Acta Scientific Microbiology (ISSN: 2581-3226)

Volume 9 Issue 4 April 2026

Research Article

A Preclinical Study on Probiotic Intervention in Alcohol-Induced Hepatic Injury

Prabu Rajagoapalan, Shri Vidya Shyam Prasad, Gokul Raj, Nithish Kumar C, Shyamprasad Kodimule and Raksha Sunhare*

Development and Research Centre-Probiotics, Vidya Herbs Pvt Ltd, No. 102B & 105B, Pharmaceuticals SEZ Industrial Area, KIDB, Hassan, 573201 Karnataka, India

***Corresponding Author:** Raksha Sunhare, Development and Research Centre-Probiotics, Vidya Herbs Pvt Ltd, No. 102B & 105B, Pharmaceuticals SEZ Industrial Area, KIDB, Hassan, 573201 Karnataka, India.

DOI: 10.31080/ASMI.2026.09.1591

Received: March 03, 2026

Published: March 31, 2026

Abstract

This study aimed to investigate the hepatic protective and therapeutic potential of *Bacillus coagulans* VHBAX in a zebrafish model of liver cirrhosis. Adult zebrafish were exposed to 0.5% ethanol to induce liver cirrhosis, and the treatment group received probiotic supplementation, with outcomes compared to control and ethanol-induced groups. The effects of supplementation were evaluated through liver histopathology, liver function markers, hepatic lipid accumulation, gut permeability markers, and inflammasome-related parameters. Probiotic administration resulted in marked improvement in liver histology, significant reduction in liver injury biomarkers, decreased lipid accumulation within hepatic tissues, modulation of gut permeability markers indicating restoration of intestinal integrity, and regulation of inflammasome-associated inflammatory responses. Biochemical analyses further confirmed reduced hepatic injury and inflammation, reflecting improved liver function and enhanced lipid metabolism. Overall, the findings suggest that *Bacillus coagulans* VHBAX exerts a protective effect against ethanol-induced liver damage and may play a beneficial role in mitigating fatty liver disease by reducing inflammation, improving hepatic health, and restoring gut barrier function.

Keywords: *Bacillus coagulans*; Gut Dysbiosis; Liver Cirrhosis; Liver Function Markers; Probiotics; VHBAX; Zebrafish Model

Abbreviations

ALD: Alcoholic Liver Disease; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; cDNA: Complementary Deoxyribonucleic Acid; dNTP: Deoxy Nucleotide Triphosphate; DPX: Dibutylphthalate Polystyrene Xylene; F11R: Junctional Adhesion Molecule-A; HCC: Hepatocellular Carcinoma; IL: Interleukin;

LDH: Lactate Dehydrogenase; LPC: Lysophosphatidylcholine; M-MLV: Moloney Murine Leukemia Virus; NAFLD: Nonalcoholic Fatty Liver Disease; NOX4: Nicotinamide Adenine Dinucleotide Phosphate Oxidase 4; PYCARD: Apoptosis-Associated Speck-Like Protein Containing a CARD; qPCR: Quantitative Polymerase Chain Reaction; RNA: Ribonucleic Acid; SPSS: Statistical Package for Social Sciences.

Introduction

Liver cirrhosis is a progressive hepatic disease and ranking as the 14th most prominent cause of death worldwide, and increasingly contributing to morbidity and mortality, with a 1-year

Citation: Raksha Sunhare., *et al.* "A Preclinical Study on Probiotic Intervention in Alcohol-Induced Hepatic Injury". *Acta Scientific Microbiology* 9.4 (2026): 20-26.

mortality rate varying from 1% to 57% depending on the disease stage [1]. The obesity, fatty liver disease, alcoholism, hepatitis B or C infection, autoimmune disorders, cholestatic diseases, and excess iron or copper exposure are the major factors causing the chronic hepatic loss [2]. This leads to portal hypertension due to inflammation resulting in fibrotic tissue and regenerating nodules that replaces healthy liver parenchyma [3]. As the disease progresses, it leads to hospitalization, reduce quality of life, and increases mortality [4].

Recent studies emphasize the vital connection of gut and liver, a bidirectional communication system between the gastrointestinal tract microbiota, and liver [5]. This axis in the gut causes bacterial translocation, endotoxemia, and hepatic inflammation, all of which aggravate liver injury [4]. Frequent alcohol use exacerbates hepatic fibrosis by further impairing the integrity of the intestinal barrier and microbial diversity. As a result, altering the gut-liver axis has become a viable therapeutic target for chronic liver conditions like cirrhosis, hepatitis, alcoholic liver disease (ALD), and hepatocellular carcinoma (HCC).

In this context, microbiota-directed interventions, including probiotics, prebiotics, synbiotics, and faecal microbiota supplementation, are being evaluated in conjunction with the therapy to restore intestinal microbial homeostasis and mitigate hepatic injury [6,7]. *Bacillus coagulans* has garnered attention as a probiotic candidate due to its spore-forming ability, resilience to gastrointestinal stress, and purported hepatoprotective and anti-inflammatory effects. Despite these potential benefits, the role of *Bacillus coagulans* in mitigating alcohol-induced liver cirrhosis and its mechanistic impact on the gut–liver axis necessitates extensive investigation to elucidate the precise mechanism.

The Danio rerio- zebra fish are knows as a versatile and compatible vertebrate model for hepatic disease research due to its conserved hepatic architecture and genetic homology with humans [8]. Ethanol-induced liver injury in zebrafish mimics the histopathological and biochemical features of human alcoholic cirrhosis, making it an ideal system for evaluating hepatoprotective compounds and exploring underlying molecular mechanisms.

Objective of the study

This study designed to understand the hepatoprotective potential of the probiotic VHBAX *Bacillus coagulans* versus alcohol-induced liver cirrhosis in zebrafish, which hypothesis that supplementation with VHBAX would reduce alcohol-induced hepatic injury by restoring healthy gut microbes, suppressing inflammatory responses, and promoting hepatic regeneration in zebrafish.

- To develop an ethanol-induced liver cirrhosis reproducible model with zebrafish.
- To understand the effects of probiotics VHBAX *Bacillus coagulans* supplementation on biochemical, molecular, and histopathological markers of hepatic injury.
- The effect of VHBAX in gut–liver axis interaction, with brief understanding on oxidative stress and inflammatory signalling pathways.

Materials and Methods

Zebrafish experiments were approved by CPCSEA (SU/CLATR/IAEC/XXI/08/2023). Insights from a zebrafish Animal Model has been approved by the IAEC of Satyabama Institute of Science and Technology, Chennai 600119, India. The model was maintained in a semi-static system with charcoal-filtered tap water at 28 ± 0.5 °C under a 14 h light/10 h dark cycle and fed commercial food twice daily. Fish were randomly divided into three groups (n=10 each):

- **Control:** Normal oxygenated water and standard feed.

- **Induction:** Exposed to 0.5% alcohol for 28 days to induce liver damage.

- **Treatment:** Exposed to 0.5% alcohol and given *Bacillus coagulans* VHBAX (500 µg/kg body weight) for 28 days.

After 28 days, livers were excised, weighed, fixed in 10% buffered formalin for histology, and the remaining tissue used for biochemical and gene-expression analyses.

Histological analysis

Zebrafish were euthanized by immersion in a freshly prepared buffered tricaine methane sulfonate (MS-222- RM2178 Himedia) solution (200–300 mg/L, pH 7.0–7.5) until opercular movement ceased, followed by decapitation to confirm death before tissue collection. Whole zebrafish from control and experimental groups were fixed for whole-mount analysis. Paraffin-embedded sections were deparaffinized in xylene at 65 °C for 20 min, cleared twice in xylene (10 min each), passed through a graded alcohol series (100%→90%→70%→50%→30%), and rehydrated in distilled water (10 min each). Sections were stained with Mayer's hematoxylin (15 min), rinsed in running water (10 min), counterstained with eosin (2 min), dehydrated through ascending alcohols (30%→100%), cleared twice in xylene, and mounted with DPX for light-microscopy. Frozen liver sections were also stained with Oil Red O for quantitative analysis.

Biochemical analysis

To measure other biochemical parameters, fish liver sample were used for estimating liver function markers. To measure liver function markers, the liver sample was homogenized in phosphate-buffered saline (0.1 M, pH 7.2), and after centrifugation at 12000 rpm for 5 min and 4 °C, the supernatant was collected. Liver function markers Aspartate transaminase, Alanine Transaminase and Lactate dehydrogenase were analyzed by auto biochemistry analyser BS-120 (Mindray) as per manufacturer's instruction. The results were expressed as IU/L.

Quantification of hepatic lipid content using Oil Red O

Lipid extraction followed Folch., *et al.* [9]. Extracted lipids were washed with 2 ml 60% isopropanol (5 min, room temperature), air-dried, and stained with 1 ml Oil Red O for 10 min. After four ddH_2O washes, the dye was eluted with 1 ml 100% isopropanol (10 min, gentle shaking). Optical density was measured at 500 nm using 100% isopropanol as the blank [10].

Quantitative real-time PCR analysis of Gap junctional genes and Inflammosomes markers of control and experimental groups

The isolation and quantification of total RNA from liver tissue followed the method outlined by Chomczynski and Sacchi, 1987 [11]. Briefly, total RNA extraction from zebrafish samples was executed using a suitable RNA extraction kit, with careful attention to prevent RNA degradation. Subsequently, cDNA synthesis from the total RNA was accomplished through reverse transcription (RT) using a cDNA conversion kit from Thermo Scientific Inc. This conversion of RNA into cDNA renders it amenable for qPCR analysis. The reaction mixture, comprising 0.5-1.0 µg total RNA, underwent incubation at 72°C for 10 min with 1.5 µL of random hexamer primer followed by immediate chilling.

- GAPDH F-5'AACTTTGGCATTGTGGAAGG 3'

R-5' GGATGCAGGGATGATGTTCT 3'

- IL-1β F-5'CCTGTGTTGCTGAAGGAGAG3'

R-5' GCTGTGAGGGTGTTGAAGAA3'

- TNF-α F-5'ATGAGCCAGGAGGGAGAAT3'

R-5'AGCAGGTTGACCTCAGAGTT3'

- IL-10 F-5' CAGGACTTTAAGGGTTACTTGG3'

R-5'AAGGCTTGGCAACCCAAGTA

- Occludin F-5'TTGCTGCTTGGTTTATCAGC3'

R-5'AGGAGGTGAGGAAGTAGAGG3'

- Claudin F-5'TGCTGTTTGCTTCTTCTGGA

R-5'GGTAGTGGTGTTGTAGTTGG)

Addition of 5.0 µL premixed 10 mmol/L deoxy nucleotide triphosphate (dNTP) solution, 3.0 µL 10x Moloney Murine leukaemia virus (M-MLV) RT buffer, and 1.0 µL of M-MLV RT constituted the subsequent steps, with the final volume adjusted to 50 µL using RNase/DNase-free water. The subsequent real-time PCR was conducted on a BioRad CFX96 with SYBR green fluorescent labeling, utilizing GAPDH as an internal control. Throughout the cycling process, fluorescence emitted by SYBR Green was monitored, proportional to the amplified DNA amount. The fluorescence data obtained during each cycle facilitated the generation of amplification curves. Analysis of the qPCR data employed specialized software accompanying the BioRad CFX96, with calculation of cycle threshold (Ct) values for both the target and reference genes in each sample.

Statistical analysis

Statistics were compared the treatment group and the induction group using Student's t-tests. The study data was reported as means ± standard deviation (S.D) with a sample size (n) of 6. The data was analyzed using SPSS 16.0 and a Student's t-test. A significance criterion of $p < 0.05$ was used for all analyses.

Results

This study evaluated the curative and protective effects of probiotics VHBAX *Bacillus coagulans* supplementation alongside the ethanol-induced liver of zebrafish model. The results showed that the probiotics VHBAX supplementation improved hepatic cellular lining, reduced fibrosis, and lowered hepatic enzyme levels (AST and ALT) compared with the control group. These findings suggest that VHBAX may mitigate alcohol-induced liver damage and support hepatic recovery.

VHBAX *Bacillus coagulans* improves liver tissue degeneration in liver cirrhosis-induced zebrafish model

The present study delved into the impact of VHBAX *Bacillus coagulans* probiotics on ethanol-induced liver fibrosis utilizing a zebrafish animal model (Figure 1). This histological examination of liver tissue from both control and experimental cohorts yielded insightful findings regarding the repercussions of probiotic intervention on hepatic cellular architecture. A notable outcome was the discernible reduction in fibrosis evident in the liver tissue of the probiotic-treated group. Fibrosis, caused by extracellular matrix protein buildup, can distort tissue architecture and impair organ function. The improved hepatic cellular architecture in the probiotic-treated group implies that probiotics may affect liver-healthy cellular activities. These mechanisms may regulate inflammation, oxidative damage, and apoptosis. Probiotics may restore liver tissue shape and function by positively modulating these variables. Figure 2 shows quantitative examination of lactate dehydrogenase (LDH) levels in control and probiotic-treated zebrafish. 0.5% alcohol induced group had significantly higher LDH levels than control. The VHBAX-treated group had significantly lower LDH levels than the induction group. The hepatoprotective effects observed in this study can be attributed to multiple mechanisms involving the gut–liver axis. *B. coagulans* is known to modulate intestinal barrier integrity, immune signaling, and lipid metabolism. In the present study, ethanol exposure downregulated claudins, key components of tight junctions, while probiotic treatment restored gap junctional protein expression. This suggests that VHBAX may protect against ethanol-induced intestinal permeability and endotoxin leakage—factors known to exacerbate hepatic inflammation and fibrosis.

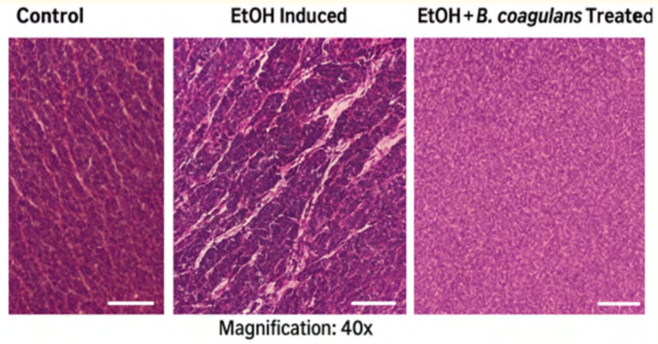

Figure 1: Microscopical Observation of the control liver showed normal histological features, whereas, alterations induced by Alcohol were shown in the figure. VHBAX *Bacillus coagulans* treated group showed normal hepatocytes as compared to the control group.

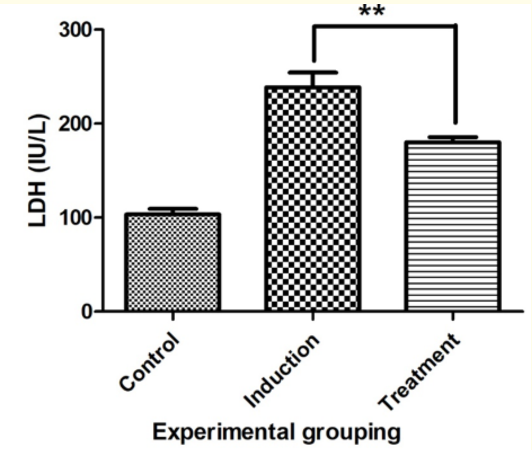

Figure 2: Shows LDH (Lactate Dehydrogenase) levels in the liver. LDH is a marker of tissue damage and cellular stress (**P < 0.01).

VHBAX *Bacillus coagulans* treatment improves liver functions

As shown in figure 3, in alcohol-induced liver fibrosis, the levels of two liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), are commonly measured to assess liver damage and function. These enzymes are released into the bloodstream when liver cells are damaged or inflamed. Elevated ALT and AST levels can indicate liver injury, including liver fibrosis, which is the formation of excess scar tissue in the liver due to ethanol induction. On the other hand, the probiotic-treated group exhibited significantly lower levels of AST and ALT compared to the control group. These markers are commonly used to assess liver function, and their reduction suggests improved liver function in the probiotic-treated group.

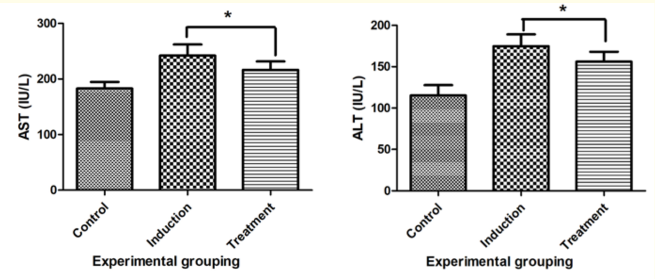

Figure 3: Displays AST (Aspartate Aminotransferase) and Alanine Transferase levels in liver tissues. Elevated AST & ALT can indicate liver damage in the induction group (*P < 0.05).

VHBAX *Bacillus coagulans* reduces lipid accumulations

Quantification demonstrated higher lipid accumulation in the ethanol-induced group (Figure 4). Complex connections between molecular mechanisms and pathways may explain this. In ethanol-induced liver fibrosis, poor lipid metabolism, transport, lipogenesis, oxidation, and inflammation cause lipid buildup. The probiotic group showed less lipid buildup than the control group, according to the results. This shows that probiotics may reduce liver lipid buildup, avoiding or alleviating fatty liver disease.

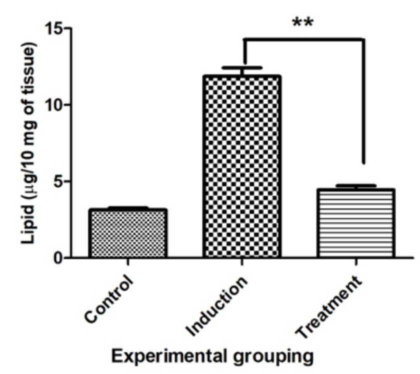

Figure 4: Bar graph displaying lipid quantification results in the liver tissue of Zebra fish with Alcohol-induced cirrhosis. Data represents mean lipid levels (± standard error) in cirrhotic liver tissue, highlighting the significant lipid accumulation compared to the control group (*P < 0.05, **P < 0.01).

The mRNA expression of Claudin 5 and F11R

Studies have shown that alterations in Claudin expression can contribute to liver fibrosis. For instance, downregulation or loss of certain Claudin proteins in the liver can disrupt the integrity of tight junctions, leading to increased permeability and the passage of harmful substances (Figure 5). This disturbance can activate hepatic stellate cells and cause inflammation, which contributes to liver fibrosis. Our results showed that the ethanol-induced group had a Studies suggest F11R may cause liver fibrosis. Alcohol-induced liver fibrosis may be linked to F11R expression changes in liver tissue from alcoholic liver disease patients. F11R expression was upregulated in fish fed probiotics compared to controls. The results from this study revelated that probiotic VHBAX supplementation for alcohol-induced liver cirrhosis. Long-term alcohol use causes liver damage and cirrhosis. Upregulation of PYCARD and IL-1β in alcohol-induced zebra zebrafish activates the inflammasome and releases pro-inflammatory cytokines, contributing to liver fibrosis development and progression. However, alcohol induction downregulated IL-10, which was restored by probiotic supplementation. Compared to control, probiotic-fed fishes had upregulated gap junctional proteins and downregulated claudins.

Similarly, VHBAX treatment altered the expression of F11R (junctional adhesion molecule-A), a factor implicated in liver fibrosis. The upregulation of F11R in the probiotic-treated group may reflect improved cellular adhesion and tissue remodeling processes that counteract fibrogenesis. Furthermore, probiotics reduced the expression of inflammatory markers such as PYCARD and IL-1β, which play central roles in inflammasome activation and fibrotic progression. Correction of IL-10 downregulation also indicates immune modulation toward a balanced anti-inflammatory response.

Comparable findings have been reported in mammalian models. For instance, studies in mice have shown that *Bacillus coagulans* and other probiotics such as Lactobacillus rhamnosus GG and Bifidobacterium longum can restore gut barrier function, reduce oxidative stress, and suppress hepatic stellate cell activation [12]. Clinical evidence also supports these effects: randomized trials in patients with nonalcoholic fatty liver disease (NAFLD) have reported improved mucosal immune function, reduced intestinal

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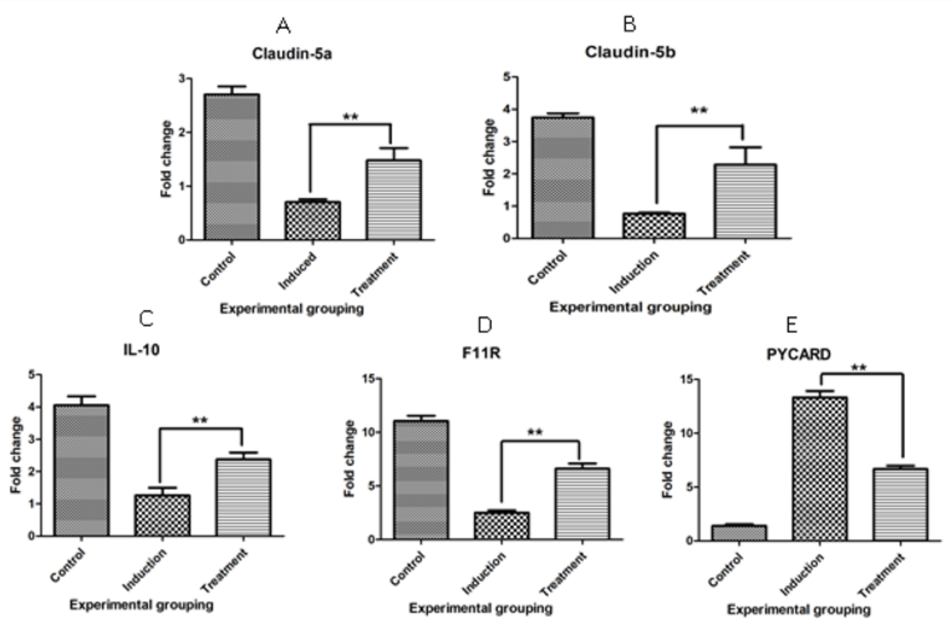

Figure 5: (A) Shows that the alcohol induction has caused an increase in the Claudin 5a compared to the control group. Treatment with VHBAX *Bacillus coagulans* has reduced the value similar to the control group. (B) shows that the induction of alcohol elevated the gut permeability marker Claudin 5b which was reduced after treatment with *Bacillus coagulans*. (C) shows a reduction of IL-10 in the induction group indicating the reduced anti-inflammatory response of the immune system against liver damage. An improvement with an increase in the value of IL-10 in the group is seen. (D) shows that F11R which may be involved in the pathogenesis of liver fibrosis increased compared to the treated group. (E) shows a sharp increase in the value of PYCARD showing the inflammation activity against liver damage. This is reduced in the treatment group (**$P < 0.01$).

permeability, and decreased liver enzyme levels following probiotic supplementation [5]. Collectively, these findings suggest that probiotic-mediated modulation of the gut–liver axis contributes to reduced fibrosis and the zebrafish model offers unique advantages for studying liver fibrosis, including transparent embryonic development, genetic similarity to humans, and high-throughput feasibility. Transgenic zebrafish models have been successfully used to evaluate lipid metabolism, liver regeneration, and hepatocyte differentiation [13-15]. Moreover, previous studies, such as those by Zou., *et al*. [16], have demonstrated that zebrafish accurately recapitulate features of hepatic steatosis, oxidative stress, and inflammatory responses seen in mammalian models.

However, the translational gap between zebrafish and human liver pathophysiology must be acknowledged. While zebrafish exhibit conserved pathways of fibrosis, bile acid signaling, and lipid metabolism, differences in immune complexity and gut microbiota composition limit direct extrapolation to humans. Therefore, findings from this model should be validated in mammalian systems—particularly in preclinical mouse models and clinical trials—to confirm.

The concurrent administration of *B. coagulans* VHBAX with ethanol exposure in this study demonstrates a protective effect during ongoing injury rather than true prevention of liver damage. Future investigations should therefore include pre-treatment and post-treatment designs to distinguish between preventive, protective, and therapeutic roles of the probiotic. Mechanistic studies should also explore the interaction of *B. coagulans* with bile salt export pumps, fibroblast growth factor 15 signaling, and hepatic stellate cell activation pathways to better define the molecular basis of its effects.

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Conclusion

In summary, *B. coagulans* VHBAX supplementation ameliorated ethanol-induced liver fibrosis in zebrafish by improving hepatic architecture, reducing lipid accumulation, restoring tight junction integrity, and modulating inflammatory signaling. These results align with existing evidence from rodent and human studies indicating that probiotics can beneficially influence the gut–liver axis. While promising, further research is required to clarify the mechanistic pathways and evaluate translational potential in human liver disease.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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