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Anti-inflammatory effect of luteolin is related to the changes in the gut microbiota and contributes to preventing the progression from simple steatosis to nonalcoholic steatohepatitis

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ABSTRACT

Increasing intestinal barrier function is one of the basic methods to suppress inflammation in the progression from simple steatosis (SS) to nonalcoholic steatohepatitis (NASH). Luteolin exists widely in vegetables, fruits and natural herbs and has various biological activities, including benefits on nonalcoholic fatty liver disease (NAFLD). However, its regulatory effects on the gut microbiota and involvement in its biological activities remain to be investigated. We fed rats a high-fat diet containing 0.5% luteolin for 12 weeks and determined the effects of luteolin on lipid metabolism, inflammation, and the gut microbiota. Supplementation with luteolin for 12 weeks significantly reduced blood lipids and hepatic lipid levels and improved liver fat accumulation and inflammation. Moreover, supplementation with luteolin led to the significant enrichment of more than 10% of gut bacterial species, which contributed to increase the abundance of ZO-1, reduce intestinal permeability, reduce plasma lipopolysaccharide, and inhibit the TLR4/NF-κB pathway. In summary, the anti-inflammatory effect of luteolin might be related to changes in the gut microbiota and contribute to preventing the progression from SS to NASH. Our research provides new insights into the anti-inflammatory mechanism of luteolin and supports its use as a dietary supplement for NAFLD patients.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) has become a common chronic liver disease [1]. NAFLD is characterized by the accumulation of fat in the liver in the absence of competing causes of steatogenic liver disease other than metabolic syndrome and its individual features and includes simple steatosis (SS), nonalcoholic steatohepatitis (NASH), liver fibrosis and cirrhosis [2,3]. The most common explanation of the complex pathogenesis at present is the "multiple hit" hypothesis. It is believed that lipid accumulation, insulin resistance, mitochondrial dysfunction, endoplasmic reticulum stress, inflammation and other factors are involved in the occurrence and development of NAFLD [4]. The progression from SS to NASH is a key stage of the development and treatment of NAFLD, and inflammation plays an important role in this progression. Increased amounts of circulating and intracellular non-esterified fatty acids are also associated with an increase in nuclear factor kappa-B (NF-κB), eventually leading to the expanded and dysfunctional adipose tissue overproducing multiple pro-inflammatory cytokines and under-producing anti-inflammatory adipokines, such as adiponectin which, collectively, may further dictate NAFLD progression

Abbreviations: ANOVA, one-way analysis of variance; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FDA, Food and Drug Administration; HDLC, high-density lipoprotein cholesterol; HE, hematoxylin-eosin; HFD, high-fat diet; IκB-α, nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor alpha; IL-6, Interleukin-6; LDLC, low-density lipoprotein cholesterol; LPS, lipopolysaccharide; LSD, least significant difference; NAFLD, Nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OUT, Operational taxonomic units; PCoA, principal coordinates analysis; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; NAFL, simple nonalcoholic fatty liver; TBST, tris-buffered saline; TC, total cholesterol; TLR-4, Toll-like receptor; TNFα, tumor necrosis factor α; TG, total triglycerides; ZO-1, zonula occludens-1 tight junction.

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[5].

Recently, an increasing number of researches have indicated that the gut microbiota is closely related to the occurrence and development of NAFLD. The liver and intestine communicate in two directions via the bile duct, portal vein and systemic circulation [6]. Intestinal permeability has a vital impact on this two-way communication, especially by directly affecting the systemic immune response and chronic inflammation. When the gut microbiota becomes imbalanced, intestinal permeability increases, which leads to an increase in antigens such as lipopolysaccharide (LPS). LPS, an endotoxin, is the main component of gram-negative bacteria. When LPS passes through the intestinal barrier and enters the circulatory system, antibody responses to these antigens occur, causing immune activation and various inflammatory reactions [7,8]. Therefore, improving the intestinal barrier and reducing intestinal permeability is one of the main methods to suppress inflammation from SS to NASH [9].

The search for drugs to treat NAFLD is in progress, but no significant results have been achieved. There are currently no Food and Drug Administration (FDA)-approved first-line drug for the treatment of NAFLD or NASH, and the efficacy of indirect drugs is also very limited [10]. Therefore, there is an urgent need to develop effective and safe drugs and foods that could prevent the progression from SS to NASH. Luteolin (3′,4′,5,7-tetrahydroxyflavone), a natural flavonoid compound, exists in a variety of vegetables, fruits, and natural herbs, including pepper, wild chrysanthemum, honeysuckle and Perilla [11]. Luteolin has a variety of biological activities, such as anti-inflammatory, anti-allergic, uric acid-lowering, antitumor, antibacterial, and antiviral activities [12–17]. Chen *et al.* reported that luteolin suppresses inflammation-associated gene expression in mouse alveolar macrophages [18]. However, luteolin shows low bioavailability (<5%) *in vivo* [19,20]. The relationship between the low bioavailability and diverse biological activities of luteolin remains elusive. Therefore, the underlying mechanism of the action of luteolin, including the anti-inflammatory effect, remains to be further elucidated. A large amount of luteolin could escape absorption in the intestine and exist in the intestine [21]. Meanwhile, many researchers have reported that luteolin has a broad-spectrum of antimicrobial activity [22,23] and Li *et al.* reported that luteolin modulate gut microbiota in ulcerative colitis rats [24]. Besides, it has been reported that a variety of flavonoids could significantly improve related diseases by regulating the gut microbiota [25,26]. For example, baicalein could treat diabetes by regulating the gut microbiota [25]. Therefore, based on these previous studies, whether luteolin could improve NAFLD by regulating the inflammatory response through the interaction between the liver and the intestine is worthy of further study and how the gut microbiota modulation by luteolin contributes to its effects needs to be further clarified.

In this study, we performed a trial with 12-week supplementation with luteolin in high-fat diet (HFD)-fed rats to determine the effect of luteolin on the gut microbiota and the progression from SS to NASH.

2. Materials and methods

2.1. Animal experiments

As male rats tend to store more fat in the visceral adipose tissue than female rats, male SPF-grade SD rats (160 g − 200 g) were used to induce NAFLD, which were purchased from Shandong Provincial Animal Experiment Center, China [27]. Luteolin (greater than98%, structural information in Table S1, Table S2, and Fig. S1) was purchased from Nantong Feiyu Co., Ltd. (Nantong, Jiangsu, China). The animals were placed in a thermoneutral housing under a 12-h day/night alternating cycle [28]. The study protocol was approved by the Institutional Animal Care and Use Committee of Institute of Biomedical Research at Shandong University of Technology. Six rats were fed a standard diet and composed the normal group (NM). Twelve rats were fed a HFD (D16492, SYSE Co., Ltd., Changzhou, Jiangsu, China) and a HFD containing 0.5% luteolin and composed the model group (MD) and the luteolin treatment group (LT). In the 12th week of the experiment, the body weight and food intake were measured, and the plasma and liver tissue were collected for physiological and biochemical tests.

2.2. Biochemical indicators

The levels of total cholesterol (TC), total triglycerides (TG), high-density lipoprotein cholesterol (HDLC), low-density lipoprotein cholesterol (LDLC), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma were measured by using commercial kits from Nanjing Jiancheng Biological Co., Ltd. (Nanjing, Jiangsu, China). The levels of TG and TC in the liver were also measured by using commercial kits from Nanjing Jiancheng Biological Co., Ltd. The levels of Interleukin- 6 (IL6) and tumor necrosis factor α (TNFα) in the plasma, liver and colon were measured by using commercial kits purchased from CUSBIO Co., Ltd. (Wuhan, China). The level of LPS was also measured by using a commercial kit purchased from CUSBIO Co., Ltd. (Wuhan, China).

2.3. Liver tissue pathological sections

The obtained liver tissue was immersed in formalin for more than 24 h, embedded in paraffin and sliced into 3-μM slices. The sections were stained with hematoxylin-eosin (HE) or Sirius red and observed under a 240 × microscope.

2.4. Immunoblotting

Primary antibodies against nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor alpha (IκB-α; 1:1,000, 9242S), and Toll-like receptor 4 (TLR-4, 1:1000, 9242S) were purchased from Cell Signaling Technology Co. Ltd., Massachusetts, USA. Primary antibody against zonula occludens-1 tight junction (ZO-1 1:1000, Ab190085) was purchased from Abcam Co. Ltd., USA. Primary antibody against β-actin was purchased from Solarbio Co. Ltd., Beijing, China. Secondary antibodies against rabbit and goat were purchased from Solarbio Co. Ltd., Beijing, China.

The protein was extracted from the colon tissue or liver tissue by using a commercial kit from Nanjing Jiancheng Biological Co., Ltd. (Nanjing, Jiangsu, China). After preliminary separation by 12% SDS-PAGE electrophoresis, the protein was transferred to a polyvinylidene difluoride (PVDF) membrane and then blocked with 5% skimmed milk for 4 h. The PVDF membrane was incubated with 1:6000 anti-β-actin or 1:1000 ZO-1 antibody overnight at 4 °C. The next day, the PVDF membrane was washed 3 times with TBST for 10 min each time. After incubating the PVDF membrane with a secondary antibody of 1:6000 at room temperature for 1 h, the PVDF membrane was washed 3 times with tris-buffered saline (TBST) for 10 min each time again. We used an ECL kit for color development and grayscale analysis software for protein grayscale analysis. Moreover, fresh liver tissue was taken for determination of TLR4 (1:1000) and IκB (1:1000) using a method similar to that described above. Each immunoblotting experiment was repeated 3 times.

2.5. Fecal DNA extraction, PCR amplification and illumina MiSeq sequencing

Genomic DNA from stool samples was extracted by a soil DNA kit (Omega Bio-Tek Co. Ltd., Norcross, GA, USA). Primers 338F (5′-barcode-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGAC-TACHVGGGTWTCTAAT-3′) were used to amplify the V3-V4 region of the bacterial 16S rRNA gene by polymerase chain reaction (PCR) (denaturation at 95 °C for 30 s, annealing at 55 °C for 40 s and extension at 72 °C for 1 min, amplification was performed in 27 cycles). The PCR system was as follows: 4 μL 5 × FastPfu buffer, 2 μL 2.5 mM dNTP, 0.8 μL

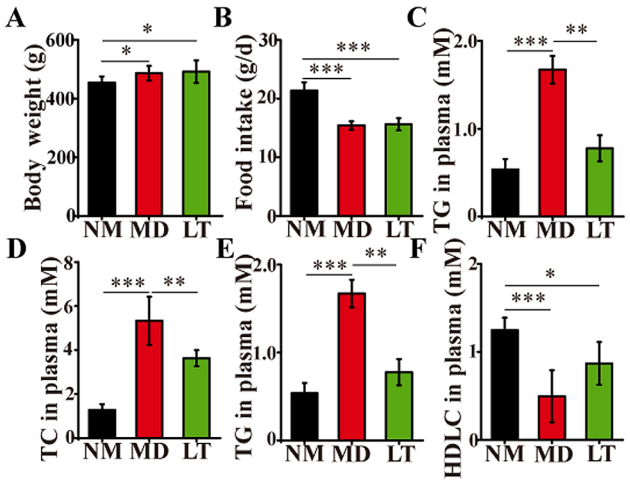

Fig. 1. Effects of luteolin on body weight, food intake, and plasma lipid profiles in HFD-fed rats. A: body weight B: food intake C: TGs in plasma D: TC in plasma E: LDLC in plasma F: HDLC in plasma. The values are shown as the means ± standard deviations, n = 6 in each group. Significant differences (*P < 0.05, **P < 0.01, and ***P < 0.001) were identified using one-way ANOVA followed by the LSD test for multiple comparisons.

of each primer (5 μM), 0.4 μL FastPfu polymerase and 10 ng template DNA. The resulting PCR product was analyzed using a 2% agarose gel and further purified using an AxyPrep DNA gel extraction kit, and quantitative analysis was performed using QuantiFluor-ST. 16S rRNA sequencing was performed by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) according to standard procedures.

2.6. Processing and analysis of DNA sequencing data

Raw files were demultiplexed and quality-filtered using QIIME (version 1.9.0) with the following process: (i) 300 bp reads truncated at any site over a 50 bp sliding window received an average quality score < 20, and the truncated reads that were shorter than 50 bp were discarded; (ii) sequences with exact barcode matching, 2 nucleotide mismatches in the primer matching, and reads containing ambiguous characters were removed; and (iii) only sequences with overlapping regions longer than 10 bp were assembled according to their overlapping sequence. Reads that could not be assembled were discarded.

Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by the RDP classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database using a confidence threshold of 70%. Refraction and alpha diversity analyses were performed using Mothur b.1.30.1. Community diversity was evaluated by indices. A heatmap based on the relative OTU abundance was generated using R package 2.15. The principal coordinates analysis (PCoA) was performed using Mothur. Microbial difference, correlation, and cooccurrence network analyses were performed using I-Sanger (Majorbio Bio-Pharm Technology Co., Ltd. Shanghai, China; www.i-sanger.com).

2.7. Data analysis and processing

All data in this study were processed using SPSS 17.0 software. All the results are expressed as the means ± standard deviations. Differential analyses were performed using one-way analysis of variance (ANOVA). Post hoc testing was performed using the least significant difference (LSD) method. *P < 0.05, **P < 0.01, and ***P < 0.001 are considered as significant differences.

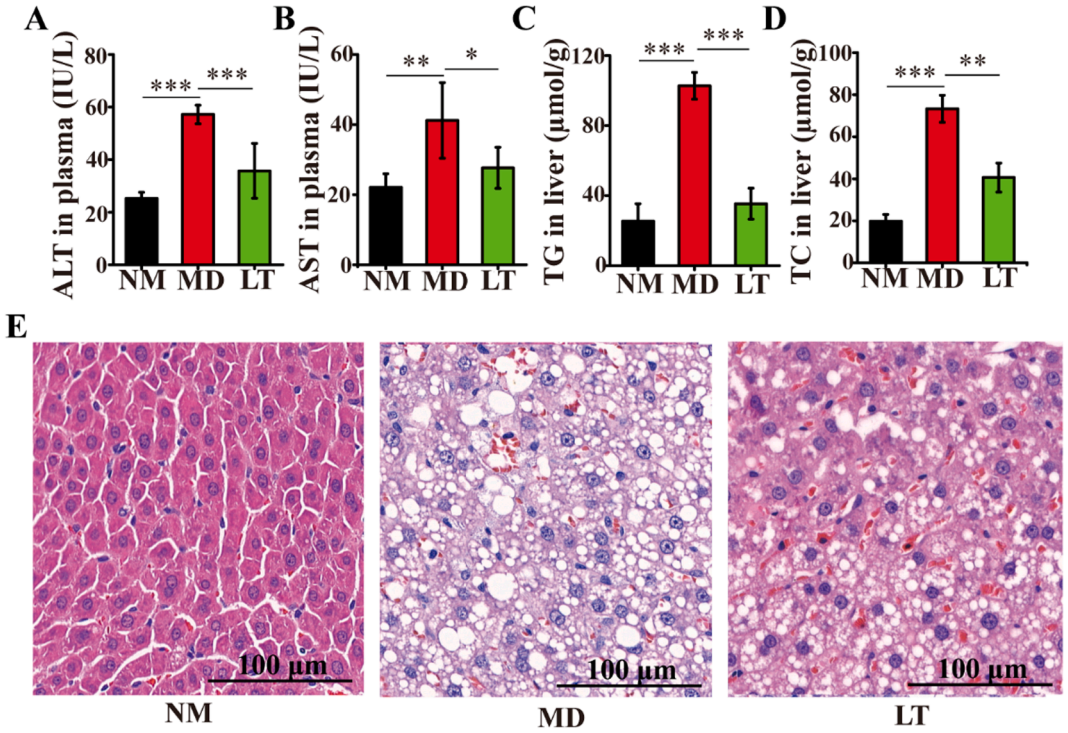

Fig. 2. Effects of luteolin on hepatic lipid profiles, function, and morphology in HFD-fed rats. A: ALT in plasma; B: AST in plasma; C: TGs in liver; D: TC in liver; and E: HE staining. The values are shown as the means ± standard deviations, n = 6 in each group. Significant differences (*P < 0.05, **P < 0.01, and ***P < 0.001) were identified using one-way ANOVA followed by the LSD test for multiple comparisons.

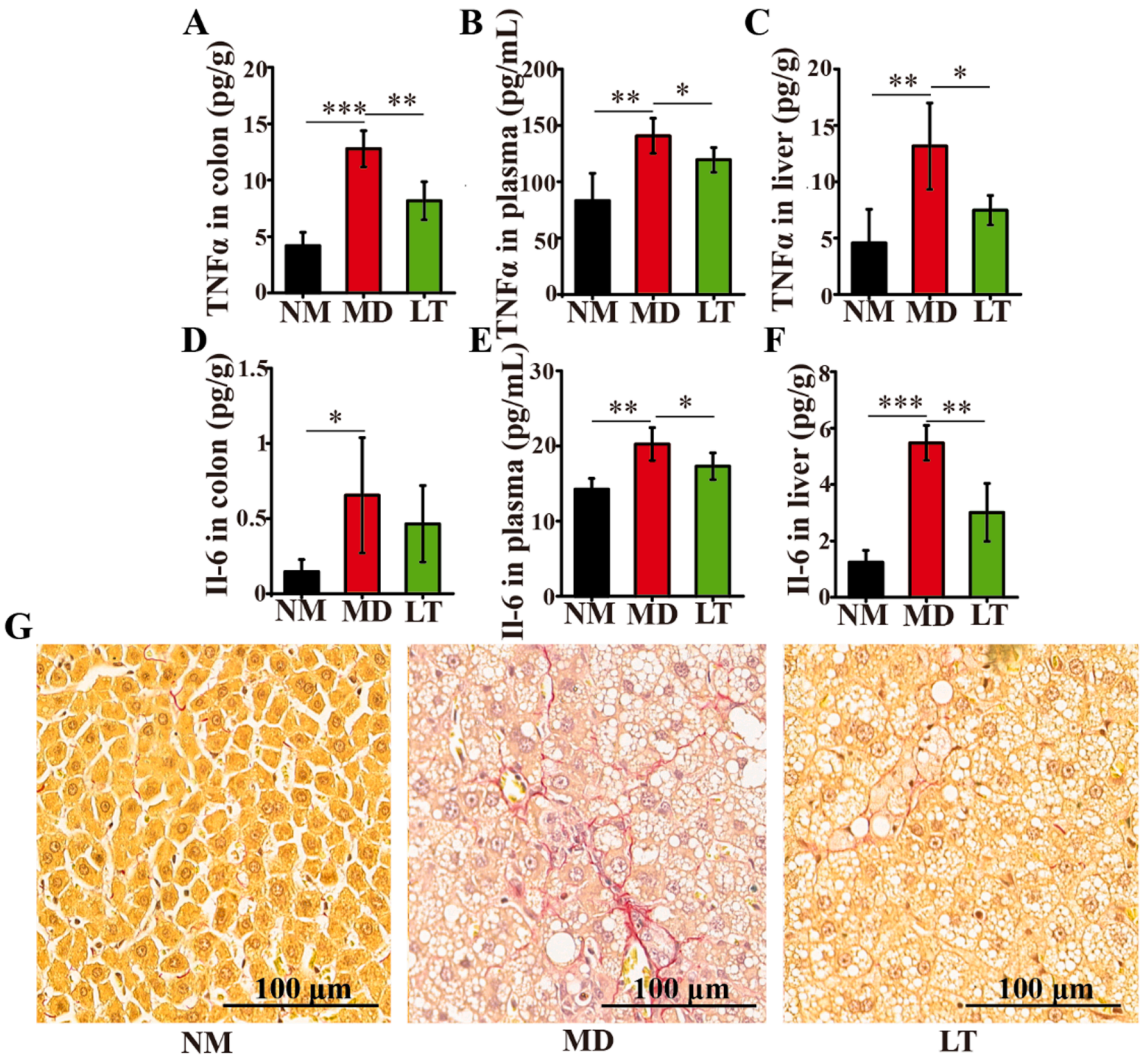

Fig. 3. Effect of luteolin on inflammatory factors and liver fibrosis in HFD-fed rats. A: TNFα in colon; B: TNFα in plasma; C: TNFα in liver; D: IL-6 in colon; E: IL-6 in plasma; F: IL-6 in liver; E: Sirius red staining. The values are shown as the means ± standard deviations, n = 6 in each group. Significant differences (*P < 0.05, **P < 0.01, and ***P < 0.001) were identified using one-way ANOVA followed by the LSD test for multiple comparisons.

3. Results

3.1. Effects of luteolin on body weight, food intake, and plasma lipid profiles in HFD-fed rats

Luteolin did not significantly change the body weight or food intake of rats fed a HFD (Fig. 1A and 1B). Compared with normal rats fed a standard diet, the levels of TC, TGs and LDLC in HFD-fed rats increased significantly, while the level of HDLC decreased. Compared with the rats fed a HFD, the TC, TG and LDLC levels of HFD rats fed with luteolin decreased significantly, but the HDLC levels did not change significantly (Fig. 1C-F).

3.2. Effects of luteolin on hepatic lipid profiles, function, and morphology in HFD-fed rats

Compared with normal rats fed a standard diet, the plasma ALT and AST and hepatic TC and TG levels of HFD rats were significantly higher. Compared with the rats fed a HFD, the plasma ALT and AST of HFD-fed rats supplied with luteolin were significantly lower, as were the hepatic TC and liver TGs (Fig. 2A-D). Additionally, compared with the rats fed a HFD, cellular swelling was improved, fat hollow spaces were reduced significantly, and the cell morphology became relatively complete in HFD-fed rats supplied with luteolin (Fig. 2E).

3.3. Effect of luteolin on inflammatory factors and liver fibrosis in HFD-fed rats.

Compared with normal rats fed a standard diet, the levels of TNFα and IL6 in the colon, plasma and liver of HFD rats increased significantly in the rats fed a HFD (Fig. 3A-F). Compared with the rats fed a HFD, TNFα levels in the colon, plasma and liver of HFD-fed rats supplied with luteolin were significantly lower, and IL6 in plasma and liver was also significantly lower (Fig. 3A-F). Although the IL6 levels in the colon of HFD rats supplied with luteolin did not change significantly, there was a certain downward trend (Fig. 3D). Additionally, the liver fibrosis in HFD-fed rats supplied with luteolin was significantly improved, and the cell morphology was restored (Fig. 3E).

3.4. Effect of luteolin on the structure of the gut microbiota in HFD-fed rats

Through the comparative analysis of bacterial 16S rRNA (V3-V4 region) in stool samples, the effects of luteolin on the structure of the gut microbiota were detected. After removing low-quality sequences from 18 samples in 3 groups, a total of 781,510 reads were produced, with an

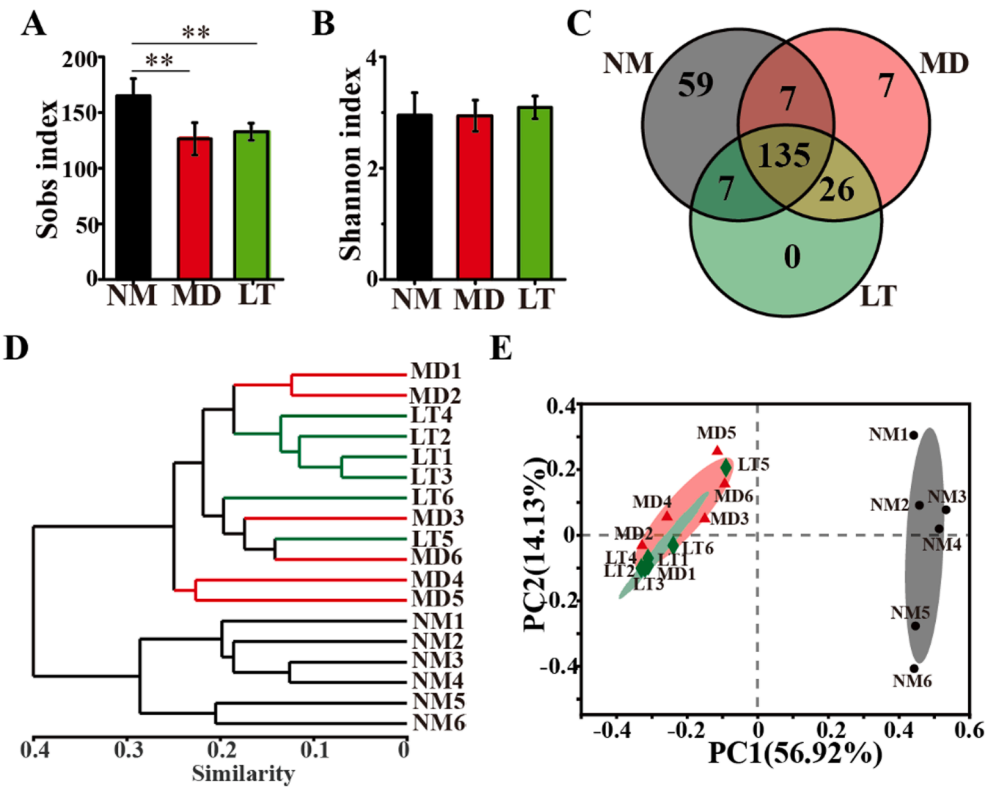

Fig. 4. Effect of luteolin on the structure of the gut microbiota in HFD-fed rats. A: Comparison of the Sobs index at the species level B: Comparison of the Shannon index at the species level C: Venn diagram D: Hierarchical cluster analysis at the species level. E: UniFrac PCoA analysis at the species level. The values are shown as the means ± standard deviations, n = 6 in each group. Significant differences (*P < 0.05, **P < 0.01, and ***P < 0.001) were identified using one-way ANOVA followed by the LSD test for multiple comparisons.

average number of effective reads of 43417 ± 4460. The Sobs and Shannon diversity indices are shown in Fig. 4A and 4B. Compared with normal rats fed a standard diet, the Sobs index of HFD rats in HFD-induced rats was significantly reduced, while the Shannon index remained basically unchanged. Moreover, after supplementation with luteolin, neither the Sobs index nor the Shannon index changed significantly. Additionally, the Venn diagram showed that the number of species was basically unchanged after supplementation with luteolin (Fig. 4C). PCoA and species-level hierarchical clustering can be used to analyze the overall structural changes of the gut microbiota. Hierarchical cluster analysis indicated that compared with normal rats fed a standard diet, the structure of the gut microbiota in HFD-fed rats was significantly different, and after supplementation with luteolin, the structure of the gut microbiota might migrate away from that of normal rats, but the difference was not obvious (Fig. 4D). The results of PCoA analysis also suggested that after supplementation with luteolin, the structure of the gut microbiota might become different from that in HFD-fed rats, but the difference was not obvious (Fig. 4E).

3.5. Effect of luteolin on the key phylotypes of gut microbiota in HFD-fed rats

As shown in Fig. 5, compared with rats fed a standard diet, most key phylotypes of the gut microbiota of HFD-fed rats showed significant changes. After supplementation with luteolin, we found that more than 80% of the bacterial species had basically no change in abundance compared with the abundance in HFD-fed rats, which is consistent with our previous analysis of the gut microbiota structure in Fig. 4. Moreover, it is worth noting that, for more than 10% of bacterial species, such as *Parvibacter, Faecalitalea, Allobaculum* sp., and *Bacteroides dorei*, the abundance of bacterial species increased significantly.

3.6. Correlation analysis of the gut microbiota in HFD-fed rats

As shown in Fig. 6A, bacteria such as *Faecalitalea, Allobaculum* sp. and *Parvibacter* were negatively correlated with LPS levels, and bacteria such as *Anaeofustis stercorihominis DSM 17244* and *Anaerotruncus* were positively correlated with LPS levels. Furthermore, the correlation analysis between different species levels is shown in Fig. 6B. For instance, *Parvibacter* was positively correlated with *Staphylococcus* and *Lachnospiraceae* but negatively correlated with *Clostridium sensu stricto 1* and *Christensenellaceae R-7* group.

3.7. Effect of luteolin on LPS, ZO-1 and TLR/NF-κB pathways in HFD-fed rats

Compared with normal rats fed a standard diet, plasma LPS levels in HFD-fed rats were significantly higher (Fig. 7A), and the abundance of the intestinal permeability marker protein ZO-1 was lower (Fig. 7B-C). After supplementation with luteolin, the plasma LPS level was significantly reduced, and the reduction in the ZO-1 level was reversed (Fig. 7A-C). Furthermore, we found that luteolin could reduce the abundance of TLR4 and increase the abundance of IκB-α in HFD-fed rats (Fig. 7D-F).

4. Discussion

In the present study, compared with the effects observed in HFD-fed rats, luteolin supplementation enriched more than 10% of bacterial species, reduced intestinal permeability, reduced plasma LPS, and inhibited the TLR4/TLR/NF-κB pathway to reduce hepatic inflammation and prevent the progression from SS to NASH.

Previous studies have reported that compared with normal littermates, the composition of the gut microbiota in rats with NAFLD induced by HFD was significantly changed, and the alpha diversity was reduced [29,30]. Many studies have indicated that a large variety of flavonoids can increase the alpha diversity of rats fed a HFD [31]. However, our research revealed that the effects of luteolin on alpha diversity were not consistent with the effects of these flavonoids, and luteolin could not significantly increase the Sobs index and Shannon

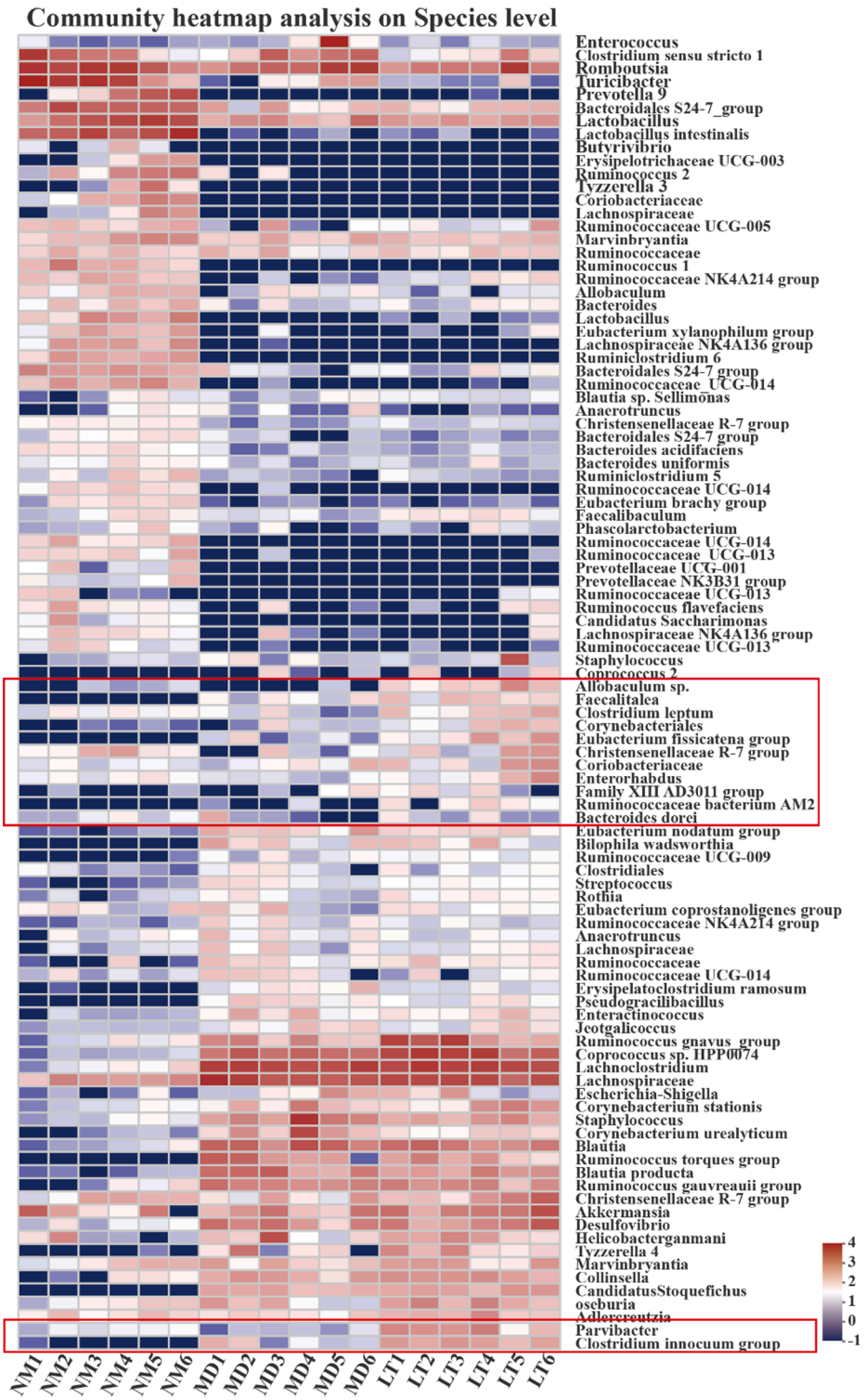

Fig. 5. Effect of luteolin on the key phylotypes of the gut microbiota in HFD-fed rats. Heatmap of the top 100 strains according to abundance changed after supplementation with luteolin. The species of interest are in the red frame. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

index. The Sobs index mainly represents the number of species, and the Shannon index represents the species abundance and uniformity, which showed that luteolin did not increase species richness nor did it increase the alpha diversity. We also found that luteolin did not reverse the gut microbiota structure of HFD-induced rats to that of normal rats significantly. The hierarchical clustering and PCoA both showed that luteolin did not restore the imbalance in the gut microbiota caused by the HFD. Interestingly, when we focused on the top 100 abundant species, more than 10% of the species in HFD-fed rats supplied with luteolin showed a significant increase compared with the levels found in HFD-fed rats and normal rats. Moreover, except for these strains, luteolin basically did not significantly change other species in HFD-fed rats. In our opinion, it is an advantage that luteolin could change only specific species, not most species, which might have a lower probability of causing side effects.

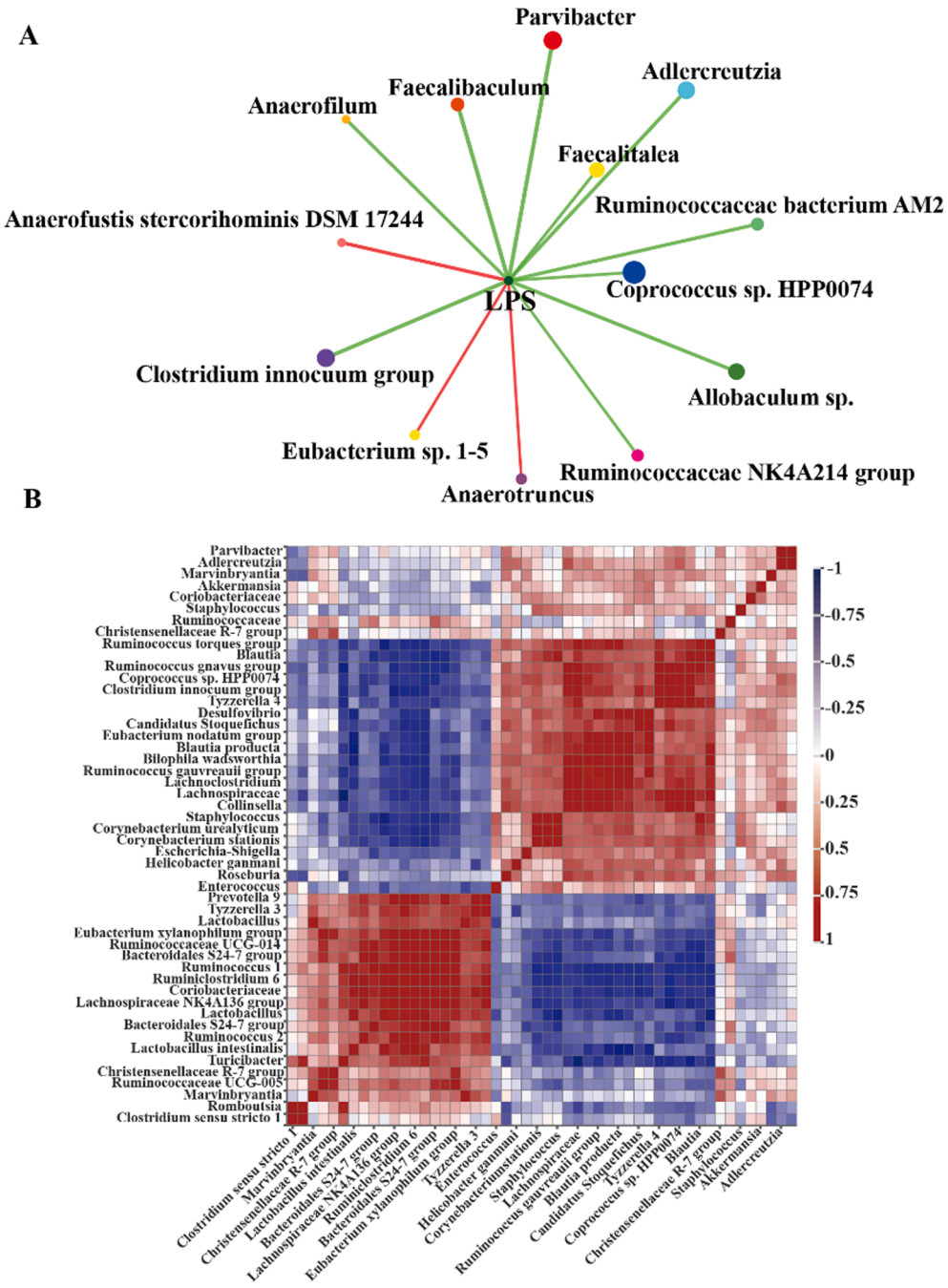

Fig. 6. Correlation analysis of the gut microbiota in HFD-fed rats. A: Analysis of LPS-related strains of the top 100 strains according to abundance. The red line segment indicates a positive correlation, and the green line segment indicates a negative correlation. B: Correlation analysis between the top 50 bacterial species according to abundance. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Certainly, further research is needed, and information on side effects related to the gut microbiota is currently lacking. Further analysis revealed that luteolin can significantly increase bacterial species such as *Parvibacter, Faecalitalea, Allobaculum* sp., *Bacteroides dorei,* and *Christensenellaceae R-7 group*. Previous studies indicated that most of these bacteria, such as *Allobaculum* sp., *Bacteroides dorei*, and *Parvibacter*, could increase intestinal barrier function, increase the expression of the intestinal barrier function marker protein ZO-1, and reduce the entry of LPS into the body [32,33]. This might be the main mechanism of luteolin acting on the gut-liver axis. Our results further indicated that luteolin reduced intestinal permeability in HFD-fed rats, increased the abundance of ZO-1, and decreased the level of LPS in plasma. Increased levels of LPS in the blood could activate the TLR4 signaling pathway [34,35]. When TLR4 is activated, the content of IκB is reduced, and the NF-κB signaling pathway is activated to mediate systemic inflammation [36]. Further experiments indicated that luteolin could inhibit the TLR/NF-κB pathway and reduce the inflammatory factors TNF-α and IL-6 in the plasma, liver and ileum. Therefore, in summary, luteolin might increase the content of some species in HFD-fed rats, reduce intestinal permeability, reduce plasma LPS, and inhibit the TLR4 TLR/NF-κB pathway to reduce liver inflammation and inhibit progression from SS to NASH.

Based on the correlation analysis, we found that the level of LPS might be related to many species; for example, LPS might be negatively correlated with *Faecalitalea, Allobaculum* sp., and *Parvibacter*. These species could be increased significantly by luteolin, which might be the main reason that the level of LPS decreased as describe above.

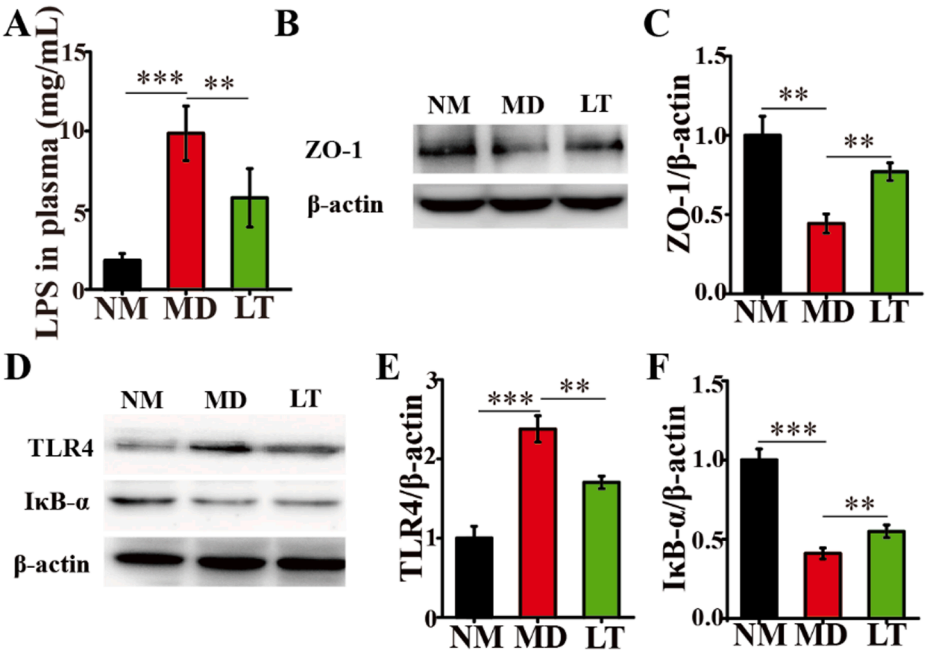

Fig. 7. Effect of luteolin on inflammatory factors and liver fibrosis in HFD-fed rats. A: LPS in plasma; B: immunoblotting analysis of ZO-1/β-actin C: Densitometric analysis of ZO-1/β-actin D: Immunoblotting analysis of TLR4/IκB-α/β-actin E: Densitometric analysis of TLR4/β-actin F: Densitometric analysis of IκB-α/β-actin. The values are shown as the means ± standard deviations, n = 6 in each group. Significant differences (*P < 0.05, **P < 0.01, and ***P < 0.001) were identified using one-way ANOVA followed by the LSD test for multiple comparisons.

Additionally, LPS is also positively related to *Anaeofustis stercorihominis* DSM 17244, *Anaerotruncus,* and *etc.*, which might be the source of LPS. In fact, the relationship between different bacterial species in the gut microbiota is very complicated. One species of bacteria might interact with many species of bacteria, and for us, it is difficult to isolate specific species to determine their interactions. Moreover, the functions and effects of many bacteria, such as *Anaerotruncus*, have not yet been determined [37,38]. So, it is also difficult to determine the specific mechanism of luteolin on the gut microbiota. Although luteolin could improve the intestinal barrier, it is difficult for us to explain exactly how it does so, which needs to be accomplished in the future. When focusing on the effect of luteolin on intestinal barriers, we should not ignore the other effects of luteolin-modified species. For example, *Allobaculum* sp. and *Bacteroides dorei* are common butyric acid bacteria, and whether they also play a corresponding role also needs to be determined [39]. In fact, we determined the level of butyric acid in the feces of the colon but did not find that luteolin could change the level of butyric acid (data not shown). The reason might be that the butyric acid produced by these bacteria was absorbed and used by other bacteria. Nevertheless, more effort is needed to determine the changes in other intestinal metabolites and further elucidate the effect of luteolin on the gut microbiota in HFD-fed rats.

This study reveals the mechanism of luteolin's effect on the gut microbiota and explains the anti-inflammatory mechanism of luteolin to a certain extent, which is involved in the prevention of the progression from SS to NASH. Future well-designed randomized clinical trials of luteolin for NAFLD are warranted.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

LS and HFJ designed the study. WLS and JWY performed the experiments. WLS, LS and HFJ analyzed the data, wrote and revised the manuscript. XYL helped within the animal experiments. HYD and GQL helped within the WB experiments. All authors read and approved the final manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2021.104966.

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