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Lacticaseibacillus rhamnosus CCFM1060 Modulates gut microbiota and intestinal barrier Function: Alcoholic liver disease Mitigation through Nrf2/HO-1 and NF-κB Pathways

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ABSTRACT

Long-term and/or excessive ethanol intake can lead to alcoholic liver disease (ALD). Gut dysbiosis contributes to a critical role in the pathogenesis of ALD. Here, we explored the impact of viable and dead *Lacticaseibacillus rhamnosus* CCFM1060 on the gut microbiota of alcohol-treated mice. The findings indicated that CCFM1060 V and D (viable and dead) administration improved the gut microbiota composition. Specifically, CCFM1060 D restored the abundance of butyric acid-producing bacteria, Alistipes, Lachnospiraceae, and Ruminococcaceae in ethanol-induced mice; CCFM1060 V improved the abundance of *g_Dubsiela*, *g_Bifdobacterium*, *g_Ruminococcaceae_UCG_014_2*, the SCFA-producing bacteria which were decreased in alcoholic hepatitis. Furthermore, the interventions improved the microstructure of the ileum, including the villi and crypts. They reinforced the intestinal barrier by increasing the RNA and protein expression of claudin-1, occludin, and ZO-1 in the colon. Also, they have been shown to enhance the liver's antioxidant function by suppressing the synthesis of MDA and boosting the concentrations of GSH and SOD through the Nrf2/HO-1 pathway. In addition, interventions can suppress mice's levels of TNF-α and IL-6, IL-1β in serum, presumably via the TRL4/MyD88/NF-κB pathway. Together, these results suggest that CCFM1060 can modulate the gut microbiota structure, reinforce the intestinal barrier, improve intestinal homeostasis, enhance liver antioxidant capacity, and mitigate inflammation response, eventually protecting the liver from ethanol-induced injury.

1. Introduction

Alcoholic liver disease is a major global healthcare issue that is of widespread concern (Esser & Jernigan, 2018). Excessive alcohol consumption has been identified as a significant health risk factor that demands attention (Bellentani, Bedogni, & Tiribelli, 2018). This disorder can have a profound impact on an individual's physical and mental health, as well as their social functioning, making it a matter of considerable concern (Esser & Jernigan, 2018). Sustained alcohol misuse leads to hepatic disorder and dysfunction, including fatty liver, hepatitis, fibrosis, cirrhosis, cancer, and other serious complications, which have a substantial influence on the overall quality of life and well-being of patients (Sugimoto & Takei, 2017). The development of ALD is intricately linked to the health of the gut microbiota. Chronic alcohol abuse can disrupt the balance of the gut microbiota, a condition known as dysbiosis. The gut microbiota plays a crucial role in the development and severity of ALD, and alterations in the gut microbiota have been observed in individuals with alcohol-related liver diseases (Hsu & Schnabl, 2023).

In recent years, emerging studies have shown that probiotics have an essential impact on the balance between the intestinal flora and human well-beings (Beck et al., 2022; Negata et al., 2019; Singh et al., 2017). Probiotics can modulate the structure of the gut microbiota, protect the functional and structural integrity of the intestinal tract, and reduce inflammation reactions; thus, they can help protect against a range of intestinal and metabolic disorders (Chopyk & Grakoui, 2020; Kayama,

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Okumura, & Takeda, 2020; Ma et al., 2020). Over the past few decades, probiotics have been widely studied and have shown promising effects in ameliorating and preventing many disorders. Several studies indicate that probiotics can potentially prevent ALD by influencing the liver's metabolic function. (Fuenzalida et al. 2021; H. X. Liu et al., 2023; Xiao et al., 2017). A strong correlation exists between gut microbiota and liver health in the progression of alcoholic liver disease (ALD). Prolonged alcohol consumption can induce dysbiosis in gut microbiota, which is associated with the deterioration of intestinal barrier integrity and an escalation of inflammatory responses. These elements collectively facilitate the advancement of liver damage (Cheng, Yang, & Chu, 2024). The supplementation of probiotics can reduce inflammation and improve intestinal barrier function by regulating the composition of gut microbiota, restoring the balance of gut microbiota, which has potential benefits for the treatment of ALD (Fuenzalida et al. 2021).

Therefore, further research is required to reveal the exact mechanism and potential clinical applications of probiotics in the treatment of ALD. The present study aimed to examine the possible mechanism of action of CCFM1060 in ALD and to develop an effective probiotic. We anticipate that our research will enhance sufferers' prognosis and quality of life by offering a fresh theoretical basis and therapeutic approaches for ALD.

2. Materials and methods

2.1. CCFM1060 and its cultivation

Lacticaseibacillus rhamnosus (CCFM1060) was cultivated under aerobic conditions in an MRS liquid medium at 30 °C for 12h. Next, it was centrifuged at 8,000 × g for 10 min, mixed with a 30 % glycerol solution and kept at −80 °C. Microbiological biomass was conducted at a concentration of 5×10^9 CFU/mL and resuspended in PBS before subsequent gavage.

2.2. Mice model and feces collection

Adult male C57BL/6 mice, aged eight weeks and weighing 22 ± 2 g, were acquired from the Animal Center of Jiangnan University in Jiangnan, China (SYXK(SU)2016–0045). Under specified pathogen-free (SPF) conditions, all mice were kept at 21 ± 1 °C and 50 ± 10 % (RH). Following a seven-day period of adjustment, the animals were grouped into two groups: one group that had ad libitum access to the control liquid diet and the other group that had an alcohol diet. The body weight was weighed every week; the feces were gathered and stored at −80 °C before investigation. Apart from the control mice, the animals were administered 5 g/kg ethanol and euthanized 9 h later on the last day (Fig. 1a). All experiments followed the ARRIVE guidelines (JN.No20220615c3840930[222]).

2.3. Serum biochemical parameters

Serum was prepared (3000 g, 15 min, 4 °C) after euthanasia. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), and triacylglycerol (TG) were examined by an automatic biochemical analyzer (Mindray, China). A commercial ELISA kit (R&D, Inc. Minneapolis, MN, USA) was used to quantify the concentration of cytokines (IL-1β, IL-6, TNF-α) based on the instructions.

2.4. Histopathological examination

The ileum and liver tissues were treated with a 4 % paraformaldehyde solution, then embedded in paraffin and processed with hematoxylin and eosin (H&E) staining. The pictures were digitized by a high-specific resolution microscope (Leica, Bensheim, Germany).

2.5. Liver TC and TG assay

Following the weighing of the liver tissues, they were homogenized with phosphate bisulfite. Plasma triglyceride (TG) and cholesterol (TC) concentrations were measured using a commercial testing kit (Nanjing, China) based on the instructions.

2.6. RNA extraction and qPCR

Extraction of total RNA from the colon tissues was performed using TRIzol reagent from Thermo Fisher Scientific. The reverse transcription process was conducted using the HiScript II 1st Strand cDNA Synthesis

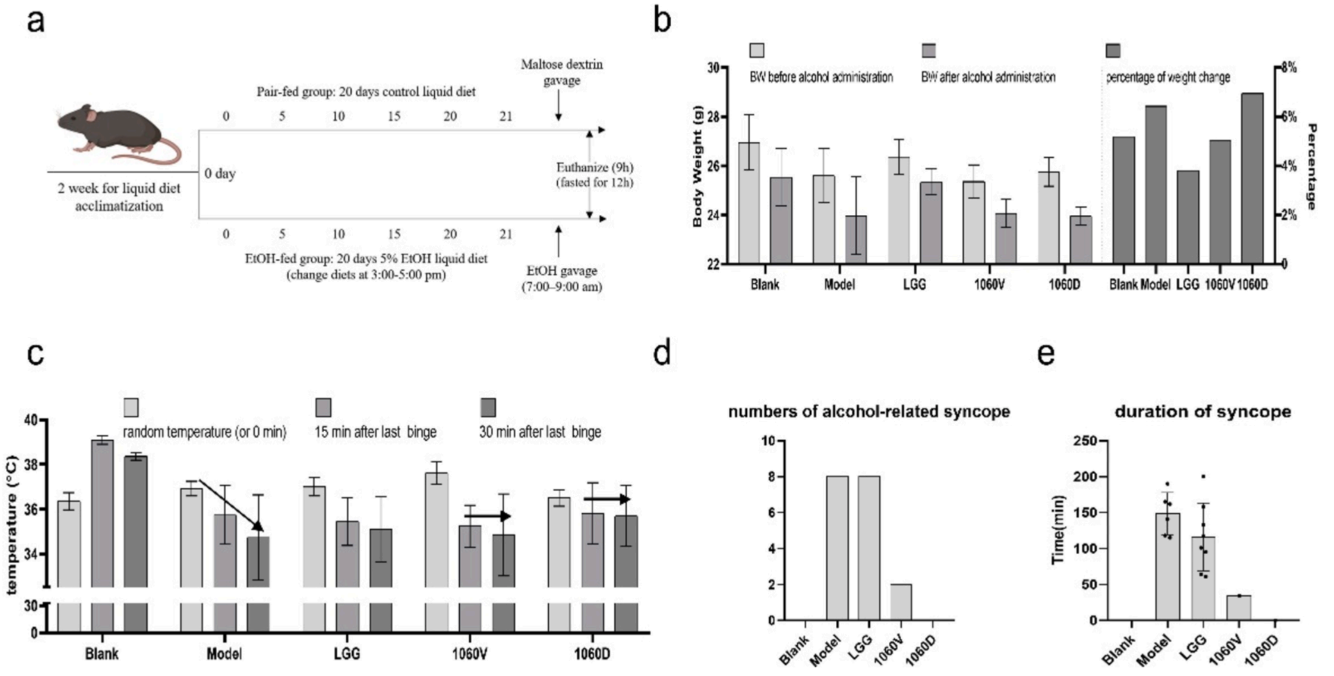

Fig. 1. Illustration of experimental procedures. NIAAA model (a); Analysis of mouse's body weight changes pre- and post-modeling (b); Alterations in the body temperature of mice during the most recent episode of binge eating (c); Analysis of ethanol-induced syncope in mice following ethanol gavage (d-e).

Kit. The relative quantification of mRNA was done in triplicate using the real-time PCR System (Bio-Rad Laboratories, Inc). The cycle threshold (Ct) values were equalized to *GAPDH*.

2.7. Western blot

Lipid lysis of liver and colon tissue was achieved using RIPA (including protease inhibitors), followed by protein collection by centrifugation. Then, the concentration was determined using the BCA analytical technique. Once denaturation, electrophoresis, membrane transfer, and blocking were performed, antibodies targeting MyD88, TLR4, P65, Claudin, Occludin, ZO-1, HO-1, and Nrf-2 (1:1000) were added and incubated aseptically at 4 ℃ overnight on a shaking table. Remove the primary antibody and allow the bands to incubate at 4 ℃ for 1.5 h in the secondary antibody β-actin, which should be diluted to a concentration of 1:5000. Post-washing, apply ECL developing solution and proceed with development using an advanced chemiluminescence detection equipment (Tanta, Beijing, China). Greyscale values were analyzed using Image J (National Institutes of Health).

2.8. Characterization of gut microbiota

Fecal samples were subjected to a FastDNA SPIN Kit (MP, Santa Ana, CA, USA). Specific bacterial primers targeting the variable region 3–4 (V3–V4) of the 16S rRNA gene were used for the PCR. The primers used were 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTATCTAAT). Subsequently, sequencing libraries were prepared, and paired-end sequencing was conducted on an Illumina MiSeq platform (San Diego, CA). The acquired data were analyzed using QIIME2, a widely used software platform for microbiome analyses (Niu et al., 2024). Construction of sequencing libraries, execution of paired-end sequencing on an Illumina MiSeq platform (Illumina, San Diego, CA, USA), and analysis of the results using QIIME2 were conducted.

2.9. Analytical statistics

Genomic sequence was conducted by an internet-based program available at https://www.microbiomeanalyst.ca. GraphPad 8.0 was employed to analyze additional data, and statistical significance was determined using a one-way ANOVA methodology. All data in this paper are reported as the mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ denote statistically significant differences.

3. Results

3.1. Mouse model of ALD and the impact of CCFM1060 on ALD

The duration of a chronic model of ALD is 4–8 weeks, sometimes more than eight weeks; here, we conducted chronic and binge ethanol feeding that includes liquid diet acclimatization for 14 days before continuing with a 21-day period with a 5 % alcohol liquid diet. Specifically, the model groups were accessible to 21 days of 5 % alcohol liquid diet while the control mice were given the control diet according to the paired-fed method (Fig. 1a) (Bertola, Mathews, Ki, Wang, & Gao, 2013). Weekly weighings of the mice revealed a little drop in body weight following alcohol treatment, which then remained constant, fluctuating approximately 3–7 % of the baseline weight (Fig. 1b). Since ethanol intake can influence the animal's body temperature (Murphy & Lipton, 1983; Myers, 1981), we observed its temperature during the last binge and found that the those in control experienced a noticeable decrease in temperature, while the temperature of the subject group (both the viable and dead CCFM1060) did not change (Fig. 1c). Meanwhile, ethanol gavage induced syncope in all the model mice, whereas CCFM1060 offset this phenomenon. The revival time for the CCFM1060 and LGG-administered mice was significantly shorter than that for model one (Fig. 1d and e).

3.2. CCFM1060 mitigates liver damage caused by alcohol

Ethanol-induced a significantly higher liver index in the model mice than in control ($p < 0.001$). Probiotic-treated mice had a lower liver index than that of model mice ($p < 0.05$), which might be ascribed to the short experimental period (Fig. 2a) (Kim, Heo, Rho, Yang, & Jeong, 2018; Z. Wang, Su, Fan, Fei, & Zhao, 2015). The H&E-stained slide showed a visually distinct and normal control, whereas the model was characterized by obvious steatosis, including tiny and large fat vesicles and an uneven distribution of hepatocytes. The lesions were improved significantly after the administration of CCFM1060 V and D. We further calculated the area of fat bubbles using Image Pro Plus 6.0. The results showed that the model achieved significantly higher scores than the control, and LGG; CCFM1060 V and D improved liver injury, and even the results of the CCFM1060D treatment were similar to the untreated ones (Fig. 2d and e).

Regarding the liver's TC and TG, the intervention ameliorated the high level of TC, which indicates an improvement in lipid metabolism disorders. Drinking alcohol interferes with bile acid production and elimination, triggering cholestasis. This may result in an increase in plasma cholesterol levels, a precursor of bile acids. CCFM1060D can improve fat accumulation and lipid metabolic abnormalities caused by ethanol intake, whereas its viable counterpart had little effect on triglyceride accumulation (Fig. 2b and c).

3.3. CCFM1060 improves the serum biochemical indicators

ALT and AST are common transaminases in liver cells. High transaminase content is usually associated with hepatocyte damage. In the ALD model, alcohol intake causes hepatocyte damage and inflammation, leading to increased ALT and AST levels. High serum levels of ALT and AST signify damage and dysfunction of the hepatocytes, respectively. Probiotics lowered the concentrations of AST and ALT induced by ethanol, and the reduction of ALT induced by CCFM1060 was compared with that induced by LGG (Fig. 3a and b).

TC is the total cholesterol content in serum, and TG is the total content of triacylglycerol. In this model, liver injuries and conditions related to lipid metabolism involve an increase in TC and TG concentrations, which may affect fat storage and lipid metabolism dysfunction. The ethanol increased the TC and TG content and caused liver malfunction in the model, whereas the probiotic administration dampened the ethanol-induced liver injury (Fig. 3d and e).

3.4. Impact of CCFM1060 on the gut microbiota's composition and structure

The commensal microbiota is a major contributor to host health and function. Gut dysbiosis is associated with the pathogenesis of ALD, which may accelerate its development and progression. Thus, we sequenced the V3-V4 region of 16S rRNA to investigate alterations in the gut microbiota. Alpha diversity, assessed using the Chao1 index, was significantly different among all six groups ($p = 0.030201$). The composition of the intestinal microflora was altered by ethanol intervention, as evidenced by beta diversity based on an unweighted UniFrac distance. Probiotics were able to partially restore this composition (Fig. 4a–c). The heatmap (Fig. 4d) provides a clear visual representation of the variations in color across different regions of the image. It reveals that certain areas have become darker while others have become lighter, reflecting changes in the framework and composition of the intestinal microflora before and after the administration of ethanol.

To further appreciate the alterations in the gut microbiota, we conducted a comparison of the bacterial community structure affected by ethanol and by CCFM1060 V or D. The interventions partially modify β diversity of the gut microbiota in mouse induced with ethanol (Fig. 4e and f). In addition, LEfSe analysis was performed; the CCFM1060 can restore the *g_Lachnospiraceae*, *g_Dubsiela*, *g_Lactobacillus*,

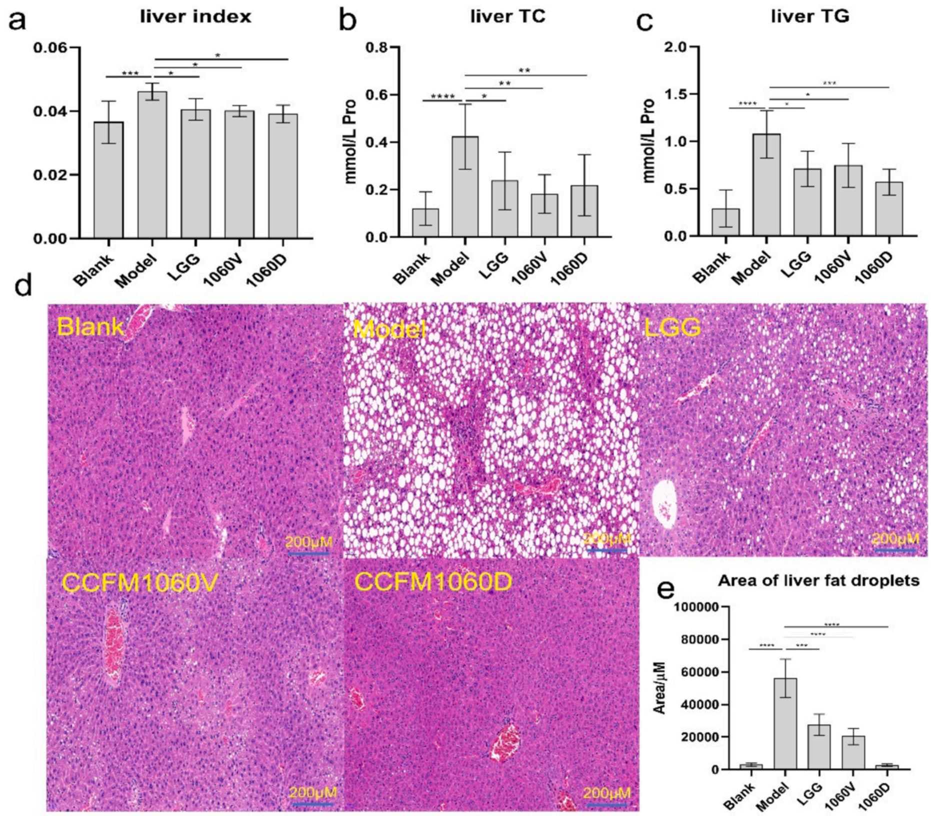

Fig. 2. CCFM1060 V and D induce remission of ALD mice. Index of liver function (a); Total cholesterol, triglyceride levels (b-c); The hepatic H&E staining (d); Fatty droplet area determined using Image Pro Plus.

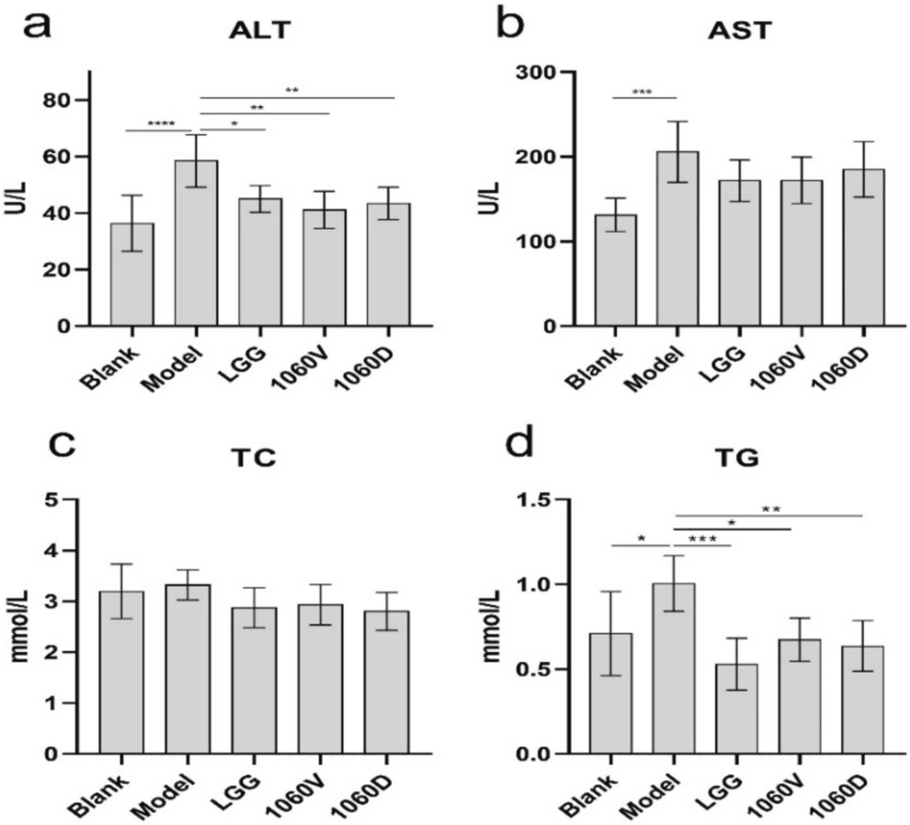

Fig. 3. The serum parameters following the treatment of CCFM1060 V and D. Quantification of ALT and AST concentrations in mice serum (a-b); Measurement of TC and TG concentrations in mice serum (c-d).

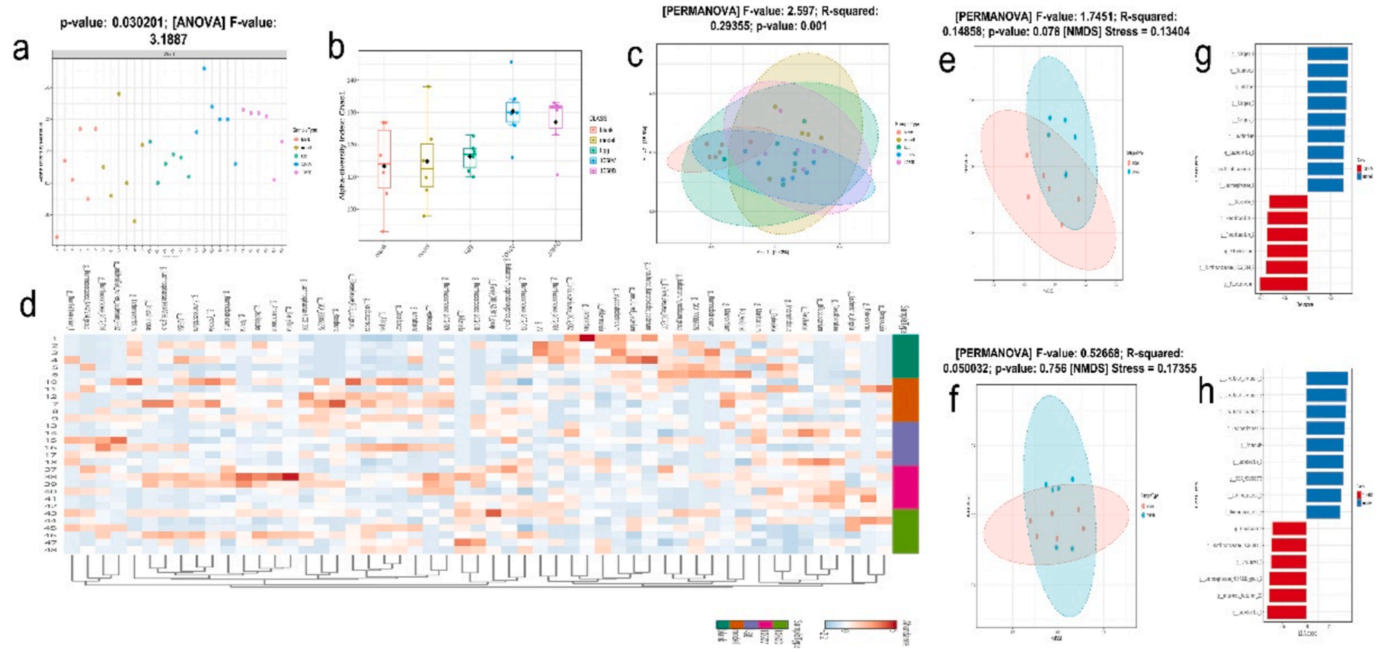

Fig. 4. Modulation of intestinal microflora by probiotics. Alpha Diversity, beta Diversity (a-c); The general alterations in the intestinal microflora following the probiotic treatment (d); Alterations in intestinal microflora before and post administration of CCFM1060V (e) and the analysis of LEfSe (g); Alterations in intestinal microflora pre and post administration of CCFM1060D (f) and the analysis of LEfSe (h).

g_Ruminococcaceae, *g_Alistipes*, *g_Odoribacter*. Specifically, in the CCFM1060D-treated groups, mice were found to have a higher concentration of *g_Odoribacter*, a bacterium that has the ability to clear succinic acid or ameliorate blood sugar and inflammation in non-alcoholic liver disease, (Huber-Ruano et al., 2022) *g_Lactobacillus*,(Hao et al., 2023) and *f_Lachnospiraceae*, and *g_Ruminococcaceae,* which are all SCFA-producing bacteria and reported beneficial for the recovery of ALD (Bajaj et al., 2021; Silvia et al., 2020; Smirnova et al., 2020). All of these are beneficial probiotics for the host. Conversely, the CCFM1060V group had a substantial abundance of *g_Dubsiela*, *g_Bifdobacterium*, *g_Ruminococcaceae_UCG_014_2*, the SCFA-producing bacteria which were decreased in alcoholic hepatitis (Bajaj et al., 2021; Silvia et al.,

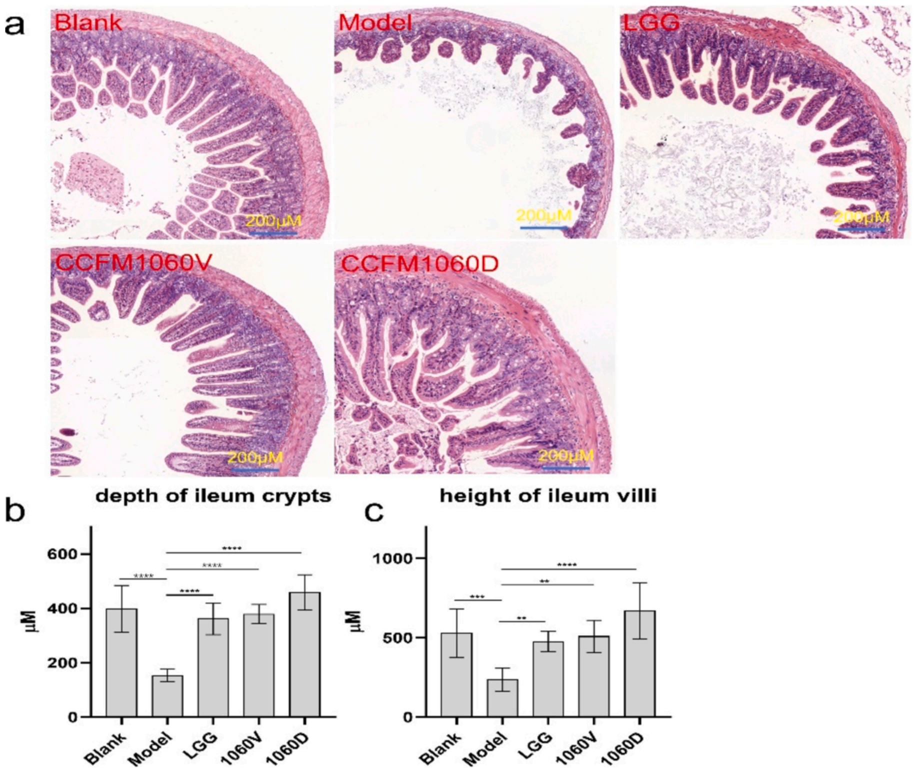

Fig. 5. Interventions with CCFM1060 V and D aid in the restoration of the mouse's intestinal barrier. Images of the ileum using H&E staining (a); Villi height and crypt depth determined by Image Pro Plus (b-c).

2020; Smirnova et al., 2020), and the g_*Faecalibaculum*, that reportedly harmful bacteria that can disrupt TH17 immune-mediated metabolic balance in non-alcoholic liver disease models (Kawano et al., 2022) (Fig. 4g and h).

3.5. CCFM1060 can improve intestinal barrier integrity

It has been reported that *Lachnospiraceae*, *Ruminococcaceae*, and *Lactobacillus* are the SCFA-producing bacteria beneficial bacterium, especially produce butyric acid. (Bajaj et al., 2021; Silvia et al., 2020; Smirnova et al., 2020) The butyric acid has been reported to be beneficial for the recovery of the intestinal barrier (Dubinkina et al., 2017; Ko, Seo, & Kim, 2020; Richa Singhal et al., 2021). Consequently, we employed HE staining to examine the microstructure of the mouse ileum. As illustrated in Fig. 5a, the control had an intact undamaged structure of the intestinal tract, whereas the slide in the model depicted a compromised and fragmented arrangement of crypts and microvillis. Both LGG and CCFM1060 relieved the intestine lesion induced by ethanol. Furthermore, we measured the depth of crypts and the height of microvillis on the ileum by Image Pro Plus. Our findings indicate that the probiotics consistently yielded elevated measurements for villi height and crypt depth, demonstrating improved intestinal integrity and a more uniform arrangement compared to the model (Fig. 5a–c).

Mucosal epithelia and their intercellular bridges are essential components of the intestinal barrier, which are responsible for preserving intestinal function. The colon was used to investigate the expression of genes and proteins associated with tight junction proteins. Occludin, claudin-1 and ZO-1 are crucial complements of tight junction proteins that play a vital role in the formation and maintenance of intercellular connections. The levels of RNA and protein expression of occludin, claudin-1 and ZO-1 were significantly high in both LGG and the tested probiotics (Fig. 6a–g).

3.6. CCFM1060 protects liver lesion from oxidative stress

Superoxide dismutase (SOD) is a crucial antioxidant enzyme found in cells particularly in hepatocytes. The mechanism involves the conversion of O_2 and H_2O_2 (Lu, Zhang, Chen, & Yang, 2018). Ethanol lowers the host's capacity to eliminate superoxide free radicals, and the inactive CCFM1060 stimulates strong antioxidant defense mechanisms in hepatic system (Fig. 7a). The tripeptide reduced glutathione (GSH) is composed of glutamic acid, cysteine, and glycine and is especially present in hepatocytes (Tsuchiya & Kurihara, 2022). Inviable *L. rhamnosus* can bring about high levels of non-enzymatic antioxidant substances in hepatic cells, which bind to oxidizing substances, which have the ability to attach to oxidizing agents, counteract free radicals, and shield the cells from oxidative damage (Fig. 7b). Malondialdehyde (MDA) is a byproduct of lipid peroxidation that critically contributes to oxidation-related damage and cell injury (Wu, Wang, Wang, Zhang, & Lai, 2022). Compared with the model, both LGG and CCFM1060 V and D resulted in insignificant oxidative damage that manifested as a lower MDA content (Fig. 7c).

Research suggests that the Keap1/Nrf2 pathway regulates the production of a multitude of detoxifying enzymes (Lv et al., 2024; Palliyaguru et al., 2016). Gene expression of HO-1 in hepatic cell is strongly correlated with antioxidant capacity (Luo et al., 2014; Zhao et al., 2023). To clarify the antioxidant impact mechanism in ALD, we investigated the Nrf2 and HO-1 expression in hepatic cells. Fig. 7d–f indicates that too much ethanol intake results in a considerable decrease in expression of the detoxification enzymes. This decrease is counteracted by LGG and CCFM1060 V and D adminisration. These findings indicate intervetion of probiotics enhances the movement of Nrf2 into the nucleus and subsequently boosts the production of HO-1 protein, thus enhancing the antioxidant capacity in hepatic cells.

3.7. CCFM1060 involves inflammation via TLR4/MyD88/NF-κB pathway

Disruption of the intestinal barrier often leads to gut leakage, and pathogen-associated molecular patterns (PAMP) like lipopolysaccharide (LPS) would diffuse via the portal vein to the liver, eventually causing a series of inflammatory reactions. An increase in the expression of TNF-α,

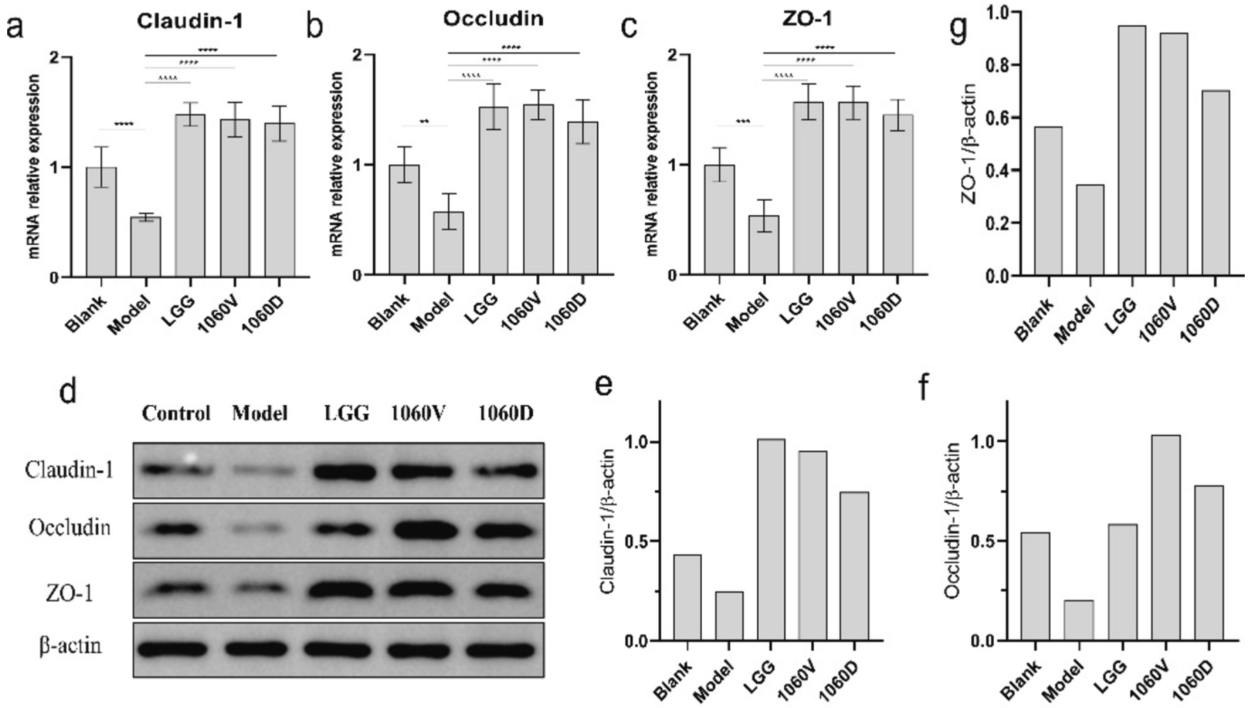

Fig. 6. Probiotics intervention can improve the intestinal barrier in mice. Measurements of RNA expression for tight junction protection (ZO-1, Claudin-1, and Occludin) (a-c); and its protein expression and the results determined using image pro plus (d-f).

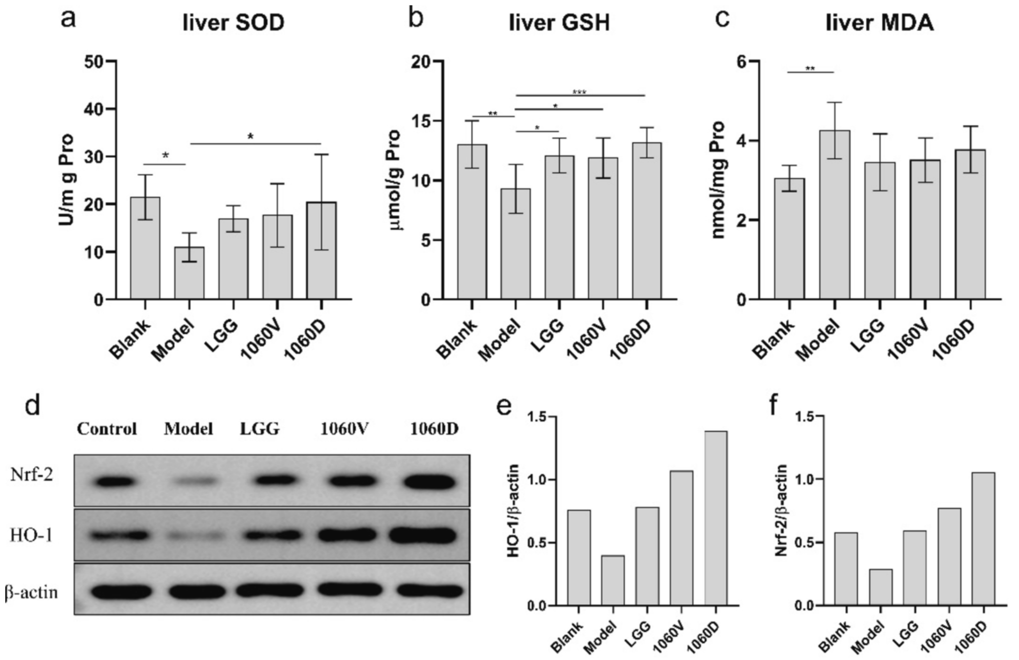

Fig. 7. Experimental administration of CCFM1060 V and D enhances the mouse's antioxidant capacity. The liver levels of SOD, GSH, and MDA were measured (a-c). Western blot pictures of important antioxidant proteins and the results determined using image pro plus (d-f).

IL-6 and IL-1β is detected following exposure to alcohol. In contrast, mice receiving probiotic intervention could mitigate the increase of pro-inflammatory cytokines induced by alcohol (Fig. 8a–c). Results indicate that CCFM1060 may modulate inflammatory cytokines in mice exposed to ethanol, thereby ameliorating liver inflammation.

Inflammatory cytokines (TNF-α, IL-1β, and IL-6), have a direct role in the development of ALD (Dong, Wu, Gai, & Han, 2023; T. Wang et al., 2023) and are strongly linked to the stimulation of NF-κB. We further investigated the process by analyzing key protein expressions using western blot. In comparison to the control, the hepatic cells of the model mice exhibited a notable rise in the expression of MyD88, TLR4, and p65. On the contrary, the probiotics administrations have selectively reduced protein phosphorylation to a various extent (Fig. 8d–g). Thus, the above findings suggest the impact of CCFM1060 on alleviating liver

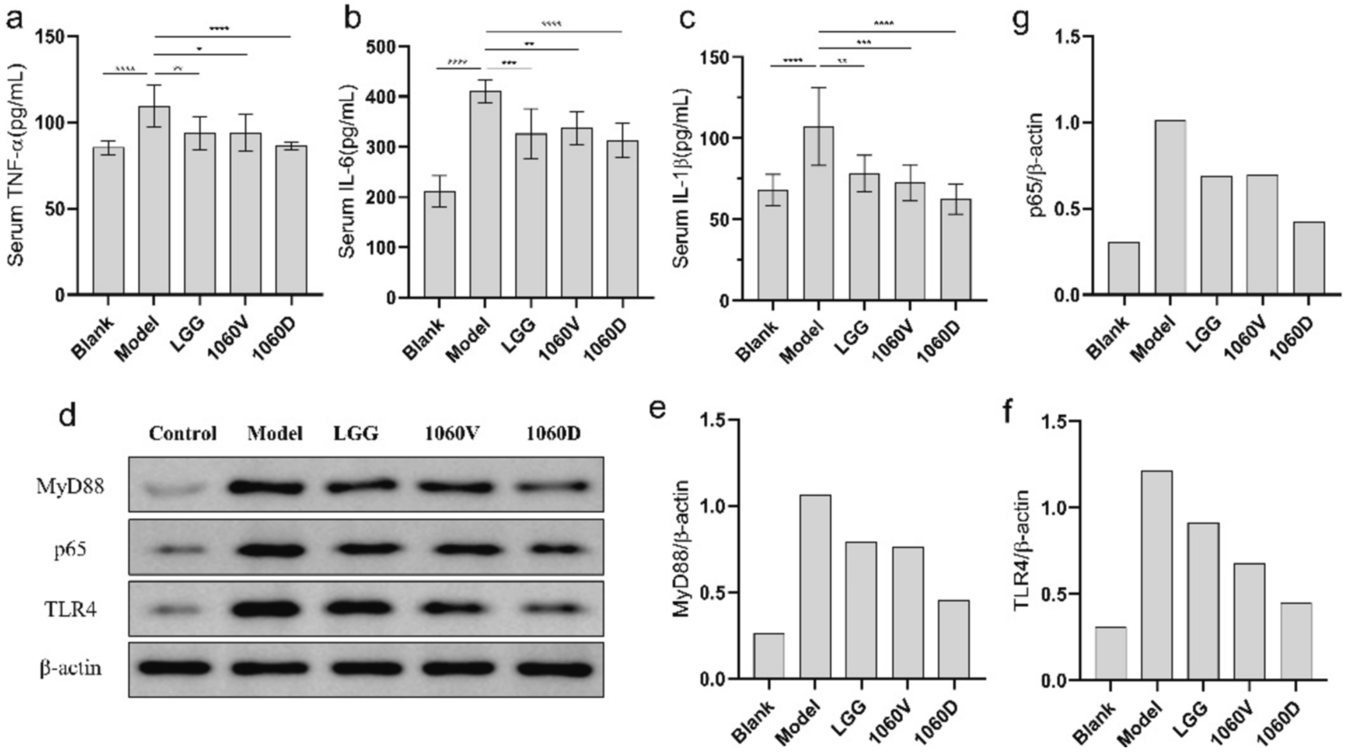

Fig. 8. The regulation of inflammation is mediated by CCFM1060. Measurements of TNF-α, IL-6, and IL-1β concentrations in mouse serum (a-c); Western blot pictures of antioxidant important proteins (MyD88, p65, and TLR4) and the results determined using image pro plus (d-g).

injury is attributed to the inhibition of the TLR4/MyD88/NF-κB pathway stimulation.

4. Discussion

ALD is a hepatic disorder resulting from excessive and/or long-term ethanol consumption and is the leading cause of liver disorders and mortality associated with liver disease on a global scale (Bellentani et al., 2018; Esser & Jernigan, 2018). There are no FDA-approved effective drugs for ALD except for abstinence. Alcohol consumption usually contributes to disturbances in the microbiota, and gut dysbiosis is crucial in the initiation and progression of several diseases. Modifying the daily food is the most straightforward and practical approach to quickly disrupt the gut microenvironment (David et al., 2014). Probiotic therapy is a beneficial dietary lifestyle that has encouraged researchers' attention to developing novel probiotics for alleviating illness.

A chronic binge model was used to investigate the protective impact of CCFM1060 on mouse livers. The excess ethanol consumption could lead to syncope in humans (R. X. Wang, Lee, Campbell, & Colgan, 2020). The number and duration of ethanol-induced syncope episodes were significantly decreased by CCFM1060 intervention. Ethanol intake lowers body temperature in the host; sometimes, death may be attributed to hypothermia caused by alcohol consumption (Murphy & Lipton, 1983; Myers, 1981). Probiotics partly alleviated temperature loss in mice to circumvent death. Ethanol intake is typically associated with the development of liver lesions. Both viable and dead CCFM1060 mitigated ethanol-induced liver injury and shared a lower fat droplet area score. TC and TG levels are critical indicators of liver lipid metabolism and physiological activity. Hepatic TC and TG concentrations instantly dictate the extent of liver injury and lipid metabolism abnormalities. In addition, the level of TC and TG in serum is a bit lower compared to the value in the model. Transient ethanol exposure may account for the minor variations in the serum rather than in the hepatocytes (Kim et al., 2018; Z. Wang et al., 2015). ALT and AST are key liver transaminases. The rupture or increased permeability of the hepatocyte membrane releases transaminases into the blood, causing an increase in serum. Lower concentrations of ALT and AST indicated that CCFM1060 alleviated alcohol-induced liver lesions. The probiotic-treated mice seemed to have better lipid metabolism than the model one.

The concept of the so-called gut–liver axis refers to the interaction between the two organs, including the microbiome, diet, environment, and other factors (Pabst et al., 2023). Gut dysbiosis triggers inflammation by destroying the intestinal barrier and increasing the translocation of bacterial products, thereby exacerbating the pathological process of ALD (Bajaj, 2019). Therefore, we performed sequence analysis of 16S rRNA gene, which showed that alcohol exposure had a substantial impact on the composition and structure of the intestinal flora, which was partly restored by probiotics. Moreover, we performed LEfSe analysis to characterize certain bacterial taxa following these treatments. In mice administered with viable strains, *Alistipes* identifies as the genus of bacterium that produce butyric acid (Trebicka, Bork, Krag, & Arumugam, 2021) and is negatively correlated with AST and MDA content (Bajaj et al., 2021; Wenwen et al., 2022). *Odoribacter* is a common SCFA-producing microorganism found in the human intestinal tract. Its abundance is negatively correlated with the occurrence of ALD (Trebicka et al., 2021), non-alcoholic fatty liver disease (Williams-Nguyen et al., 2020), cystic fibrosis, and inflammatory intestinal diseases (Hiippala et al., 2020). A low abundance of *Lachnospiraceae* and *Ruminococcaceae* has been observed in patients with ALD (Bajaj et al., 2021; Bajaj et al., 2017; Trebicka et al., 2021). These symbiotic bacteria produce SCFA, particularly butyric acid (Chen et al., 2017; Guo et al., 2022; Reichardt et al., 2014; Sun et al., 2020). A reduction in the population of bacteria that produce butyric acid is a crucial characteristic in the pathogenesis of ALD (R. Singhal et al., 2021). This reduction actively controls the balance of nutrients in the intestines and strengthens the integrity of the intestinal barrier (R. X. Wang et al., 2020; R. Y. Wang et al., 2023). Interestingly, researchers have discovered that *Ruminococcaceae* has the ability to decrease the preference and desire for ethanol in mice (Wolstenholme et al., 2022). The abundance of *Dubosiella* increased in the acute alcohol intake model after *Antrodia A.* administration, which is related to the improvement in ALD (Z. Yi et al., 2021). It exhibits a weak negative correlation with AST, ALT, and MDA levels (Z. W. Yi et al., 2020). Evidently, *Bifidobacterium* is beneficial to the host. Combined, CCFM1060 can inhibit microorganisms that exacerbate the progression of ALD and thrive the ones that relieve the pathological process.

The disruption of the gut barrier, especially the epithelial tight junction protein, is a prerequisite for ALD(Cassard & Ciocan, 2018). The qPCR and western blot results show both CCFM1060 V and D substantially upregulated the expression of ZO-1, claudin-1, and occludin genes and proteins in the colon. In addition, the H&E slide clearly illustrated the ileal damage caused by alcohol, whereas probiotics relieved the injury. The crypt depth and villi height calculated using Image-Pro Plus further illustrated this effect, demonstrating that CCFM1060 has the potential to ensure the integrity of the gut structure. A healthy and complete gut architecture prevents endotoxemia induced by the escape of endotoxins and the immune response.

Apart from gut leakage, alcohol-induced oxidative stress is an additional element that exacerbates liver lesions. Ethanol intake decreases the concentrations of critical antioxidant enzymes, including SOD and GSH. SOD converts O_2^- into more stable O_2 and H_2O_2, therefore mitigating oxidative stress in cells. GSH counteracts intracellular oxidative stress by interacting with O_2^- and other detrimental oxidants. CCFM1060 intervention enhanced antioxidant capability by increasing the levels of SOD and GSH, eventually alleviating the harm induced by oxidative damage to the liver by ethanol metabolism. Oxidative stress damage can also be reflected by MDA, which increases once oxygen free radicals increase and antioxidant capacity decreases. CCFM1060 shares the ability to decrease MDA content in the liver. Experimental evidence has demonstrated that alcohol exposure suppresses the expression of HO-1, while the nucleotide transversion of Nrf2 could stimulate the expression and provide liver protection against ethanol-induced damage. Prolonged exposure to alcohol suppresses the expression of HO-1 (Lamlé, Marhenke, Borlak, Wasielewski, & Vogel, 2008; Liu et al., 2018). Nrf2 transversion stimulates its expression and safeguards the liver from ethanol-induced damage (D. Wang, Wang, & Wu, 2023). Based on the western blot shown in Fig. 7d–f, the protein of HO-1 and Nrf2 were suppressed in hepatic cells after alcohol intake, as compared to the control. After intervention with probiotics, the decrease can be reversed. Thus, we suggest that CCFM1060 stimulates the antioxidant defense mechanism, maybe by activating the Nrf2/HO-1 pathway. (Fig. 9).

Ethanol intake usually triggers lesions in the intestinal mucosa and increases gut permeability and leakage. Bacteria, bacteria-associated components, and PAMPs, like LPS, easily traverse the portal vein and enter the liver to attach to pattern recognition receptors, including endotoxin receptors, to activate the TLR4 pathway in liver Kupffer cells, which releases reactive oxygen species (ROS) and results in an inflammatory reaction. Concentrations of pro-inflammatory factors (TNF-α, IL-1β, and IL-6) in the serum rise dramatically when exposed to ethanol. Nevertheless, CCFM1060 can mitigate this predicament. In order to comprehend the possible processes, we analyzed the protein expression of markers associated with inflammation. Our observations suggest that the expression of MyD88, TLR4, and p65 were notably elevated in the hepatic cells of the model compared to the control. Protein phosphorylation was reduced as a consequence of the administration of probiotics. The PAMP and LPS molecules produced in the gut transit via the portal vein and interact with TLR4 receptors found in hepatic Kupffer cells. This binding leads to the recruitment of Myd88 protein and the subsequent activation of NF-κB signaling, which activates the innate immune response and causes damage to the liver. Hence, the capacity of CCFM1060 to induce remission of ALD can be partially attributed to its ability to inhibit the triggering of the TLR4/MyD88/NF-κB pathway.

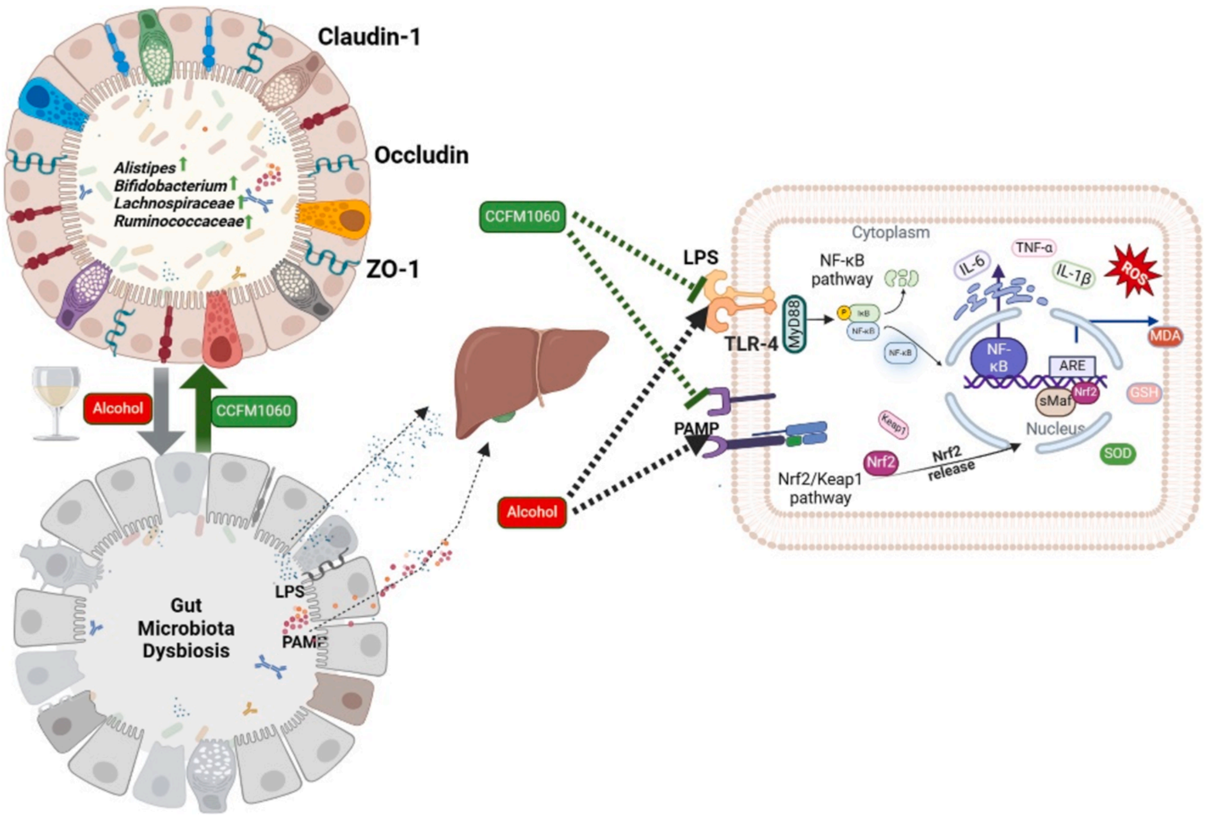

Fig. 9. Potential mitigating mechanism of CCFM1060 treatment in mice with alcohol-caused ALD.

(Fig. 9).

5. Conclusion

In conclusion, we demonstrated that CCFM1060 (especially in the inactive form) has therapeutic potential for mitigating liver injury caused by ethanol intake in mice. It can partially restore the composition and structure of the intestinal microflora, improve antioxidant capacity, and enhance intestinal barrier function, thereby maintaining immune homeostasis and preventing liver steatosis and pathogenic processes. These findings provide further evidence that probiotics have the potential to ameliorate ALD by improving gut microbiota dysbiosis through the gut-liver axis.

CRediT authorship contribution statement

Ben Niu: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Lijuan Huang:** Methodology. **Xu Cheng:** Methodology. **Zhangming Pei:** Methodology. **Hongchao Wang:** Methodology. **Fengwei Tian:** Methodology. **Wenwei Lu:** Visualization, Validation, Supervision, Project administration.

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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Data availability

No data was used for the research described in the article.

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