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Polyphenols from foxtail millet improve non-alcoholic fatty liver disease by regulating intestinal flora

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic hepatic manifestation of metabolic dysfunction for which effective interventions are lacking. The burden of NAFLD is increasing at an alarming rate. NAFLD is frequently associated with morbidities such as dyslipidemia, type 2 diabetes mellitus and obesity, etc. The current study explored the potential role of bound polyphenol from foxtail millet (BPIS) in treating NAFLD mice induced by the high-fat diet (HFD). The results exhibited the critical role of BPIS treatment for NAFLD by effectively restoring gut microbiota in C57BL/6 mice that received a high-fat diet (HFD) for 12 weeks. At the same time, 16S rRNA analysis demonstrated that BPIS remodeled the overall structure of the gut microbiota from fatty liver diseases towards that of normal counterparts, including ten phylum and twenty genus levels. Further study found that the expression of tight junction proteins was up-regulated in the BPIS-treated group. This study provides new insights into the potential NAFLD protective effects induced by polyphenols of foxtail millet.

Keywords: BPIS; gut microbiome; Foxtail millet; NAFLD
**Introduction**

Non-alcoholic fatty liver disease (NAFLD) has become rapidly increasingly prevalent worldwide. It affects approximately 25-30% of individuals globally, and the prevalence and incidence of NAFLD have increased significantly [1-3]. NAFLD is categorized by excessive accumulation of hepatic triglycerides and incorporates a range of disease states, from steatosis to non-alcoholic steatohepatitis, distinguished by the presence of lobular inflammation and hepatocyte ballooning with increasing fibrosis stage, to cirrhosis and hepatocellular carcinoma [4]. There are no FDA-approved pharmacology therapies to treat NAFLD [5], further studies are required for novel approaches to NAFLD prevention and treatment. NAFLD is closely related to demographic differences (aging of society), an unnatural lifestyle (overeating, lack of exercise), gut dysbiosis, and cardiovascular-metabolic diseases. Obesity is a key factor in NAFLD and is significantly associated with other NAFLD risk factors [6]. Mice lacking gut microbiota are resistant to diet-induced obesity and hepatic steatosis, and germ-free mice that receive fecal microbiota transplantation from donor mice with metabolic syndrome develop liver steatosis independent of dietary intake, demonstrating that NAFLD occurrence may be associated with gut flora [7, 8].

Polyphenols are plants' secondary metabolites [9]. Many natural compounds are abundant in polyphenols such as green tea, coffee, cocoa, and plant-sourced foods (vegetables, grains, and fruits), and have positive effects on health (anticancer, hypoglycemic, and anti-inflammatory) [10, 11]. There have been studies showing that Polyphenol exerts favorable effects on NAFLD, with positive outcomes related to insulin resistance, liver fat accumulation, oxidative stress, proinflammatory status, and mitochondrial dysfunction [12]. Foxtail millet is extensively grown globally. Foxtail millet bran is the hard outer sheet of Foxtail millet, and as a by-product of
milling processing, it is often used for livestock and poultry feed [13]. Foxtail millet bran is reported rich in vitamins, minerals, essential amino acids, and, especially Polyphenols [14]. Several studies explored BPIS link with the treatments of diseases, reducing the risk of inflammatory bowel diseases, and human colorectal cancer through the intestinal flora [15, 16].

The gut microbiome, a diverse microbial community comprised of trillions of bacteria, fungi, viruses, archaea, and protists that encode several orders of magnitude, more functional genes than the human genome, can modulate human health and will likely be an integral component of the personalized medicine [17]. The diverse microbes that colonize the gut, which are collectively known as the gut microbiota, provide key health benefits. One of the key benefits of colonization is the ability to restrict colonization of the pathogens that trigger different diseases when dysbiosis occurs in gut microbiota [18]. Under normal circumstances the relationship between the human host and gut microbiome is mutually beneficial, perturbations of the gut microbiota have been associated with many chronic diseases [19]. The intestinal epithelial cells and mucus are the first line of defense and limit the translocation of harmful antigens. Possible mechanistic links between transformed microbiome and fatty liver are emerging and include the potential for bacterial protein to function as ligands for G protein-coupled receptors [20].

Recently it become possible to investigate the effect of phenolic compounds and their effects on gut microbiota [21, 22]. Many studies suggested that phenolic compounds might affect the gut microbiota to improve hepatic fat deposition, obesity, intestinal inflammation, insulin resistance, oxidative stress, and remodeling of the gut microbiome [23, 24].

In this regard, the NAFLD preventive effect of BPIS from millet bran is unclear, and the molecular mechanism involved requires further investigation. The effects of BPIS on lipid metabolism, and inflammation in HF-fed mice were evaluated. Subsequently, we assessed
whether BPIS-prevented NAFLD was related to the restoration of the intestinal barrier and gut
diagnostic composition in HF-fed mice. The BPIS is linked to the alleviation of HF-induced
NAFLD through the regulating of the gut microbiota, effect on liver and colon tissue, and
intestinal barriers has not been explained. This study aimed to evaluate the potential role of BPIS
for the treatment of NAFLD in mice, with a focus on a possible connection between alterations
in lipid metabolism, lipid accumulation effects on liver and colon tissue, and gut microbiota
restoration in NAFLD mice.

Material and Methods
Materials

RPMI 1640 medium and fetal bovine serum (FBS) were from Dcell biologics. Standard samples,
including BPIS, were purchased from Victory Biological Technology Co., Ltd. (Sichuan, China).
PCR kit purchased from Vanzyme Biotech Co., Ltd (Beijing, China). HiPerFect Transfection
Reagent and EpiTect@Bisulfite Kit (48) purchased from QIAGEN (Duesseldorf, Germany).
Ezup Column Animal Genomic DNA Purification Kit obtained from Sangon Biotechnology
(Shanghai, China). PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) and
Epi- Taq™ HS (for bisulfite-treated DNA) purchased from Takara. qPCR SuperMix was
obtained from TransGen Biotech (Beijing, China).

Animals

Male healthy C57BL/6N mice (5 weeks old) were purchased from (GemPharmatech Co., Ltd.,
Nanjing, China). Mice were placed in a specific-pathogen-free (SPF) feeding facility in the
Laboratory Animal Center and Animal Laboratory of Nephrology, Shanxi Provincial People’s
Hospital (Shanxi, China) at a temperature of 23±2 °C and a humidity of 50±15%, under a 12-h light-dark cycle condition and ad libitum access to food and water. The health status of mice was determined via daily observation by technicians under veterinary care. Under the permission of the Committee on the Ethics of Animal Experiments of Shanxi University (Shanxi, China) (Clinical Trial Number; SXULL2020046), all experiments were conducted by the American guide for the management and use of laboratory animals.

After a week of adaptation, a total of 44 mice with minimal difference were randomly assigned into three groups: 12 weeks mice the normal group (n = 11) were fed on ND (270 kcal per 100 g, 10% from fat, 20% from protein, and 70 % from carbohydrate Lab Diet) and the model-making group (n = 33) were fed with HFD (521 kcal per 100 g, 60% from fat, 20% from protein and 20% from carbohydrate, research diet) for 12 weeks. BPIS-treated groups were given BPIS (10 mg kg⁻¹ or 5 mg kg⁻¹ daily) by gavage treatment and were continued for 12 weeks.

**Sample collection**

After the 12-week feeding period, the mice were euthanized. Liver, intestine tissue, and feces were quickly frozen and stored at -80 °C for further analysis.

**Histopathology and Immunohistochemistry examination**

According to the previous method (Li et al., 2021), liver and colon tissue was rapidly immobilized in 4% paraformaldehyde for 24 h, inserted in paraffin wax, and made into 5-μm-thick sections. Sections were dewaxed after hydration. Sections of the colon and liver were stained with hematoxylin-eosin (H&E) for histological analysis, Oil Red O (ORO) stain, and immunohistochemistry (IHC) assay. After H&E and IHC staining of the sections, the staining
solution was flushed out. After dewatering and sealing, the sections were visualized under a motorized fluorescence microscope (Olympus, Tokyo, Japan) and pictures were saved.

**Serum biomarkers**

**Automatic determination by fully automated biochemistry**

(https://www.servicebio.cn/goodsdetail?id=2234)

**RNA isolation, cDNA synthesis, and RT-qPCR**

The colons of mice were comminuted using an automatic freeze grinder (Jing Xin, Shanghai, China). Total RNA was extracted from tissue using Triazole Reagent (Takara, Japan) according to the manufacturer’s protocol. For cDNA synthesis, RNA was reverse transcribed using the reagent Kit with gDNA Eraser (Takara, Japan) and the concentration of RNA used for the synthesis of cDNA was 500 ng/μL. Primer synthesis was completed by Sangon Biotech (Shanghai, China) as listed, RT-qPCR was using Tip Green qPCR SuperMix (TransStart, China). Quantification of total RNA was performed by running 1 μl each sample on Nanodrop. Complementary DNA was prepared by reverse transcription. The qPCR mixture contained 100 ng of tissue cDNA, primer1 (10 μM) 0.4 μl, primer2 (10 μM) 0.4 μl, Template DNA/cDNA × μl, and 2× ChamQ Universal SYBR qPCR Master Mix 10.0 μl, ddH₂O to 20.0 μl. PCR amplifications were performed using the following cycling parameter: The PCR reaction is in the following conditions: stage 1 at 95°C for 30 s, followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s, and 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s. The cDNA level for each gene was normalized to GAPDH mRNA levels. The primer sequences were as follows:
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cldn1</td>
<td>TGGCTATGGAGGCGGCTATGG</td>
<td>CCTGAGCGGTCACGATGTTGTC</td>
</tr>
<tr>
<td>Ocll</td>
<td>TGGCTATGGAGGCGGCTATGG</td>
<td>AAGGAAGCGATGAAGCAGAAGG</td>
</tr>
<tr>
<td>Zo1</td>
<td>CCACCTCGCACGCATCACAG</td>
<td>TGGTCCTTCACCTCTGAGCAGCACTAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACCCACTCCTCCACCTTTGA</td>
<td>AAGGAAGCGATGAAGCAGAAGG</td>
</tr>
</tbody>
</table>

The copy number was determined from standard curve generation using a synthetic template. Genetic relative quantification was achieved by the 2-ΔΔCT method utilizing GAPDH as an internal reference. The mRNA levels of genes were analyzed by Real-time PCR thermocycler (Bio-Rad, California, USA) according to the instructions of BlasTaqTM 2× RT-qPCR.

**16S rRNA gene sequencing analysis of microbiota in the fecal contents**

16S rRNA gene sequencing was applied to the intestinal flora in the fecal contents. Total DNA was isolated by the QIAamp Rapid Fecal DNA Extraction Kit (QIAGEN, German). Diluted genomic DNA was used as a template for the amplification of the V3-V4 hypervariable region using specific primers with barcodes. PCR was performed using Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs, Salisbury, The United Kingdom). After the libraries were qualified, the libraries were sequenced using NovaSeq6000. The 16S rRNA sequencing data of the intestinal flora were analyzed using QIIME2 software. Venn diagrams were presented by the R (Version 3.5.3) Venn Diagram package. Species abundance clustering heat maps were shown by the R (Version 3.1.0) heatmap package. NMDS analysis used the vegan package with R (Version 3.5.3). Differential species analysis was presented by LEfSe.
software (Version 1.0). Spearman correlation analysis was made with 157 the R (Version 2.15.3) psych package 158 and the heatmap package.

**Statistical Analysis**

The mean ± SEM was exploited to represent the data. One-way ANOVA and Student's t-test analyses were carried out by GraphPad Prism (Version 9.2). Student’s t-test was used for single variable comparisons. Comparisons of means of ≥3 groups were performed by analysis of variance (ANOVA), followed by Tukey’s posthoc test. The data were represented as the mean ± standard deviation (±SD) from at least three independent experiments. The p values of less than 0.05 and 0.01 were considered the difference significant and highly significant compared with the control.

**Results**
Fig 1 The design of the animal experiment and macroscopic structure of the liver and colon (A) C57BL/6J mice and body weight. (B) Serum TBIL. ALT. TCHO. (C) images of the H&E-stained and oil-red O-stained liver. (D) IHC-stained liver tissue (Significant difference)

**BPIS attenuates NAFLD and reduces lipid accumulation**

To investigate the effect of BPIS on the progression of NAFLD, C57BL/6N mice were grouped and orally treated with BPIS (5mg kg\(^{-1}\)) for 12 weeks. The normal chowed diet fed C57BL/6J mice were used as a control. The design of the animal experiment is displayed in Fig 1 A. The food intake of the control mice was observably greater than that of other mice, which consumed 30% HFD (35.27 ± 1.20–35.93 ± 1.40 mL/day/group) than the control mice (32.31 ± 1.31 mL/day/group. Mice's body weight was reduced to normal weight in BPIS-treated mice as compared to the control group (Figure 1 A). HFD ingestion for 12 weeks in mice significantly induced increases in total bilirubin (TBIL), alanine aminotransferase (ALT), and total cholesterol level (TCHO) (Figure 1 B).

Next, histopathological observation of hematoxylin and eosin (H&E), Oil red O stain (ORO-stain) (Figure 1 C), and Immunohistochemistry (IHC) of the liver tissue (Figure. 1 D) were performed. There were abundant red lipid vacuoles in hepatic tissue in the HFD group, unlike in the control group. Co-consumption of HFD together with BPIS prevented hepatic injury in mice, and the ORO mean density decreased, indicating that hepatic steatosis was enhanced, in H&E-stained liver tissue showing a near-normal appearance with little cytoplasmic vacuolation, legible cell boundaries, a clear nucleolus, and a detectable nucleus (Figure 1 C).

While IHC staining was performed for the functional markers for fatty acid translocase (CD36) also known as fatty acid transporter involved in the pathogenesis of NAFLD. With the treatment
of BPIS to HFD, the contents of fat were reduced in liver tissue with the appearance of nearly like control group of mice. CPT1 transports fatty acids from the cytosol to mitochondria, and in that way catalyzes the rate-limiting step of fatty acid oxidation. The HFD group showed more lipid contents, while BPIS group tissue displayed lower lipids with enhanced tissue appearance. Fatty acid binding protein 1 (FABP1) is involved in free-fatty acid uptake. Samples displayed a significant ameliorating effect on liver tissue in the BPIS-treated group with a similar appearance to the control group as shown in Figure. 1 D. The model group serum markers presented NAFLD, while the BPIS-treated group presented NAFLD ameliorate effect. The above results suggest that BPIS significantly ameliorates fatty liver, preventing HFD-induced NAFLD.

**Fig 2** BPIS effect on colon and colonic tight junction proteins (A) images of the H&E-stained colon tissue. (B) HFD induces changes in gut permeability, and relative mRNA expression of ZO-1, claudin, and Occludin. (C) protein abundance of Zo-1, Claudin, and Occludin in the colon, with GAPDH, applied as a loading control.
Data represented as means ± SD (n = 3); *p < 0.05, **p < 0.01

**Protection of intestinal epithelium by BPIS**

Tight junction proteins play crucial roles in maintaining gut health and preventing the entry of harmful substances into the body. A leaky gut allows the translocation of bacteria and bacterial products. Dysbiosis can disrupt the intestinal barrier function and contribute to the progression of liver diseases. The data of histopathological showed that the epithelial architecture of the model group was destructed in the model group, while after BPIS treatment colon architecture was completely restored and similar appearance to the control group (Figure. 2 A). Tight junction proteins, including ZO-1, Occludin, and Claudin1. qRT-PCR and western blot results showed that tight junction proteins were significantly inhibited in HFD mice colon, and decreased levels were observed after BPIS treatment (Fig 2 B). The gene expression of Zo-1, Claudin1, and Occludin in mice colon tissue was upregulated after BPIS treatment, the results were confirmed three times. The current results indicate that BPIS treatment improved intestinal barrier dysfunction.

**BPIS regulates the gut microbiome in C57BL/6N mice**

To determine the effect of BPIS on the gut microbiota of NAFLD mice, high-throughput sequencing of 16S rRNA in the cecal content was performed. In this study, gut microbiota in fecal samples from three groups, Control, model, and BPIS were analyzed for the effect of BPIS on ameliorating NAFLD. According to the results of OTUs cluster analysis, there were 1664 unique OTUs in the Control group, 639 in the Model group, 932 in the BPIS group, and 290 OTUs shared among triple groups (Figure 3A). The diversity and community richness of
intestinal microbiota were assessed by an alpha-diversity analysis using Chao1 and Shannon indices. A comparable change was found in the value of Shannon and Simpson indices indicating that BPIS could effectively inhibit the excessive proliferation of gut microflora induced by the chronic consumption of HFD. A series of analyses were accomplished to reveal the critical intestinal microorganisms, which BPIS suppressed in HF-fed mice (Figure 3 B) (Chao1 index, \( p < 0.05 \); Shannon index, \( p < 0.05 \)). The richness and diversity of intestinal microbiota were evaluated by an \( \alpha \)-diversity analysis using Chao1 and observed species (Figure 3. B). In addition, the species rarefaction curve (Figure. 3 C), also presented the same difference in the richness (Chao1) and observed species between the Model and other groups. It demonstrated that the current sequencing depth is sufficient to reflect the microbial diversity of the samples. Therefore, BPIS treatment is beneficial to regulate gut microbiota diversity.
Fig 3 α-Diversity and β-Diversity analysis of the fecal microbiota communities based on OTUs

(A) Venn diagram of different groups. (B) Comparison of the diversity indices among different groups; Chao1 index; Simpson; Shannon; observed species. (C) Rarefaction curve of Chao1 description; (D) Rarefaction curve of Shannon description
Data represented as means ± SD (n = 3); *p < 0.05, **p < 0.01.

**BPIS modulates the complete structure of the gut microbiome**

To investigate the effect of BPIS treatment on the gut microbiome of NAFLD mice, the β-
analysis employing several unsupervised multivariate statistical assessments, including PCA,
UniFrac NMDS, and UniFrac UPGMA were applied. As shown in PCA (Figure. 4 A) and
UniFrac NMDS (Figure. 4 B), the samples of each group are distinctly clustered. In contrast, the
model group indicated low gut community structure compared to the control group, while the gut
community structure was recovered by BPIS treatment. In line with (Figure. 4 C&D, UniFrac
UPGMA indicated that significant separation appeared between the control, model, and BPIS-
treated group (Figure. 4 C). These analyses confirmed the effect of BPIS on microbiome
structure remodeling in NAFLD mice.
**Fig 4** β-Diversity analysis of the fecal microbiota communities based on OTUs. (A) Principal component analysis (PCA). The percentage variation explained by each principal coordinate is indicated on the axes. (B) UniFrac distance-based nonmetric multidimensional scaling (NMDS). (C) UniFrac distance-based unweighted pair-group method with arithmetic means (UPGMA)
UniFrac distance-based unweighted pair-group method with arithmetic means (UPGMA) analysis; the shorter the branching length between samples, the more similar the two samples. (D)

BPIS regulates the abundance of certain bacteria in mice

To evaluate the effect of BPIS on the microbiome makeup remodeling, we examined changes in bacterial abundance at the phylum and genus levels by taxon analysis. At the phylum level, a total of ten phyla were shared by all samples and the most abundant were Firmicutes and Bacteroidetes (Figure 5 A). BPIS treatment significantly increased the relative abundance of *Firmicutes* (p < 0.01), but reduced *Bacteroidetes* (p < 0.05) (Figure 5 B). At the genus level, as shown in (Figure 5 C&D) four genera that positively affected the prevention of NAFLD, including *Lactobacillus* (p < 0.05), *Bifidobacterium* (p < 0.01), *Ruminococcus* (p < 0.05), *Allobaculum* (p < 0.01) and Bacteroides. Next, the co-occurrence or co-exclusion analysis revealed the correlation between different microbiota compositions. The above data demonstrate that BPIS modulates the overall structure of the gut microbiome by controlling the abundance of certain bacteria. The genus level *Lactobacillus, Bifidobacterium, and Allobaculum* were the most abundant recovered genus (Figure. 5. C and D). The effect of BPIS treatment on intestinal flora composition was mainly explained by the differences in the relative abundance of *Lactobacillus, Bifidobacterium, Allobaculum, and Ruminococcus*. The mechanism of BPIS improves NAFLD Meanwhile reversing gut microbiota as it provides rational support for the use of BPIS as adjuvant therapy for NAFLD.
Fig 5 Taxonomy analysis of microbiome components. (A) Relative abundance of the top 10 phyla from each sample, compared by Metastatic analysis. (B) Significant intergroup differences in two phyla. (C) Relative abundance of genera ranking the top 20 from each sample was shown. Significant intergroup differences were found in four genera. Data represented as means ± SD (n = 4); *p < 0.05, **p < 0.01 versus the model groups.

Discussion

Recently, polyphenols have gained the attention of researchers in the prevention and treatment of diseases due to their high availability of bioactive compounds and high biological activities. Plant secondary metabolites can transform intestinal microbial components and produce intestinal metabolites such as methane, hydrogen, and vitamin B complex after microflora fermentation [25]. NAFLD is highly prevalent worldwide; identifiable risk factors include diabetes type 2, hyperlipidemia, and obesity [26]. Collecting the evidence suggests that polyphenols possess promising effects against NAFLD through a variety of molecular mechanisms including activation of β-oxidation, adipocyte differentiation, and inhibition of free fatty acid uptake and lipogenesis [27]. However, the mechanism by which polyphenols affect NAFLD through gut flora is unclear.

Several studies revealed that gut microbiota is associated with numerous human diseases. Gut microbiota plays a significant role in disease prevention, such as NAFLD, and hepatic encephalopathy-associated diseases. Researchers have proved that dysbiosis of the intestinal flora is closely associated with lifestyle and leads to the development of NAFLD. The liver can be greatly affected by changes in gut microbiota due to the entry of gut microbiota metabolites.
and gut bacteria [28]. The current study possesses the medicinal value of BPIS on NAFLD and regulates gut microbiota in mice models. The results displayed a significant ameliorating effect of BPIS on NAFLD. Previous studies reported, at the phylum level, a shift toward an increase in *Firmicutes* and *Bacteroidetes* was found in obese and high-fructose-fed mice [29] and humans, while weight loss was accompanied by an already lower abundance of Bacteroidetes in obese objects [30, 31]. Currently, there is debate on the validity of the *Bacteroidetes/Firmicutes* ratio as a marker of metabolic changes in mice and humans [32, 33]. In the present study, *Firmicutes* were increased, while *Bacteroidetes* were decreased compared to the control group after the treatment of BPIS (Figure. 5. A).

*Allobaculum* presented a low detection in the HFD group, quercetin supplementation in HFD significantly increased the relative abundance of these genera in NAFLD mice, [30], while our results displayed BPIS increased *Allobaculum* increased with BPIS treatment as compared to a control group of mice, and it correlates positively with NAFLD. In the current study *Lactobacillus, Bifidobacterium, Allobaculum, and Ruminococcus* genus were increased with treatment of BPIS to NAFLD mice as compared to the control group, other studies also reported *Lactobacillus, Bifidobacterium* genera species administered to C57BL/6 mice significantly improved HFD related hepatic steatosis, liver damage, increased hepatic expression of peroxisome proliferation activated receptors, and insulin resistance [34-36].

NAFLD is studied as the excessive accumulation of fats in hepatocytes. Unlike other prevalent diseases, NAFLD gains little attention. The liver is a crucial organ in the human body responsible for an array of functions including metabolism, detoxification, immunity, and vitamin storage. If the liver does not process or break down the fats will accumulate in hepatocytes. NAFLD is strongly associated with metabolism syndrome and is the cause of
chronic liver disease and cirrhosis [37]. The tight junctions of intestinal epithelial cells, as a dynamic permeability barrier, have double functions to prevent potential toxic substances and allow nutrients to enter the body [38]. In the current study, H&E stains displayed lesions in liver and colon tissue with a high-fat diet and the colon has tight junctions’ dysfunctions are another cause of “leaky gut” which develops inflammatory progression in mice. The tight junction is gathered by transmembrane such as Occludin, different Claudin, and Zo proteins in the peripheral membrane [39]. However, the liver was repaired after BPIS treatment and leaky colon tissue was significantly restored (Fig.2 D&E). We found that the mRNA and protein expression levels of the tight junction proteins (ZO-1, cloud1, and Occludin) in the colon were increased following BPIS administration in BPIS-treated mice, indicating that BPIS could avoid endotoxin accumulation in blood by protecting the gut's physical barriers. Previous studies reported that the gut microbiota of NAFLD patients reduced the diversity compared to healthy individuals. Under dysbiosis conditions, homeostasis is not capable of being maintained by gut microbiota, as a result, disruption of the intestinal barrier occurs [40, 41]. A study reported the effect of synbiotics combination of probiotics and prebiotics improves biochemical such as triglyceride (TG), total cholesterol (TC), modification of lipid metabolism and inflammation by activating the AMPK and NFκB signaling pathway and the intestinal barrier dysfunction and inflammation caused by a high-fat diet [36] these results are similar to our BPIS treatment of NAFLD mice group.

The current study findings strongly suggest that BPIS can be used to maintain homeostasis of the gut microbiota, serve as a marker in various pathologies, and treat NAFLD. In summary, we suggest that BPIS treatment mediates increased beneficial bacteria and decreased pathogenic
bacteria in the gut microbiota of fatty liver-diseased mice model and is a key contributor to ameliorating NAFLD.

**Conclusion**

In conclusion, we investigated that BPIS is an effective agent for reducing lipid buildup in the liver, improving inflammation in fatty liver mice, and enhancing the gut microbiome, providing firm evidence that BPIS treatment significantly mitigated NAFLD in high-fat diet-induced mice. BPIS plays a significant role in ameliorating NAFLD and paving the way for a novel therapeutic agent.

**Author contributions**


**Conflict of interest**

There are no conflicts of interest.

**Funding Sources**

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Data availability statement

Data is enclosed within the article and can be available upon request.

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35. Hayashi, H., et al., *The effect of heat-killed Lactobacillus brevis SBL88 on improving selective hepatic insulin resistance in non-alcoholic fatty liver disease mice without


Supplementary Files

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