microbiota composition by increasing *Anaerostipes*, *Bifidobacterium*, and *Akkermansia*, without affecting body weight [11]. Highland barley supplementation (49%, 8 weeks) enhanced the relative abundances of *Prevotella* and *Anaerovibrio*, improved dyslipidemia, and reduced body weight gain in SD rats [12]. Daily administration of common buckwheat (1,000 mg/kg/day, 8 weeks) in Kunming mice improved lipid profiles and limited weight gain. The treatment also increased the relative abundances of *Blautia*, *Lactobacillus*, *Streptococcus*, and *unclassified_Rikenellaceae*, while reducing *Desulfovibrio*, *Bacteroidales_S24-7*, and *Allobaculum* [13]. Collectively, these findings highlight the potential of whole grains in mitigating NAFLD.

However, variations in study design, intervention methods, dosage, and models make it difficult to determine which specific whole grain exerts the most pronounced beneficial effect on NAFLD. Besides, most prior research has focused on the biochemical effects of highland barley and common buckwheat, with limited investigation into the role of gut microbiota and its metabolites as therapeutic targets.

Therefore, this study compared diets containing 30% highland barley (HB) and 30% common buckwheat (BW) using the same experimental protocol to evaluate their effects on hepatic steatosis, body fat accumulation, serum lipid levels, inflammatory factors, and glucose tolerance in high-fat diet-induced NAFLD mice. Furthermore, 16S rRNA gene sequencing, untargeted fecal metabolomics, and liver transcriptome analyses were employed to elucidate the potential mechanisms underlying the protective effects of HB and BW. These findings may support future work to develop functional foods for NAFLD management.

2. Materials and methods

2.1 Preparation of HB and BW

Highland barley and common buckwheat used in the study were purchased from Shanxi Oriental Dongfangliang Life Sciences Co., LTD. Both grains were soaked in distilled water for 30 min, then mixed with distilled water in the ratio of 1:2 (w/v), and cooked in a standard commercial cooker according to the manufacture guidelines. The cooked grains were dehydrated in an air oven at 40°C for 24 h, milled, sieved through a 60-mesh screen, and stored at 4°C for subsequent use.

2.2 Animal Experimental Design and Diets

Male C57BL/6 mice, aged 6 weeks, were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). The mice were housed under specific-pathogen-free (SPF) conditions at $23 \pm 2^\circ\text{C}$, with a 12-h light/dark cycle and $50 \pm 5\%$ humidity with unrestricted access to food and water. All experimental protocols complied with National Research Council guidelines and were approved by the Animal Protection Professional Committee of China Agricultural University (approval number: AW90303202-5-1). Following a one-week acclimation, mice were randomly assigned to six groups ($n = 6$ per group): C57/BL 6J mice fed with (1) a normal control diet (NCD group; 10% fat); (2) a high-fat diet (HFD group; 60% fat); (3) a NCD supplemented with 30% HB (NCD-HB group); (4) an HFD supplemented with 30% HB (HFD-HB group); (5) a NCD supplemented with 30% cooked common buckwheat (NCD-BW group); and (6) an HFD supplemented with 30% BW (HFD-BW group). In the present study, the 30% supplementation level was chosen based on previous studies [14–17]. Diets were produced by Changzhou SYSE Bio-Tec. Co., Ltd (Changzhou, China). The composition of all diets used in this study is detailed in Table S1. The vitamin mix was formulated in accordance with the AIN-76A standards established by the American Institute of Nutrition.

At the end of the 12-week intervention, an oral glucose tolerance test (OGTT) was carried out. After fasting for 6 h with free access to water, mice were taken 20% glucose dissolved in saline orally at a dose of 2 g/kg body weight, using individual body weights measured immediately before gavage on the test day. Then blood glucose levels were measured at 0, 30, 60, 90, and 120 min post-gavage using a glucometer and test strips (Byer, Ascensia Diabetes Care Shanghai Co., Ltd.) [18]. The area under the curve (AUC) was calculated for blood glucose levels over 120 min. After an 8-h fast, mice were anesthetized, and blood was collected from the retro-orbital vein before sacrifice by cervical dislocation. The blood samples were centrifuged at 3,500 rpm for 15 min to obtain serum, which was stored at -80°C for further testing. White adipose tissue and livers were also collected. A part of the liver tissue was fixed in 4% paraformaldehyde, and the rest was stored at -80°C for subsequent experiments.

2.3 Biochemical Analysis of Serum and Liver

The levels of serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) were measured using a 3100-automatic biochemistry analyzer (Hitachi Ltd., Tokyo, Japan). TG and TC levels in liver tissues were measured with the corresponding kits purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.4. ELISA analysis of plasma inflammatory cytokines

The levels of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) were measured using ELISA kits (Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China) according to the manufacturer instructions.

2.5 Hematoxylin and Eosin (H&E) Staining

The fixed liver tissues were embedded in paraffin and sectioned at 5 μ m thickness. The sections were stained with H&E and examined under a NIKON Eclipse CI microscope (Nikon, Tokyo, Japan).

2.6 16S rRNA Gene Sequencing Analysis

Fresh fecal samples were collected at the experiment conclusion and immediately stored in liquid nitrogen. Genomic DNA from fecal samples was extracted from the E.Z.N.A.[®] soil DNA kit (Omega Bio-Tek, Norcross, GA, USA). DNA concentration and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, MA, USA) and verified through 1% agarose gel electrophoresis. The V3-V4 region of the bacterial 16S rRNA gene was amplified using ABI GeneAmp[®] 9700 thermocycler (ABI, Foster City, CA, USA) with primers 338F: 5'-ACTCCTACGGAGGCAGCAGCAG-3' and 806R: 5'-GGACTACHVGGTWTCTAAT-3'. PCR products were obtained with 2% agarose gel, purified with AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified using Quantus Fluorometer (Promega, Madison, WI, USA). Sequencing was performed on the Illumina MiSeq PE300 platform. Quality screening and filtering were carried out on the QIIME bioinformatics platform to obtain high-quality sequences. Alpha diversity was analyzed at the operational taxonomic unit (OTU) level, clustering sequences with at least 97% similarity. Beta diversity was examined by principal coordinates analysis (PCoA) to compare microbial community composition across samples. In addition, differences in relative abundance among groups were assessed using the Wilcoxon rank sum test.

2.7 Untargeted Fecal Metabolomics Analysis

For fecal metabolomics analysis, 50 mg fecal samples were measured, and the metabolites were extracted using 400 μ L of methanol to acetonitrile (4:1, v:v) containing 0.02 mg/mL of the internal standard. Then, the samples were ground with Wonbio-96c frozen tissue grinder for 6 min at 50 Hz, extracted by low-temperature ultrasonic extraction for 30 min at 40 kHz, maintained for 30 min at -20°C, and centrifuged for 15 min at 13,000 g and 4°C. Then, following previously described methods, the supernatant was transferred to an injection vial for LC-MS/MS analysis^[19]. The raw UHPLC-MS/MS data files were processed with Progenesis QI 2.3 software (Waters Corporation, Milford, USA) for baseline correction, peak identification, integration, retention time correction, and peak alignment, and finally, the retention data matrix was obtained to acquire time, mass-to-charge ratio, and peak intensity for further analysis. Metabolites were identified via HMDB (<http://www.hmdb.ca/>) and KEGG (<http://www.genome.jp/kegg>) databases. Principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were applied to evaluate overall

differences. Metabolites with $VIP > 1$, $P < 0.05$, and fold change (FC) of at least 1.5 were considered differentially expressed among different groups.

2.8 Liver transcriptome analysis

Total RNA was extracted from liver samples using Trizol reagent (Takara, Osaka, Japan). After quality assessment via an Agilent 2100 Bioanalyzer and quantification with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, MA, USA), high-quality RNA was selected for library construction. Sequencing was conducted on an Illumina HiSeq 2000 platform by Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China). Raw paired-end reads were trimmed and quality-controlled using FastQC, followed by differential expression analysis with DESeq2 (p -value < 0.05 , $FC \geq 1.5$). Fisher's exact test with Benjamini-Hochberg correction was applied for statistical significance, and functional enrichment was evaluated using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

2.9 Statistical Analysis

The data were analyzed and calculated significance using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test by GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). Results are expressed as S.E.M. (standard error of the mean). Statistical significance was considered at $P < 0.05$.

3. Results and discussion

3.1 Effects of HB and BW Supplementation on Weight Parameters, Fat Accumulation, and Glucose Metabolism in HFD-Fed Mice

Previous studies have linked obesity and diabetes to NAFLD ^[1,2]. In our study, initial body weights did not differ significantly across the six groups, confirming the validity of the grouping method. At the end of the experiment, the HFD group showed a significant increase in body weight compared to the NCD group (Fig. 1A). HB supplementation significantly reduced HFD-induced weight gain, while BW supplementation delayed weight gain during the intervention (Fig. 1B). Compared with the NCD group, the HFD group showed a significant increase in white adipose tissue weight; as expected, it was significantly decreased in the HFD-HB group and reduced in the HFD-BW group (Fig. 1C). In addition, HFD resulted in significantly higher fasting blood glucose levels and the area under the curve (AUC) value. HB supplementation significantly reduced AUC and blood glucose levels at 15 min during OGTT, while BW did not demonstrate significant effects on these indices (Fig. 1G-I). Notably, there were no significant differences in food intake among the HFD, HFD-HB, and HFD-BW groups (Fig. 1D-F). These results indicate that HB supplementation can more effectively attenuate HFD-induced body weight gain, white adipose tissue accumulation, and insulin resistance than BW without affecting appetites or energy intake.

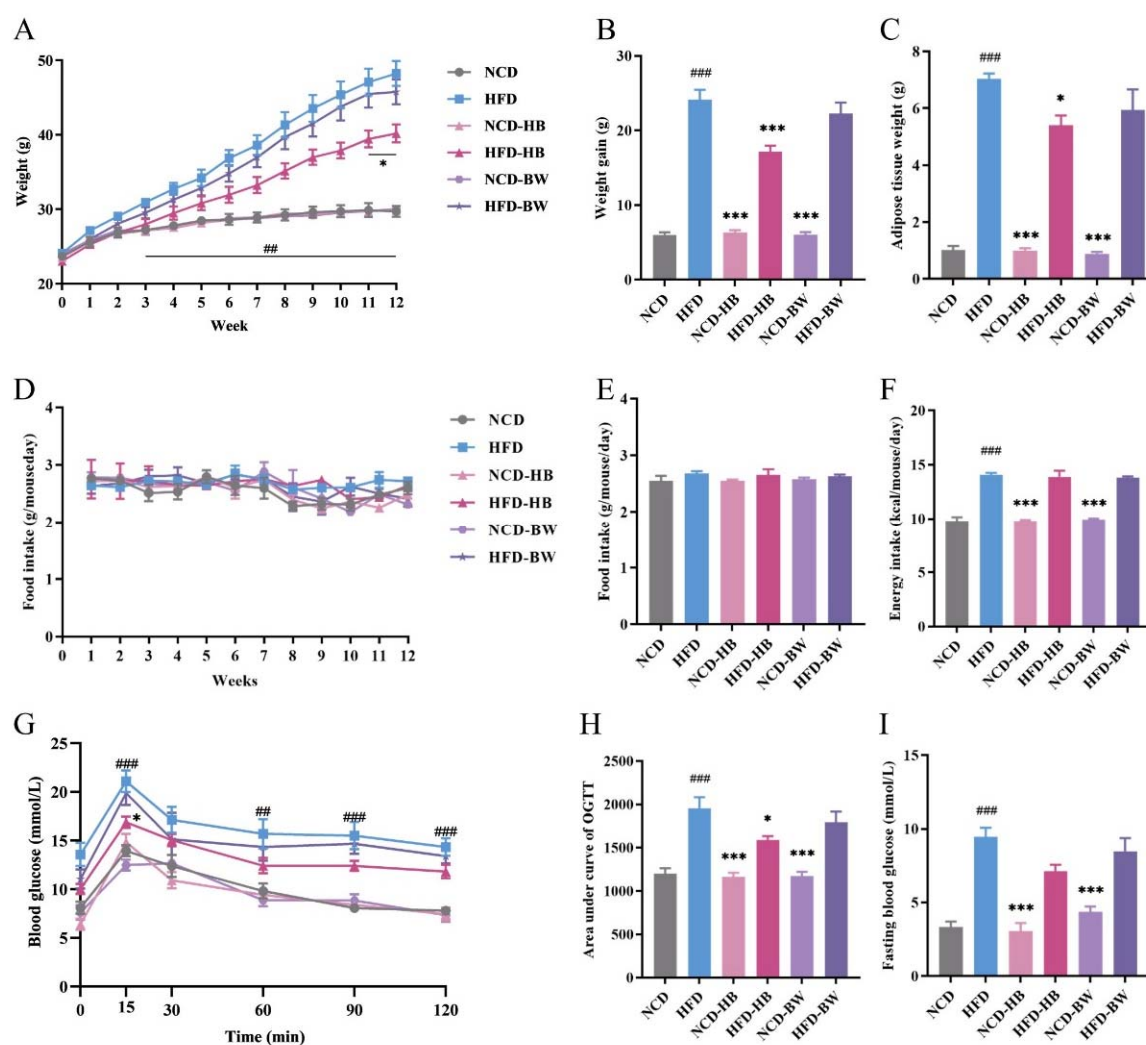


Fig. 1 After eight weeks of treatment, the effects of highland barley (HB) and common buckwheat (BW) supplementation on (A) Body weight curve; (B) Body weight gain; (C) Adipose tissue weight; (D) Food intake curve; (E) Food intake; (F) Energy intake; (G) Blood glucose at different time points during oral glucose tolerance test (OGTT); (H) Area under the curve (AUC) of OGTT; (I) Fasting blood glucose. Data are expressed as mean $\pm$ SEM ($n = 6$). * $P < 0.05$ and *** $P < 0.001$ compared with HFD group. ### $P < 0.01$ and #### $P < 0.001$ compared with NCD group.

3.2 Effects of HB and BW Supplementation on Liver and Serum Parameters and Histopathology in HFD-Fed Mice

NAFLD is marked by fat accumulation in the liver and often results in lipid metabolism dysfunction. Inflammatory response is an essential contributor to the development and progression of NAFLD [20]. In this study, HFD feeding led to a significant increase in liver weight compared to mice fed an NCD, which was lowered by BW supplementation and notably reversed by HB supplementation (Fig. 2B). H&E staining of liver sections showed well-organized hepatocytes with intact cytoarchitecture and no inflammation in the NCD group (Fig. 2A). In contrast, HFD induced severe liver damage, marked by steatosis vacuoles and infiltration of inflammatory cells. Both HB and BW ameliorated these pathological changes, with HB exhibiting a stronger protective effect. HFD feeding also elevated hepatic and serum lipid levels (Fig. 2C, D). While both supplements significantly reduced serum TG and hepatic TC concentrations, only HB supplementation resulted in a significant reduction in hepatic TG. Similarly, HFD-induced elevations of serum

TNF- α and IL-6 were significantly reduced by both supplements, with HB showing a greater efficacy (Fig. 2E, F). Notably, no significant parameter differences were found among the NCD, NCD-HB, and NCD-BW groups, indicating that the supplements had no adverse effects under normal dietary conditions.

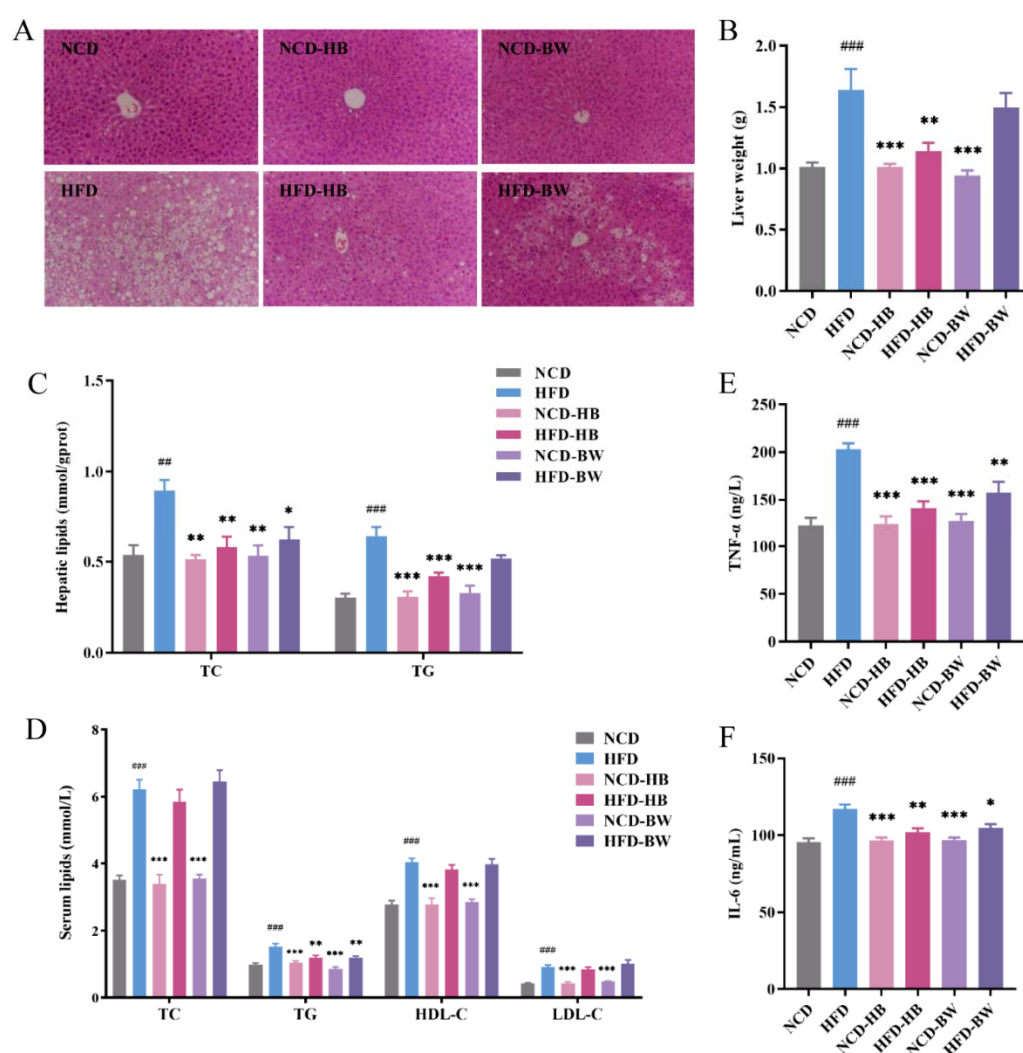


Fig. 2 Serum lipid parameters, liver function indicators and histology in mice of different groups. (A) H&E staining of liver tissues (400 $\times$ magnification); (B) Liver weight; (C) Hepatic total cholesterol (TC) and triglyceride (TG); (D) Serum concentrations of TG, TC, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C); (E-F) Serum levels of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6). Data are expressed as mean $\pm$ SEM ($n = 6$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with HFD group. ### $P < 0.01$ and #### $P < 0.001$ compared with NCD group.

3.3 Effects of HB and BW Supplementation on Gut Microbiota Dynamics in HFD-Fed Mice

Recent studies have demonstrated that gut microbiota play a crucial role in host metabolism. To further evaluate the effects of HB and BW on HFD-induced NAFLD, we conducted 16S rRNA gene sequencing of fecal samples. As shown in Fig. 3A-D, HB and BW supplementation did not significantly alter α -diversity indexes (ACE, Chao, Shannon, and Simpson indexes) compared to the HFD group. PCoA and NMDS based on OTUs abundance further revealed that gut microbial composition exhibited a response to HB and BW interventions (Fig. 3E, F). The gut microbial composition in the HFD group was clearly separated from that of the NCD group. While the BW group clustered close to the HFD group, the HB group was distinctly

separated from the HFD group, which suggested that HB supplementation induced a more remarkable change in gut microbial structure compared with the HFD group. Figure 3G shows that *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* dominated all groups with varying relative abundances. Compared with the NCD and intervention groups, an evident increase in *Deferribacterota* and a significant decrease in *Verrucomicrobia* were observed in the HFD group (Fig. 3G). At the genus level, HB supplementation significantly reduced the relative abundances of *Mucispirillum* and *Helicobacter*, while increasing *Ileibacterium* and *norank_f_Muribaculaceae* compared with the HFD group. BW supplementation notably reduced the relative abundances of *Mucispirillum* and *norank_f_Peptococcaceae*, along with increases in *Akkermansia* and *Lachnospiraceae_UCG-001*. These findings indicate that HB and BW may mitigate hepatic steatosis by promoting beneficial bacteria while suppressing certain harmful taxa associated with HFD.

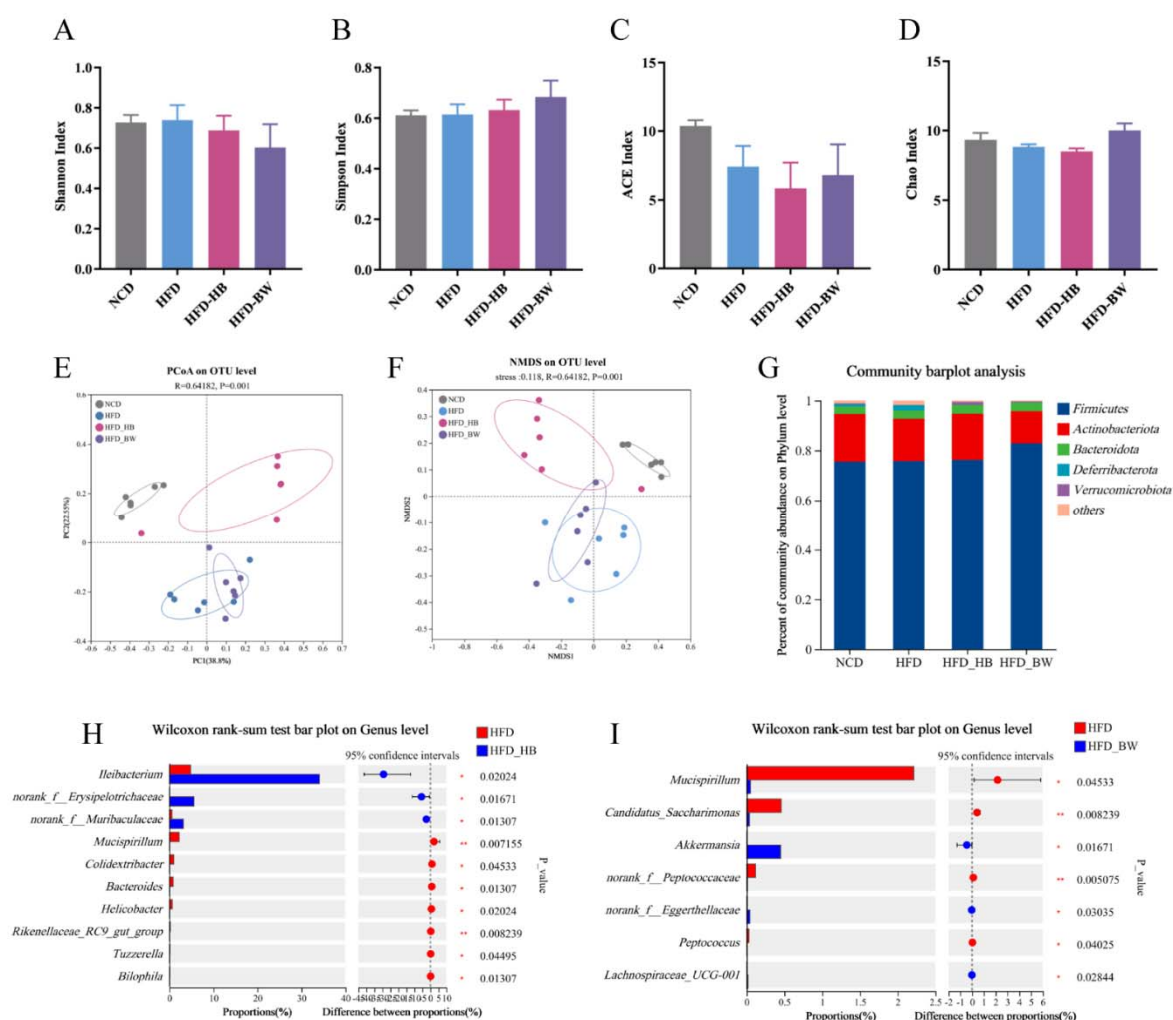


Fig. 3 HB and BW mitigated gut microbiota dysbiosis in HFD-induced NAFLD mice. (A) Shannon index; (B) Simpson index; (C) ACE index; (D) Chao index; (E) Principal coordinate analysis (PCoA) score plot; (F) Non-metric multidimensional scaling (NMDS) score plot; (G) The relative abundance of gut microbiota at the phylum level; (H-I) Mean proportions of key genera in different groups. * $P < 0.05$ and ** $P < 0.01$ compared with HFD group.

3.4 Effects of HB and BW Supplementation on Fecal Metabolomics in HFD-Fed Mice

Fecal metabolic profiles of 12th week mice were assessed by PCA under positive and negative ion modes (Fig. 4A). PC1 and PC2 explained 25.60% and 16.70% of the variance, respectively. The NCD group clustered distinctly from the HFD group, indicating that HFD altered the fecal metabolic profile. The ellipses of the HFD-HB and HFD-BW groups were separated from the HFD group and partially overlapped, suggesting their potential to mitigate metabolic disruptions in HFD-induced NAFLD mice. Differential metabolites were identified after peak alignment (VIP values > 1.0 and P -values < 0.05): HB supplementation up-regulated 269 and down-regulated 509 differential metabolites, while BW supplementation up-regulated 324 and down-regulated 520 differential metabolites compared with the HFD group (Fig. 4B, C). These metabolites can be classified into 5 KEGG pathway categories (Fig. 4D, E). KEGG topological analysis identified 7 pathways with impact value > 0.1 in HFD-HB vs. HFD: alanine, aspartate and glutamate metabolism; pyrimidine metabolism; pantothenate and CoA biosynthesis; histidine metabolism; tryptophan metabolism; tropane, piperidine and pyridine alkaloid biosynthesis; and biosynthesis of siderophore group nonribosomal peptides (Fig. 4F). HB supplementation significantly increased succinic acid, L-aspartic acid, uracil, pantothenic acid, and L-histidinol. In contrast, the levels of thymidine and phenyllactic acid were reduced greatly by HB supplementation compared with the HFD group (Table S2). Seven pathways with impact values above 0.1 were identified in HFD-BW vs. HFD: aminobenzoate degradation; sphingolipid metabolism; histidine metabolism; glycerophospholipid metabolism; caffeine metabolism; benzoate degradation; tropane, piperidine and pyridine alkaloid biosynthesis (Fig. 4G). High levels of 2,3-dihydroxybenzoic acid, p-salicylic acid, pyrocatechol, lysophosphatidylcholine (LysoPC) (17:0), gentisic acid, L-aspartic acid, and low levels of phosphatidic acid (PA) (16:0/15:0) and phenyllactic acid were observed in the HFD-BW group compared to the HFD group (Table S3). These results demonstrated that both HB and BW treatment had regulatory effects on NAFLD-induced fecal metabolites disorder.

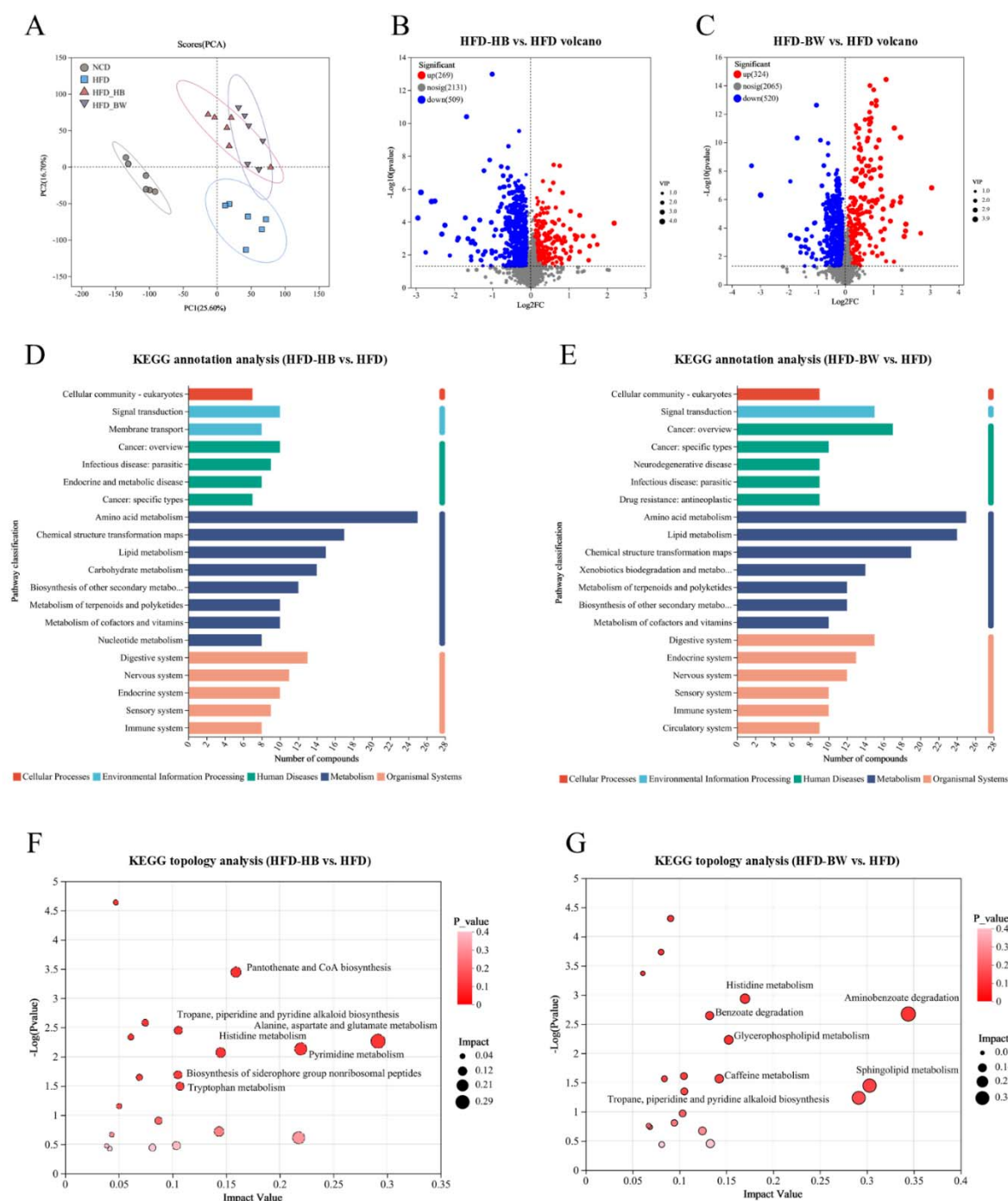


Fig. 4 Effects of HB and BW supplementation on fecal metabolite profile in mice. (A) Principal component (PCA) analysis; (B-C) Volcano plot; (D-E) Kyoto encyclopedia of genes and genomes (KEGG) annotation analysis of the changed metabolites based on the differences between the HFD and HFD-HB, HFD-BW groups; (F-G) KEGG topology analysis of metabolic pathways in the HFD-HB, HFD-BW groups compared with the HFD group. The p -value and pathway impact value are visualized by circle color and size, respectively.

3.5 Correlations between Gut Microbiota and Fecal Metabolites in HFD-Fed Mice

To investigate potential associations between gut microbiota at the genus level and fecal metabolites, the Pearson correlation analysis was conducted. In terms of HB supplementation, succinic acid displayed marked positive correlations with *Ileibacterium* and *norank_f_Muribaculaceae*, and significant negative correlations with *Helicobacter*. L-aspartic acid showed pronounced positive associations with *norank_f_Muribaculaceae*,

while thymidine exhibited strong negative links with *norank_f_Muribaculaceae*. Furthermore, phenyllactic acid showed robust positive correlations with *Helicobacter*, and negative correlations with *Ileibacterium* and *norank_f_Muribaculaceae*. Regarding BW supplementation, the analysis revealed that p-salicylic acid showed marked positive correlations with *Lachnospiraceae_UCG-001*. Moreover, 2,3-dihydroxybenzoic acid, p-salicylic acid, pyrocatechol, and gentisic acid showed robust negative associations with *norank_f_Peptococcaceae* (Fig. 5). Therefore, the gut microbiota is tightly related to fecal metabolites and host phenotypes.

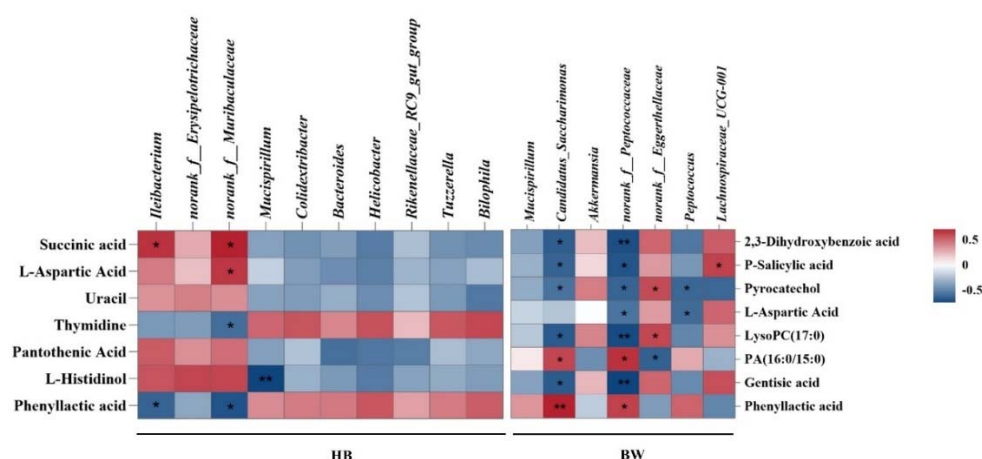


Fig. 5 Spearman correlation analysis between gut microbiota and fecal metabolites. The r value is represented by the gradient color, with red indicating a positive correlation and blue indicating a negative correlation. Significant correlations are marked by * $P < 0.05$ and ** $P < 0.01$. LysoPC, lysophosphatidylcholine; PA, phosphatidic acid.

3.6 Liver transcriptome analysis

To further investigate the molecular mechanisms underlying the hepatoprotective effects of HB and BW, liver transcriptomic profiling was performed using RNA sequencing. PCA analysis revealed a clear separation between the HFD and NCD groups (Fig. 6A). Both HB and BW supplementation groups clustered distinctly from the HFD group, indicating substantial alterations in hepatic gene expression profiles. Volcano plots illustrated differentially expressed genes (DEGs) across comparisons. Relative to the NCD group, the HFD group up-regulated 913 genes and down-regulated 826 genes (Fig. 6B). Compared with the HFD group, HB supplementation resulted in 194 upregulated and 287 downregulated genes, while BW supplementation led to 1,269 upregulated and 349 downregulated genes (Fig. 6C, D). Venn diagram analysis identified 46 genes commonly altered across all three comparisons, along with 1,411, 235, and 1,288 unique DEGs in HFD vs. NCD, HFD-HB vs. HFD, and HFD-BW vs. HFD, respectively (Fig. 6E). KEGG pathway enrichment analysis revealed that both HB and BW supplementation modulated genes associated with non-alcoholic fatty liver disease (Fig. 6F, G). Notably, HB supplementation significantly elevated the expression of leptin receptor (LEPR), AMP-activated protein kinase $\alpha 1$ (AMPK $\alpha 1$), and proliferator-activated receptor- γ coactivator-1 α (PGC-1 α). BW supplementation significantly elevated the expression of phosphoinositide 3-kinase (PI3K), Protein kinase B (AKT), and B cell lymphoma 2 (BCL-2) (Table S4).

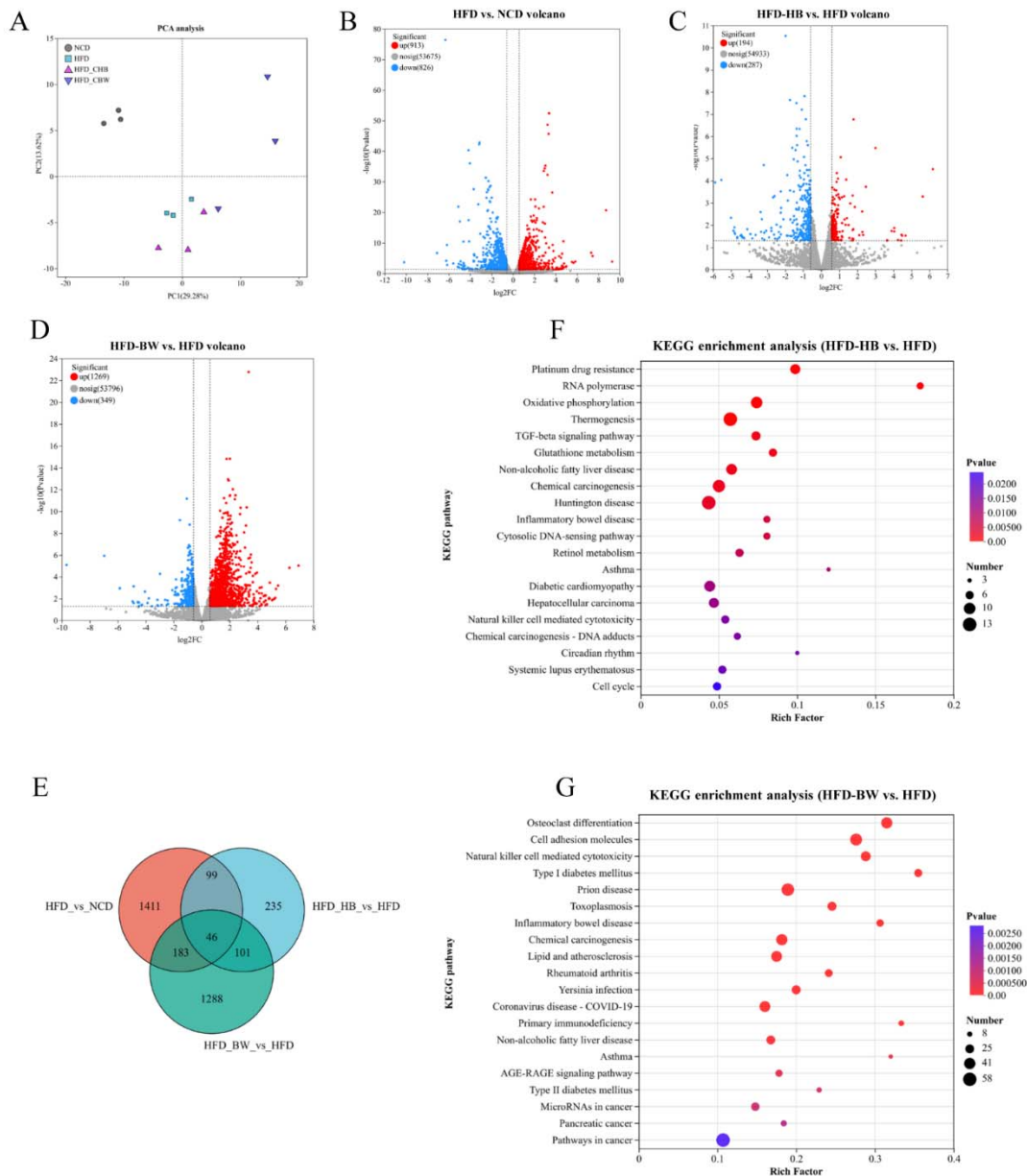


Fig. 6 Effects of HB and BW supplementation on transcriptome of mice liver. (A) Principal component (PCA) analysis; (B-D) Volcano plot; (E) Venn plot showing differentially expressed genes (DEGs) among groups; (F-G) KEGG enrichment analysis of DEGs.

4. Discussion

In this study, HB supplementation favorably altered physiological parameters in HFD-induced NAFLD mice, evidenced by significant reductions in liver weight, hepatic TG levels, and hepatic steatosis visualized through H&E staining. BW supplementation also reduced these parameters, though less pronouncedly. NAFLD progression is closely associated with body weight gain and adipose tissue accumulation. A weight gain over 7% increases NAFLD risk, while weight loss exceeding 3% reduces it [21]. Also, insulin resistance is tightly related to the buildup of liver fat [22], and elevated serum TG is a critical risk factor for NAFLD [23]. Increased fatty acid oxidation exacerbates this process by promoting the release of pro-inflammatory cytokines that drive NAFLD development and progression [20]. The present study found that HB supplementation can

significantly reduce body weight gain, fat accumulation, serum TG, insulin resistance, and systemic inflammation. BW significantly lowered serum TG and inflammation but showed no significant impact on other parameters. Overall, these findings suggest HB may be more effective than BW in improving glucolipid metabolism and inflammation in HFD-induced NAFLD mice.

Dietary patterns and nutritional profiles can alter the gut microbiota composition and attenuate obesity, type 2 diabetes, and other metabolic diseases that increase the risk of developing NAFLD [24]. As shown by PCoA and NMDS analyses, HB and BW supplementation altered gut microbial communities differently. Consistent with previous findings, HB increased *Ileibacterium* and *norank_f_Muribaculaceae* levels [25,26], while BW enhanced *Akkermansia* and *Lachnospiraceae_UCG-001* abundances [27]. *Ileibacterium* correlates with production of organic acids, influences weight management, host metabolism, and inflammatory conditions [28,29]. *Norank_f_Muribaculaceae*, a member of the *Muribaculaceae* family within the *Bacteroidetes* phylum, is also known to generate organic acids. Reduced levels of *Muribaculaceae* in the gut microbiota of NAFLD mice have been linked to increased hepatic steatosis and metabolic dysfunctions [30]. According to previous research, consumption of β -glucan can increase the populations of *Ileibacterium* and *Muribaculaceae*, potentially preventing NAFLD-related metabolic disorders [31,32]. Thus, β -glucan in HB may be partially responsible for the increased abundance of these genera and consequent hepatic protection in NAFLD mice. *Akkermansia*, well-documented for health benefits, can reduce hepatic steatosis and serum TG levels in HFD-induced mice [33]. *Lachnospiraceae_UCG-001* showed inverse correlations with body weight index and serum TG [34]. In trials where mice were fed high-fat diets supplemented with rutin, there was a significant increase in the abundance of *Akkermansia* and various *Lachnospiraceae* species [35,36], suggesting that the rutin in BW may drive the beneficial microbial changes, improving NAFLD outcomes. In addition to enhancing beneficial bacteria, HB and BW supplementation reduced the relative abundance of harmful bacteria. *Mucispirillum*, enriched in high-fat high-cholesterol diets, promotes fatty liver, steatohepatitis, fibrosis, and NAFLD–HCC [37]. *Helicobacter pylori*, more abundant in NAFLD patients compared to controls, worsens NAFLD through its impact on inflammatory cytokines, insulin resistance, and lipid metabolism [38]. *Peptococcaceae*, increased in high-fat diets, correlate positively with obesity and inflammation, contributing to NAFLD progression [39,40]. In the present research, HB significantly reduced *Mucispirillum* and *Helicobacter* levels, while BW lowered *Mucispirillum* and *norank_f_Peptococcaceae* abundances. Summarily, the findings suggest that HB and BW supplementation can favorably act on different gut microbiota that likely help alleviate hepatic steatosis in NAFLD mice. However, the specific bacterial taxa affected differ between the two interventions.

Fecal metabolomic analysis showed that HB and BW supplementation significantly alleviated the disordered fecal metabolism profiles associated with NAFLD. HB supplementation notably enriched succinic acid and L-aspartic acid, key metabolites in alanine, aspartate, and glutamate metabolism. Succinic acid is known to reduce epididymal white adipose tissue in HFD-fed mice and mitigate steatohepatitis [41,42], while L-aspartate (the dissociated form of L-aspartic acid) can inhibit CCl₄-induced liver fibrosis by modulating

AMPK activity [43]. In the context of pyrimidine metabolism, HB supplementation elevated uracil while decreasing thymidine. Elevated uracil may protect against NAFLD by enhancing hepatic fatty acid oxidation through AMPK signaling pathway [44], whereas increased thymidine levels have been linked to heightened insulin resistance [45]. Moreover, metabolites such as pantothenic acid and L-histidinol were up-regulated following HB supplementation. Pantothenic acid has been reported to suppress IL-6 and TNF- α [46], while L-histidine can significantly lower body weight, epididymal fat, and hepatic lipids in mice consumed a high saturated fat diet [47]. Conversely, phenyllactic acid, which associates with triglyceride accumulation in liver cells, was down-regulated after HB treatment [48]. Among the metabolites regulated by BW treatment, 2,3-dihydroxybenzoic acid, p-salicylic acid, and pyrocatechol were up-regulated in aminobenzoate degradation. These phenolic acids exhibit antioxidant and anti-inflammatory properties that help improve hepatic steatosis in the host [49–52]. Specifically, 2,3-dihydroxybenzoic acid has been shown to activate the PI3K/AKT pathway in cellular models [53]. In glycerophospholipid metabolism, BW supplementation increased LysoPC(17:0) while decreasing PA(16:0/15:0). The elevation of LysoPC(17:0) provides hepatoprotection during overnutrition [54], while the reduction in PA(16:0/15:0) limits the release of palmitic acid, a driver of NAFLD [55]. Furthermore, up-regulated gentisic acid was observed in benzoate degradation. Gentisic acid is another phenolic compound that can suppress the expression of inflammatory cytokines, including TNF- α and IL-6 [56,57]. In line with the effects of HB treatment, BW supplementation also upregulated L-aspartic acid and downregulated phenyllactic acid levels. Together, the findings suggest that HB may alleviate NAFLD symptoms by regulating metabolites involved in alanine, aspartate, and glutamate metabolism; pyrimidine metabolism; pantothenate and CoA biosynthesis; histidine metabolism; and the tropane, piperidine and pyridine alkaloids biosynthesis. BW appears to benefit hepatic health through aminobenzoate degradation, histidine metabolism, glycerophospholipid metabolism, benzoate degradation, and tropane, piperidine and pyridine alkaloids biosynthesis.

Plant-derived bioactive compounds can modify the composition of intestinal microbiota and reshape the fecal metabolic profile, which subsequently enters circulation and influences various physiological processes in the host. This interaction between gut microbiota and fecal metabolites is crucial for maintaining homeostasis. Nutrients containing dietary fibers, like β -glucan, can be metabolized into succinic acid, which is favorable to reduce fat accumulation and mitigate steatohepatitis [40,41]. The process by which β -glucan generates succinic acid is mediated by the gut microbiota. For example, *Muribaculaceae* encode and produce a large number of carbohydrate-active enzymes (CAZymes), which enable them to degrade complex polysaccharides like β -glucan [58]. *Ileibacterium* is also enriched in CAZymes that enable efficient carbohydrate metabolism. Furthermore, studies showed that *Helicobacter* can consume succinic acid by incorporating it into the TCA cycle during microaerophilic growth [59]. Therefore, the enrichment of *norank_f_Muribaculaceae* and *Ileibacterium*, coupled with the reduction of *Helicobacter* may have elevated succinic acid levels in the HFD-HB group, contributing to the alleviation of HFD-induced NAFLD. L-aspartate, the dissociated form of L-aspartic acid, has been shown to inhibit CCl₄-induced liver fibrosis [43].

Members of the *Muribaculaceae* family are capable of synthesizing aspartate [58]. In the present study, HB supplementation induced a bloom of *norank_f_Muribaculaceae* and increased L-aspartic acid levels, which may have contributed to the alleviation of NAFLD symptoms. Dietary phenols, like rutin, can be generated by gut microbiota to produce phenolic acids, which mitigate oxidative stress and inflammation in HFD-fed mice [41]. The *Lachnospiraceae* family of bacteria within the *Firmicutes* phylum possesses enzymes such as α -rhamnosidases and β -glucosidases that are critical for breaking down dietary phenols, leading to the production of low-molecular-weight phenolic acids (e.g., 2,3-dihydroxybenzoic acid, p-salicylic acid, pyrocatechol, and gentisic acid) for the host to absorb [60,61]. Specifically, a study on habitual polyphenol consumption identified *Lachnospiraceae_UCG-001* as a biomarker associated with polyphenol intake [62]. The *Peptococcaceae* family, on the other hand, has been demonstrated to utilize phenolic compounds as energy sources through anaerobic pathways [63]. Therefore, the increased abundances of *Lachnospiraceae_UCG-001* and decreased abundance of *norank_f_Peptococcaceae* may have elevated 2,3-dihydroxybenzoic acid, p-salicylic acid, pyrocatechol, and gentisic acid levels in the HFD-BW group, contributing to the relief of HFD-induced NAFLD. Collectively, these findings suggest that the beneficial effects of HB and BW were associated with enhanced intestinal homeostasis and restoration of normal metabolic function in the feces.

Furthermore, HB and BW supplementation distinctly modulated the liver transcriptome in HFD-induced NAFLD mice. The AMPK and PI3K/AKT pathways are critical in NAFLD development. Specifically, HB supplementation increased expression of AMPK pathway genes, such as LEPR, AMPK α 1, and PGC-1 α . AMPK, which is activated by LEPR, is a recognized target for NAFLD therapy. Bioactive compounds such as L-aspartate and uracil have been shown to treat NAFLD via AMPK-mediated pathways [43,44]. Notably, increasing AMPK activity in NAFLD suppresses lipogenesis and increases expression of fatty-acid oxidation genes including PGC-1 α . Elevated hepatic PGC-1 α expression can significantly reduce inflammation, thereby alleviating NAFLD [64]. Conversely, high-fat diet inhibits the PI3K/AKT pathway, promoting hepatocellular injury through apoptosis [65]. BW supplementation elevated PI3K, AKT, and anti-apoptotic BCL-2 expression, reducing hepatic steatosis. Additionally, 2,3-dihydroxybenzoic acid activates the PI3K/AKT pathway in cellular models [53]. Collectively, these findings suggest that HB and BW supplementation alter gut microbiota composition, enhancing metabolic function and upregulating AMPK and PI3K/AKT pathway genes, thereby improving liver fibrosis (Fig. 6).

5. Conclusion

In summary, the present study revealed that HB supplementation alleviates glucose metabolism, lipid disorders, systemic inflammation, and hepatic steatosis in HFD-induced NAFLD mice. BW supplementation had similar but less pronounced effects at the same dose. These improvements were associated with restored intestinal homeostasis and fecal metabolic function. To be specific, HB increased the abundances of *Ileibacterium* and *norank_f_Muribaculaceae* while reducing *Mucispirillum* and *Helicobacter*. BW enriched *Akkermansia* and *Lachnospiraceae_UCG-001*, and decreased *Mucispirillum* and *norank_f_Peptococcaceae*.

In addition, HB markedly elevated fecal succinic acid and L-aspartic acid levels, whereas BW raised concentrations of phenolic metabolites, including 2,3-dihydroxybenzoic acid, p-salicylic acid, pyrocatechol, and gentisic acid. These microbial and metabolic shifts may be partially attributable to the distinct bioactive compounds in HB (β -glucan) and BW (rutin). Furthermore, liver transcriptomic analysis revealed that HB upregulated LEPR, AMPK α 1, and PGC-1 α , whereas BW upregulated PI3K, AKT, and BCL-2 expression. Subsequent studies could investigate how varying doses of HB and BW influence NAFLD outcomes. Targeted validation of microbiota and metabolite alterations resulting from β -glucan and rutin would provide stronger support for their key contributions. Additionally, the functional roles of individual bacterial species could be examined through microbiota transplantation.

Conflict of interest

The authors have no conflict of interest to declare.

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