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Hepatoprotective Potential of Probiotic-Enriched Stirred Yogurt Against Metabolic Dysfunction-Associated Steatotic Liver Disease in a Rat Model

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ABSTRACT

Background: Metabolic dysfunction-associated steatotic liver disease (MASLD) represents the most prevalent chronic liver disorder worldwide, driven principally by excessive caloric intake, high-fat dietary patterns, and sedentary lifestyles. The condition is pathophysiologically characterized by hepatic lipid accumulation, insulin resistance, oxidative stress, and low-grade systemic inflammation, which may progress to steatohepatitis, fibrosis, and ultimately cirrhosis in the absence of timely intervention. Probiotic microorganisms have recently attracted considerable scientific attention as safe biotherapeutic agents capable of modulating gut microbiota, improving lipid metabolism, and attenuating hepatic inflammation through the gut–liver axis.

Aims: This study aimed to develop a cost-effective, food-based strategy for the prevention and early management of MASLD through the incorporation of a defined probiotic mixture into stirred yogurt, and to evaluate its hepatoprotective efficacy in an experimental rat model.

Material and Methods: Eleven dairy-origin probiotic strains were initially screened for acid and bile tolerance, antioxidant activity, antimicrobial potential, and technological suitability for yogurt production. Four strains — *Lactobacillus rhamnosus*, *L. plantarum*, *L. acidophilus*, and *Bifidobacterium lactis* — were selected on the basis of their superior performance profiles for the production of probiotic stirred yogurts, administered either as single-strain or multi-strain formulations. These yogurts were subsequently administered to rats maintained on a high-fat diet (HFD) to assess their preventive effects against MASLD development. Biochemical analyses encompassed the evaluation of serum lipid and metabolic profiles, including total cholesterol, triglycerides, low-density lipoprotein (LDL), high-density lipoprotein (HDL), fasting serum glucose (FSG), insulin, alanine aminotransferase (ALT), and albumin concentrations. Histopathological examination of hepatic tissues and assessment of intestinal microbiota were additionally performed to confirm hepatic protection and gut microbial modulation.

Results: The yogurt containing the multi-strain probiotic blend (T5) exhibited superior organoleptic properties and the most pronounced hepatoprotective effects among all tested formulations Administration of T5 markedly reduced serum total cholesterol (85.1 mg/dL), triglycerides (122.3 mg/dL), LDL (25.8 mg/dL), FSG (28.6 mg/dL), insulin (0.84 μIU/mL), and ALT (36.9 U/L), with values approaching those recorded in the healthy control group. Histopathological findings and microbiota analyses corroborated these biochemical improvements, confirming attenuation of hepatic steatosis and restoration of gut microbial balance.

Conclusions: The multi-strain probiotic yogurt formulation (T5) demonstrated robust hepatoprotective potential in HFD-fed rats, effectively ameliorating the lipid profile, glucose metabolism, liver enzyme activity, and body weight. Collectively, these findings highlight the considerable promise of multi-strain probiotic yogurt as a preventive and early-stage therapeutic functional food intervention for MASLD, offering an accessible, palatable and scientifically grounded functional food approach to liver health management in the context of metabolic disease.

Keywords: Probiotics; Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD); Lipid Profile; Liver Histology; Glucose Metabolism; High-Fat Diet (HFD); Gut Microbiota.

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1 INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) has emerged as the most prevalent chronic liver disorder worldwide, affecting approximately one quarter of the global population (Younossi *et al.*, 2018). The clinical spectrum of this pathology encompasses a broad continuum of hepatic pathology, ranging from simple steatosis — characterized by the aberrant accumulation of triglycerides within hepatocytes — to the more advanced and clinically significant entity of metabolic dysfunction-associated steatohepatitis (MASH), which involves inflammation, hepatocellular injury, and progressive fibrosis (Buzzetti *et al.*, 2016). In its advanced stages, MASLD may culminate in the development of cirrhosis and hepatocellular carcinoma, imposing a substantial burden on global healthcare systems and underscoring the urgent imperative for effective preventive and therapeutic strategies (Khomich *et al.*, 2019).

The gut–liver axis has gained increasing attention as a central mechanism in MASLD pathogenesis. Alterations in gut microbiota composition and function can disrupt intestinal barrier integrity, promote endotoxin translocation, and trigger hepatic inflammation. These processes exacerbate

lipid accumulation and oxidative stress in the liver, highlighting the importance of microbial modulation as a potential intervention point (Rusman *et al.*, 2025; Zhou *et al.*, 2026).

Among the broad spectrum of functional foods, fermented dairy products have attracted considerable scientific interest for their potential to improve metabolic health outcomes, principally through the enhancement of insulin sensitivity, the support of body weight regulation, and the beneficial modulation of gut microbiota composition (Mackowiak, 2013; Montemayor *et al.*, 2023). Yogurt, one of the most widely consumed fermented milk product, is traditionally produced through defined starter cultures comprising *Streptococcus Thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* (Illikoud *et al.*, 2022). However, these conventional starter cultures exhibit limited tolerance to the physicochemical conditions prevailing in the gastrointestinal tract — including low gastric pH and the detergent activity of bile salts — which significantly restricts their probiotic functionality and survival through the gut (Verma *et al.*, 2022).

Probiotics, formally defined as live microorganisms that confer health benefits upon the host when administered in adequate quantities, represent a well-established and clinically relevant intervention in this context. Probiotic species belonging to the genera *Lactobacillus* and *Bifidobacterium* have demonstrated the capacity to restore intestinal microbial homeostasis, reduce hepatic lipid deposition, and suppress pro-inflammatory signaling pathways implicated in MASLD progression. The incorporation of such strains into fermented dairy products, particularly yogurt offers an accessible, palatable, and cost-effective vehicle for probiotic delivery at the population level (Montemayor *et al.*, 2023).

Notwithstanding these promising findings, the majority of existing studies in this domain have focused on isolated probiotic strains or commercially standardized probiotic supplements, administered in non-food formats. Relatively few investigations have examined customized multi-strain probiotic yogurt designed to simultaneously satisfy the dual requirements of technological feasibility and demonstrable biological efficacy. This scientific gap is of particular relevance given the strain-specific nature of probiotic functionality, the critical importance of ensuring adequate microbial survivability throughout gastrointestinal transit, and the need to validate the sensory and physicochemical acceptability of any novel formulation intended for broad consumer uptake. Bridging this gap represents a meaningful opportunity to integrate food science innovation with the principles of medical nutrition therapy, thereby offering a practical, evidence-based dietary strategy the prevention and early management of MASLD.

The present study therefore aimed to develop a stirred yogurt formulation fortified with a targeted mixture of robust, dairy-origin probiotic strains — selected on the basis of their technological suitability and gastrointestinal resilience — and to systematically evaluate its functional, sensory, and hepatoprotective properties in a high-fat diet (HFD)-induced rat model of MASLD. By integrating biologically active probiotic mixture a widely consumed food matrix, this research seeks to provide a scientifically grounded, cost-effective, and sustainable approach to the modulation of the gut–liver axis and the mitigation of MASLD progression.

2 Materials and Methods

2.1 Materials

2.1.1 Bacterial Strains

The Probiotics strains employed in this study comprised *lactobacillus rhamnosus* (LGG), *Lactobacillus plantarum* (Lp-115), *and lactobacillus acidophilus* (La-5) and *Bifidobacterium lactis* (BL-04), *Lactobacillus salivarius* (Ls-33), *Bifidobacterium breve* (Bb-03), *Bifidobacterium longum* (Bl-05), *lactobacillus casei* (Lc-11), *Lactobacillus fermentum* (Lf-10) *lactobacillus reuteri* (Lr-10) and *Lactobacillus paracasei* (Lp-33). A commercial yogurt starter culture (YOFLEX®), comprising *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, was additionally employed. All bacterial strains were procured from Chr. Hansen; Denmark (Hørsholm, Denmark) and stored at -18 °C until required for inoculation.

2.1.2 Media and Media Constituents

De Mann, Rogosa, and Sharpe (MRS) broth, M17 broth, Staphylococcus Medium 110 (Staph 110), and Violet Red Bile Agar (VRBA) were obtained from Bio Life (Milan, Italy). Plat Count Agar (PCA) and Rose Bengal Chloramphenicol Agar media were obtained from Oxoid (Basingstoke, England).

2.2 Methods

2.2.1 Preparation of Probiotics Culture

All probiotic cultures were prepared in accordance with the recommendations of the respective culture collections. The lyophilized pellet of each strain was activated in 10 mL of sterilized MRS broth and incubated at 37 °C for 24 hours. The activated cultures were subsequently purified by plating onto MRS agar and incubating at the strain-recommended temperature until discrete colonies appeared, typically within 48 hours. A single representative colony from each plate was transferred to 10 mL of fresh MRS broth and incubated at the recommended temperature for a further 24 hours. Culture purity was verified by catalase testing and

microscopic examination of Gram-stained preparations. Confirmed pure cultures (1 mL) were transferred into sterile 1.5 mL microcentrifuge tubes containing 200 μL of sterile glycerol (20% v/v) as a cryoprotectant and stored at -18 °C. Prior to experimental use, all strains were subcultured twice in MRS broth (10 % v/v) and incubated aerobically for 24 hours at either 37 °C or 28 °C, according to the specific growth requirements of each strain.

2.2.2 Assessment of Potential Probiotic Strains

Acid Resistance

Overnight cultures were prepared by inoculating each strain into MRS broth (0.1% v/v) at 37 °C for 16 hours to achieve mid-logarithmic phase growth. MRS broth was subsequently acidified using hydrochloric acid (HCl) to yield media at pH values of 2, 3, and 4. Bacterial cultures were then inoculated into the acidified media at a ratio of 10% (v/v) and the resulting mixtures were incubated at 37 °C for 6 hours. Bacterial growth was monitored at hourly intervals by measuring optical density at 650 nm (OD_{650}) utilizing a UV/VIS spectrophotometer (Apel-pD-303UV, Japan). The growth of each strain in acidified MRS broth was compared with that recorded in standard MRS broth (pH 6.6) as the control, in accordance with the method described by Baccigalupi *et al.* (2005).

Bile Salts Resistance

The tolerance of each probiotic strain to bile salts was assessed by evaluating growth kinetics in MRS broth supplemented with varying concentrations of bile salts (Bio Life, Milano, Italy) at 0%, 0.2%, 0.3%, and 0.4% (w/v). Overnight-grown cultures were inoculated into the supplemented media and incubated at 37 °C for 6 hours, with bacterial growth monitored by optical density measurements at 650 nm (OD_{650}) at regular intervals, in accordance with the procedure described by Baccigalupi *et al.* (2005). The comparative growth performance of each strain under bile salt stress was assessed relative to the unsupplemented MRS broth control (0% bile salts).

Phenol Tolerance

The capacity of each probiotic strain to grow in the presence of phenol was evaluated according to the method described by Aswathy *et al.* (2008). Twenty-four-hour MRS broth cultures of each strain were adjusted to an initial optical density of 0.8 at 600 nm. The standardized cultures were subsequently inoculated at 1% (v/v) into MRS broth supplemented with phenol at concentrations of 0%, 0.2%, and 0.4% (w/v) and incubated at 37 °C for 24 hours. Growth was assessed by measuring the absorbance at 600 nm at the end of the incubation period, with results compared to the unsupplemented control.

2.2.3 Manufacture of Probiotic Stirred Yogurt

Bovine milk was standardized utilizing skimmed milk powder (SMP) to a solid non-fat (SNF) content of 13% (w/w). The standardized milk was heat-treated at 85 °C for 15 minutes, cooled to 42 ± 1 °C, and divided into six equal portions. Each portion was inoculated with the designated probiotic strain(s) at a concentration of 0.01 g/kg as follows: *Lactobacillus rhamnosus* (T1), *Lactobacillus plantarum* (T2), *Lactobacillus acidophilus* (T3), *Bifidobacterium lactis* (T4), and a four-strain composite mixture in equal proportions (1:1:1:1) (T5). One hour following probiotic inoculation, all portions — including the unsupplemented control (T0) — were inoculated with the commercial yoghurt starter culture (YOFLEX®) et 0.03 g/kg. Each probiotic inoculum was standardized to approximately 1×10^8 CFU/mL prior to inoculation, as verified by serial dilution and viable plate counting on MRS agar. Fermentation was monitored throughout by recording both pH and titratable acidity (expressed as % lactic acid). Incubation was conducted at 42 ± 1 °C and terminated upon attainment of a target pH of 4.6 ± 0.1, typically achieved within 4 – 6 hours depending on inoculum activity. Following fermentation, all samples were transferred into 100 mL coded plastic containers, cooled, and stored at 4 °C until further analysis.

2.2.4 Sensory Evaluation

The organoleptic properties of the freshly prepared probiotic stirred yogurt samples were evaluated by a trained consumer panel of 20 assessors (11 men and 9 women) recruited from the Department of Food Science, Faculty of Agriculture (Saba Basha), Alexandria University, Egypt, in accordance with the procedure described by Darwish *et al.*, 2021). The six treatment groups assessed were: T0 (commercial starter culture), T1 (*Lactobacillus rhamnosus*), T2 (*Lactobacillus plantarum*), T3 (*Lactobacillus acidophilus*), T4 (*Bifidobacterium lactis*), and T5 (four-strain probiotic mixture with starter culture).

Panelists were selected on the basis of their familiarity with fermented dairy products and received a standardized orientation on the utilization of the Hedonic scale prior to commencement of the evaluation session. All assessments were conducted under standardized white fluorescent lighting in a quiet sensory evaluation room to minimize extraneous sensory influences. Samples were presented in identical coded opaque containers to avoid visual bias. Samples stored at 4 °C were equilibrated to ambient temperature (25 °C) for 10 minutes prior to presentation. Panelists rinsed their mouths with still water between successive samples to minimize carry-over effects.

Each panelist independently assessed all samples through a seven-point Hedonic scale (1 = dislike extremely; 7 = like

extremely) for the following sensory attributes: color, taste, flavor, odor, texture, appearance, acidity, and overall acceptability. Scores from the 20 panelists were treated as independent observations (n = 20 per treatment group). Data were analyzed employing one-way analysis of variance ANOVA followed by Duncan's multiple range test for post hoc mean comparisons, with statistical significance set at $p < 0.05$.

2.2.5 Animal Experiment

Experimental Diets

The standard commercial chow diet employed as the basal diet for control animals was obtained from the Department of Home Economics, Faculty of Agriculture Alexandria University, Egypt. The standard diet comprised the following constituents per 100 g: soybeans (11.50 g), maize (5.00 g), starch (43.50 g), sucrose (30.00 g), mineral salt mixture (5.00 g.), cellulose (1.00 g), and vitamin mixture (1.00 g), providing approximately 300 kcal/100 g. A high-fat diet (HFD) was formulated by supplementing the standard chow with 35% (w/w) palm oil, thereby increasing the total fat content from 5.5% to 40% of the diet and raising the gross energy content to approximately 414 kcal/100 g. Forty-eight male albino Wistar rats (4 weeks of age; mean body weight: 68 ± 1.5 g) were procured from the Institute of Graduate Studies and Research, Alexandria University, Egypt.

Experimental Design

Male albino Wistar rats were selected for this study on the basis of their genetic homogeneity and well-documented physiological characteristics which render them a widely validated animal model for metabolic disease research. Animals were randomly allocated into eight experimental groups of six rats each (n = 6 per group) and housed in standard polycarbonate cages under controlled environmental conditions (temperature: 22 ± 2 °C, 12-hour light/dark cycle, relative humidity: 50–60%), with unrestricted access to food and water throughout the experimental period. Cage bedding was changed at regular intervals to maintain hygiene standards. Each animal constituted an independent biological replicate, and all data were analyzed at the individual animal level rather than as pooled group values. For all biochemical assays, measurements were performed in technical duplicate and the mean of the two replicates was utilized for statistical analysis.

During an initial two-week pre-feeding phase, all groups were maintained on the standard chow diet whilst receiving their respective treatments by daily oral gavage as follows:

- *Group I (Negative Control)*: Standard chow diet + distilled water
- *Group II (Positive Control)*: High-fat diet (HFD) + distilled water
- *Group III*: HFD + control yogurt (T0)
- *Group IV*: HFD + T1 yogurt (*L. rhamnosus*)
- *Group V*: HFD + T2 yogurt (*L. plantarum*)
- *Group VI*: HFD + T3 yogurt (*L. acidophilus*)
- *Group VII*: HFD + T4 yogurt (*B. lactis*)
- *Group VIII*: HFD + T5 yogurt (four-strain probiotic mixture in equal proportions)

The two-week pre-feeding period was incorporated into the study design to allow stabilization of the intestinal microbiota and establishment of probiotic colonization prior the induction of metabolic stress by HFD introduction. Pre-conditioning with probiotics prior to dietary fat challenge has been demonstrated to enhance their protective effects against subsequent metabolic perturbation. Following the pre-feeding phase, Groups II–VIII were transitioned from standard chow to HFD and continued receiving their respective probiotic yogurt treatments throughout the induction and treatment phases. Group I remained on standard chow for the entirety of the experimental period as the negative control.

Body weight was recorded weekly for all animals throughout the study. Blood samples were collected at two-week intervals via the medial canthus of the eye employing microhematocrit capillary tubes during an 8-hour fasting period. At the conclusion of the 12-week experimental period, animals were fasted for 8 hours, anesthetized, and euthanized by cervical dislocation. Terminal blood samples were collected for biochemical analysis. The liver, kidneys, and spleen were excised, rinsed with ice-cold physiological saline, blotted dry, and weighed individually to determine relative organ-to-body weight ratios. Segments of the small intestine were aseptically collected for microbiological analysis, and liver specimens were immediately fixed in 10% neutral-buffered formalin for histological examination.

2.2.6 Biochemical Analysis of Blood Samples

Serum was obtained by allowing blood samples to clot spontaneously in dry glass centrifuge tubes at 24 ± 3°C, followed by centrifugation at 1,400 x g for 20 minutes. The resultant clear, non-hemolyzed supernatant serum was resultant using a clean, dry disposable plastic syringe and stored at −80 °C until biochemical analysis. Serum lipid profile parameters — including triglycerides (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) — were determined using commercially available enzymatic colorimetric assay kits (SRTA City, New Borg El Arab, Egypt). Serum low-density lipoprotein cholesterol (LDL-C) concentration was calculated through the Friedewald equation: LDL-C (mg/dL) = TC − HDL-C − (TG/5).

Hepatic function was assessed by measuring serum alanine aminotransferase (ALT) activity and albumin concentration. Metabolic function was evaluated through the determination of fasting serum glucose and insulin concentrations. All biochemical analyses were performed in strict accordance with the manufacturer's instructions for each respective assay kit.

2.2.7 Microbiological Analysis of the Small Intestine

The small intestinal contents of rats from groups I, II, and VIII were collected by flushing the intestinal lumen with 20 mL of sterile physiological saline utilizing a sterile syringe into a sterile collection flask. Serial ten-fold dilutions were prepared immediately thereafter, and samples were processed using the aseptic serial dilution technique described by Klaver *et al.* (1993). Total aerobic mesophilic flora was enumerated by the conventional pour plate method on PCA, with results expressed as colony-forming units (CFU g^{-1}). Total coliforms and staphylococci were enumerated on Violet Red Bile Agar (VRBA) in accordance with Marshall (Marshall, 1993) and on Staphylococcus Medium 110 in accordance with Khan *et al.*, (2008), respectively.

2.2.8 Histopathological Analysis

Fixed liver specimens were subjected to standard histological processing, comprising sequential dehydration through ascending concentrations of ethanol, clearing in xylene, and embedding in paraffin wax. Tissue sections of 3-5 μm thickness were cut using a rotary microtome and stained with haematoxylin and eosin (H&E) according to following the method of Bancroft and Gamble (2007). Stained sections were examined under light microscopy by a qualified histopathologist for the assessment of hepatic steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis.

2.2.9 Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics software (Version 23.0; IBM Corp., Armonk, NY, USA, 2015). Data were analyzed by one-way analysis of variance (ANOVA), and statistically significant differences among group means were identified using Duncan's multiple range test as a post hoc procedure. All results were expressed as mean ± standard deviation (SD) of two independent replicates. Statistical significance was set at $p < 0.05$.

3 Results

3.1 Probiotic Potential of the Screened Lactic Acid Bacterial Strains

The functional efficacy of probiotics microorganisms is fundamentally contingent upon their capacity to remain viable and metabolically active throughout transit across the lower gastrointestinal tract. To fulfil this requirement, probiotic strains must withstand the harsh physicochemical conditions encountered in the upper digestive tract, most notably the highly acidic gastric environments and the detergent activity of bile salts in the small intestine. Several probiotic organisms, however, are unable to survive these physiological challenges. Consequently, resistance to gastric acid and tolerance to intestinal bile salts constitutes the primary and most critical criteria for the selection of functionally effective probiotic strains. Following the systematic screening of eleven lactic acid bacterial strains against acid, bile salt, and phenol stress conditions, four strains — *Lactobacillus rhamnosus* (LGG), *Lactobacillus plantarum* (Lp-115), *Lactobacillus acidophilus* (La-5), and *Bifidobacterium lactis* (BL-04) — demonstrated superior and consistent tolerance across all three stress parameters (Table 1). These strains were selected for subsequent experimental use on the basis of their well-established probiotic properties and their demonstrated capacity to survive and function under simulated rat gastrointestinal conditions, and were accordingly incorporated into the probiotic stirred yoghurt formulations.

3.2 Sensory Attributes of Probiotic-Enriched Yogurt

The results of the sensory evaluation of all probiotic yoghurt samples and the unsupplemented control are presented in Figure 1. The data indicate that the incorporation of probiotic strains — *Lactobacillus rhamnosus* LGG, *Lactobacillus plantarum* Lp-115, *Lactobacillus acidophilus* La-5, and *Bifidobacterium lactis* BL-04 — into the yogurt starter culture consistently enhanced the overall organoleptic quality of the resulting products relative to all other formulations examined. Among all treatment groups,

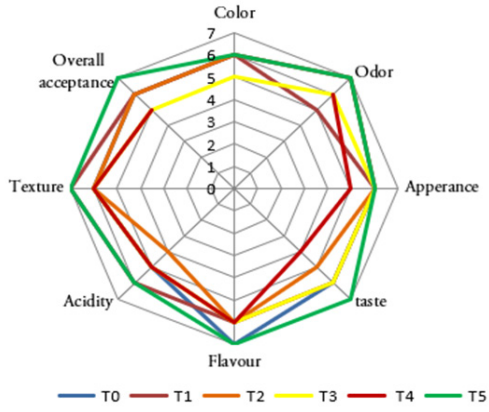

Figure 1. Sensory Evaluation of Probiotic-Enriched Yogurt

T5 — the four-strain composite probiotic mixture — received the highest mean scores across all assessed sensory attributes, with particularly notable superiority in taste and texture.

One-way ANOVA followed by Duncan's multiple range test ($p < 0.05$) confirmed the presence of statistically significant differences in sensory scores among the treatment groups, with T5 demonstrating significantly superior overall acceptability relative to both the unsupplemented control (T0) and all single-strain probiotic formulations. These findings collectively indicate that the incorporation of a multi-strain probiotic mixture not only maintained adequate microbial viability throughout yogurt production but also conferred measurable improvements in product organoleptic quality, suggesting a positive contribution of probiotic metabolic activity to the development of desirable sensory characteristics.

3.3 Effect of Probiotics Supplementation on Body Weight and Organ Mass

At the commencement of the experimental period, no statistically significant differences in body weight were observed among the experimental groups ($p > 0.05$) (Table 2). As the experiment progressed, rats in the probiotic-supplemented groups (Groups IV–VIII) exhibited significantly lower final body weights and reduced weight gain compared to the positive control group (Group II), which demonstrated pronounced increases in both parameters. Despite these improvements, body weights in the probiotic-supplemented groups remained comparable to those of the negative control group (Group I), indicating that probiotic yogurt supplementation did not induce excessive or pathological weight reduction.

Administration of the HFD to Group II resulted in marked increases in absolute organ weights relative to all other groups (Table 2). Specifically, liver, spleen, and kidney weights were elevated by approximately 85%, 59%, and 31%, respectively, compared with Group I. The administration of yogurt — particularly probiotic-enriched formulations — significantly attenuated these organ weight increases, yielding values considerably lower than those observed in Group II. Among all probiotic-supplemented groups, Group VIII — receiving the T5 multi-strain probiotic yoghurt — demonstrated the most pronounced hepatoprotective and organotropic effects, reducing liver, spleen, and kidney weights by approximately 40%, 24%, and 18%, respectively, relative to Group II. Liver weights in Group VIII approached those recorded in the negative control group (Group I), indicative of substantial protective effect against HFD-induced hepatomegaly.

3.4 Effect of Probiotic Supplementation on Lipid Profile, Hepatic Function, and Glucose Metabolism

As anticipated, the positive control group (Group II) exhibited a markedly impaired lipid profile compared with all other groups ($p < 0.05$) (Table 3). Total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) were elevated by approximately 2-, 3-, and 7-fold, respectively, relative to Group I, whilst high-density lipoprotein (HDL-C) was reduced by approximately 50%. Supplementation with plain yogurt (T0) in Group III did not produce significant improvements in these lipid parameters relative to Group II ($p > 0.05$), with TG, TC, and LDL-C remaining markedly elevated and HDL-C persistently reduced. In contrast, supplementation with probiotic-enriched yogurt in Groups IV–VIII significantly alleviated the negative impact of the HFD on lipid metabolism. In particular, the multi-strain probiotic yogurt (Group VIII) decreased TC, TG, and LDL-C by 40%, 47%, and 66%, respectively, whilst concurrently increasing HDL-C by 83% relative to the positive control group, reflecting a substantially improved and cardioprotective lipid profile.

With respect to hepatic function, HFD administration in Group II induced a significant increase in serum ALT activity and a reduction in plasma albumin concentration, with ALT activity rising by approximately 70% and albumin declining by approximately 7% relative to Group I (Table 3). Administration of plain yogurt (T0) in Group III partially mitigated hepatocyte damage, reducing ALT activity to 44.60 U/L. Probiotic yogurt supplementation further attenuated HFD-induced hepatic dysfunction across Groups IV–VIII. Plasma albumin concentrations in probiotic-treated Groups increased by approximately 4–5% relative to Group II, with no statistically significant differences observed among the probiotic formulations. ALT activity was additionally and significantly reduced in all probiotic-supplemented groups, with decreases ranging from 13–28% relative to Group II, and Group VIII exhibiting the most pronounced hepatoprotective effect among all treatments.

Regarding glycemic regulation, HFD administration significantly elevated both fasting plasma glucose and serum insulin concentrations in Group II, reaching 52.10 mg/dL and 2.17 μIU/mL, respectively, compared with 19.60 mg/dL (glucose) and 0.69 µIU/mL recorded in Group I (Table 3). Plain yogurt supplementation (Group III) did not produce statistically significant modifications in either glycemic parameter relative to the positive control ($p > 0.05$). In contrast, all probiotic yogurt treatments markedly reduced both fasting plasma glucose and serum insulin concentrations. Notably, rats in Group VIII — administered the T5 multi-strain probiotic yoghurt — exhibited the lowest

Table 1. Acid Tolerance, Bile Salts Tolerance, and Phenol Tolerance of the Probiotic Bacteria Strains

Probiotic Strains	Acid Tolerance (pH)			Bile Salts Tolerance (%)			Phenol Tolerance (%)	
	2	3	4	0.2	0.3	0.4	0.2	0.4
Lactobacillus acidophilus (La-05)	+	+	+	+	+	+	+	+
Bifidobacterium lactis (Bl-04)	-	+	+	+	+	+	+	+
Lactobacillus rhamnosus (LGG)	+	+	+	+	+	+	+	+
Lactobacillus plantarum (Lp-115)	+	+	+	+	+	+	+	+
Bifidobacterium longum (Bl-05)	-	+	+	+	+	-	+	+
Bifidobacterium breve (Bl-03)	-	-	-	+	-	-	-	-
Lactobacillus casei (Lc-11)	-	+	+	+	+	+	+	-
Lactobacillus salivarius (Ls-33)	-	-	+	+	+	+	+	+
Lactobacillus fermentum (Lf-10)	-	+	+	+	+	+	+	+
Lactobacillus reuteri (Lr-1)	+	+	+	+	+	-	+	-
Lactobacillus paracasei (Lp-33)	-	+	+	+	+	-	+	+

Note: Data are represented in means of (-) not detected.

Table 2. Effect of Probiotics on the Body and Organ Weight in Rats

Weight	Initial body weight (g)	Final body weight (g)	Mean Body weight gain (g)	Liver (%)	Kidney (%)	Spleen (%)
Group I	66.2± 1.9^{a}	214.0± 3.7^{a}	147.8±3.7^{a}	4.50±0.35^{a}	0.65±0.21^{a}	0.53±0.09^{a}
Group II	66.0± 2.0^{a}	276.0±4.5^{c}	209.8±4.5^{c}	8.30±0.89^{c}	0.85±0.38^{c}	1.03±0.12^{c}
Group III	66.3± 1.9^{a}	249.0± 2.9^{b}	182.7±2.9^{b}	6.40±0.44^{b}	0.79±0.13^{b}	0.84±0.06^{b}
Group IV	67.1± 1.7^{a}	238.0±4.7^{b}	170.9±4.7^{b}	5.80±0.39^{b}	0.74±0.24^{b}	0.71±0.08^{b}
Group V	68.3± 1.6^{a}	244.0±2.8^{b}	175.7±2.8^{b}	5.90±0.61^{b}	0.72±0.11^{b}	0.69±0.09^{b}
Group VI	67.7± 1.7^{a}	239.0±3.5^{b}	171.3±3.5^{b}	5.8± 0.54^{b}	0.71± 0.22^{b}	0.69± 0.06^{b}
Group VII	66.8± 1.9^{a}	239.0±4.1^{b}	172.2±4.1^{b}	5.9± 0.48^{b}	0.71± 0.31^{b}	0.67± 0.11^{b}
Group VIII	66.7±1.9^{a}	231.0± 3.1^{b}	164.3± 3.1^{b}	4.9± 0.58^{a}	0.70± 0.19^{b}	0.64± 0.09^{b}

Note: Data are presented as mean ± SD; Means with different lowercase letters in the same column are significantly different (p<0.05).

Table 3. Effect of Probiotics on the Lipid Profile, Liver Function and Glucose Metabolism in Rats

Groups	TC (mg/dL)	TG (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	ALT (U/L)	Albumin (mg/dL)	Glucose (mg/dL)	Insulin (µIU/mL)
Group I	70.7±3.9^{a}	88.4±6.9^{a}	42.2±7.3^{a}	10.5±3.6^{a}	30.2± 2.1^{a}	3.37± 0.17^{a}	19.6± 2.1^{a}	0.69± 0.11^{a}
Group II	141.8±2.1^{c}	231.1±7.2^{c}	19.8±5.5^{c}	74.2±6.1^{c}	51.1±4.8^{c}	3±0.23^{b}	52.10±3.00^{c}	2.17±0.19^{c}
Group III	114.6±3.3^{b}	202.8±7.7^{b}	22.8±3.1^{b}	49.5±4.9^{b}	44.6±3.9^{b}	3.03±0.22^{b}	41.80±3.40^{b}	1.45±0.08^{b}
Group IV	92.4±2.9^{b}	170.7±7.1^{b}	31.5±4.4^{b}	27.9± 4.3^{b}	41± 2.7^{b}	3.24±0.23^{b}	37.00±2.40^{b}	1.09±0.14^{b}
Group V	95.1±4.3^{b}	180.3±6.4^{b}	30.6±5.3^{b}	28.1±5.4^{b}	43.2± 3.3^{b}	3.22±0.22^{b}	36.20±1.90^{b}	1.14±0.16^{b}
Group VI	94.8±3.5^{b}	171.8±7.5^{b}	32.9±7.9^{b}	29.3±2.1^{b}	42.4± 4.2^{b}	3.23±0.19^{b}	31.10±2.80^{b}	1.04±0.10^{b}
Group VII	96.3±2.5^{b}	176.7±6.8^{b}	32.1±4.1^{b}	29.2±3.9^{b}	43.1±2.7^{b}	3.21±0.2^{b}	32.40±3.70^{b}	1.22±0.17^{b}
Group VIII	85.1±2.8^{b}	122.3±5.9^{b}	36.1±5.2^{b}	25.8±2.6^{b}	36.9±2.9^{b}	3.29±0.21^{b}	28.60±2.60^{b}	0.84±0.09^{b}

Note: Data are presented as mean ± SD; Means with different lowercase letters in the same column are significantly different (p<0.05). (TC) Cholesterol; (TG) Triglyceride; (HDL) High Density Lipoprotein; (LDL) Low Density Lipoprotein; (ALT) Alanine Aminotransferase.

recorded fasting plasma glucose (28.60 mg/dL) and serum insulin (0.84 µIU/mL) concentrations among all treatment groups, reflecting a substantial improvement in metabolic regulation.

3.5 Effect of Probiotic Supplementation on The Intestinal Microbiota

The intestinal microbiological findings for the three groups subjected to microbiological analysis are presented in Table 4. Rats maintained on the HFD (Group II) exhibited elevated counts of total coliforms and staphylococci, alongside a reduction in overall viable bacterial count, compared with rats receiving the standard chow diet (Group I). In contrast, supplementation with the T5 multi-strain probiotic yogurt in Group VIII resulted in markedly lower enumeration of both total coliforms and staphylococci, accompanied by a significantly higher total viable bacterial count relative to Group II.

Table 4. Effect of Probiotics on the Microbial Count of Rat's Intestine

Microbial Count	Total count (CFU/mL)	Coliform (CFU/mL)	Staphylococci (CFU/mL)
Group I	5.3×10^{7}	1.5×10^{6}	2.4×10^{8}
Group II	4.1×10^{7}	3.9×10^{6}	8.1×10^{8}
Group III	5.0×10^{7}	2.6×10^{6}	5.9×10^{8}
Group VIII	6.3×10^{7}	1.8×10^{6}	3.8×10^{8}

3.6 Effect of Probiotic Supplementation on Hepatic Histopathology

Histopathological examination of liver sections from Group I revealed normal hepatic microarchitecture, characterized by well-organized hepatocyte cords with healthy, structurally intact hepatic cells (black arrows) and a clearly defined central vein (red arrows) (Figure 2A). In marked contrast, chronic HFD administration in Group II resulted in profound disruption of normal hepatic architecture. Liver sections from this group exhibited hepatocytes with rounded and pyknotic nuclei (black arrows), focal areas of hepatic necrosis with mononuclear cell infiltration (blue arrows), and extensive centrilobular degeneration with congested and dilated portal tracts and hemorrhagic regions (red arrows) **(**Figure 2B**)**.

The addition of plain yogurt (T0) to the HFD in Group III marginally attenuated the hepatic histopathological changes induced by the HFD (Figure 2C). Whilst Group III liver sections continued to exhibit disruption of normal hepatocyte architecture, the severity of pathological changes was reduced relative to Group II, with fewer rounded and pyknotic nuclei (black arrows), reduced foci of hepatic necrosis (blue arrows), and less extensive degeneration (red arrows).

The hepatoprotective effects of probiotic supplementation were evident across Groups IV–VIII and exhibited a strain-dependent pattern. In Group V, supplementation with *Lactobacillus plantarum* was associated with predominantly normal hepatocytes (black arrows), with only a slight degenerated area (blue arrows) and a minor hemorrhagic region, along with a precise central vein (red arrows) (Figure 2E). Meanwhile, *Lactobacillus rhamnosus* in Group IV (Figure 2D), *Lactobacillus acidophilus* in Group VI (Figure 2F), *Bifidobacterium lactis* in Group VII (Figure 2G), and the mixed probiotic yogurt in Group VIII (Figure 2H) resulted in regular hepatocyte cords with healthy hepatic cells and active dilated sinusoids (black arrows) and clear central veins (red arrows).

4 Discussion

The present study demonstrates that stirred yogurt fortified with a multi-strain probiotic culture exerted a pronounced protective influence against MASLD in rats subjected to HFD induction. The composite probiotic blend — comprising *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *Bifidobacterium lactis* — conferred broad improvements across multiple indices of metabolic health. In comparison with single-strain probiotic formulations, the multi-strain mixture produced a wider spectrum of beneficial effects, consistent with the established principle that probiotic combinations may act synergistically within the gut ecosystem to produce effects that exceed those achievable by individual constituent strains administered in isolation (Yoo *et al.*, 2013). Importantly, the findings of the present study extend this existing body of literature by demonstrating that such synergetic effects were most pronounced in lipid regulation and hepatic protection — precisely those parameters in which single-strain preparations demonstrated comparatively limited efficacy.

Whilst the lipid profile alterations observed in the HFD-fed positive control group — specifically the elevation of serum TG, TC, and LDL-C — are consistent with the findings of Alnami *et al.* (2022) and Wang *et al.* (2004), the magnitude of improvement in lipid parameters achieved with the multi-strain probiotic yogurt in the present study exceeded that reported for individual probiotic strains in comparable animal models. This discrepancy may reflect differences in the probiotic delivery matrices employed, suggesting that fermented dairy matrices such as yogurt may potentiate probiotic bioavailability and functional efficacy relative to non-food delivery formats such as capsules or powders. Such observations highlight the importance of considering not only the selection of probiotic strains but also the formulation context and delivery vehicle when designing and evaluating probiotic interventions.

The alterations in serum lipid parameters observed in HFD-fed animals were accompanied by histological evidence of hepatocellular lipid deposition, indicative of varying degrees of hepatic steatosis — a hallmark pathological feature of MASLD progression. Sustained HFD administration is well established in the literature as a driver of intestinal microbial dysbiosis, increased intestinal epithelial permeability, and enhance translocation of microbial endotoxins — most notably lipopolysaccharides (LPS) — into the portal and systemic circulation, thereby promoting chronic low-grade systemic inflammation and progressive metabolic imbalance (Chakaroun *et al.*, 2020; Ojeda *et al.*, 2016). These interconnected pathophysiological processes collectively impair hepatic lipid processing, promote triglyceride accumulation within hepatocytes, and

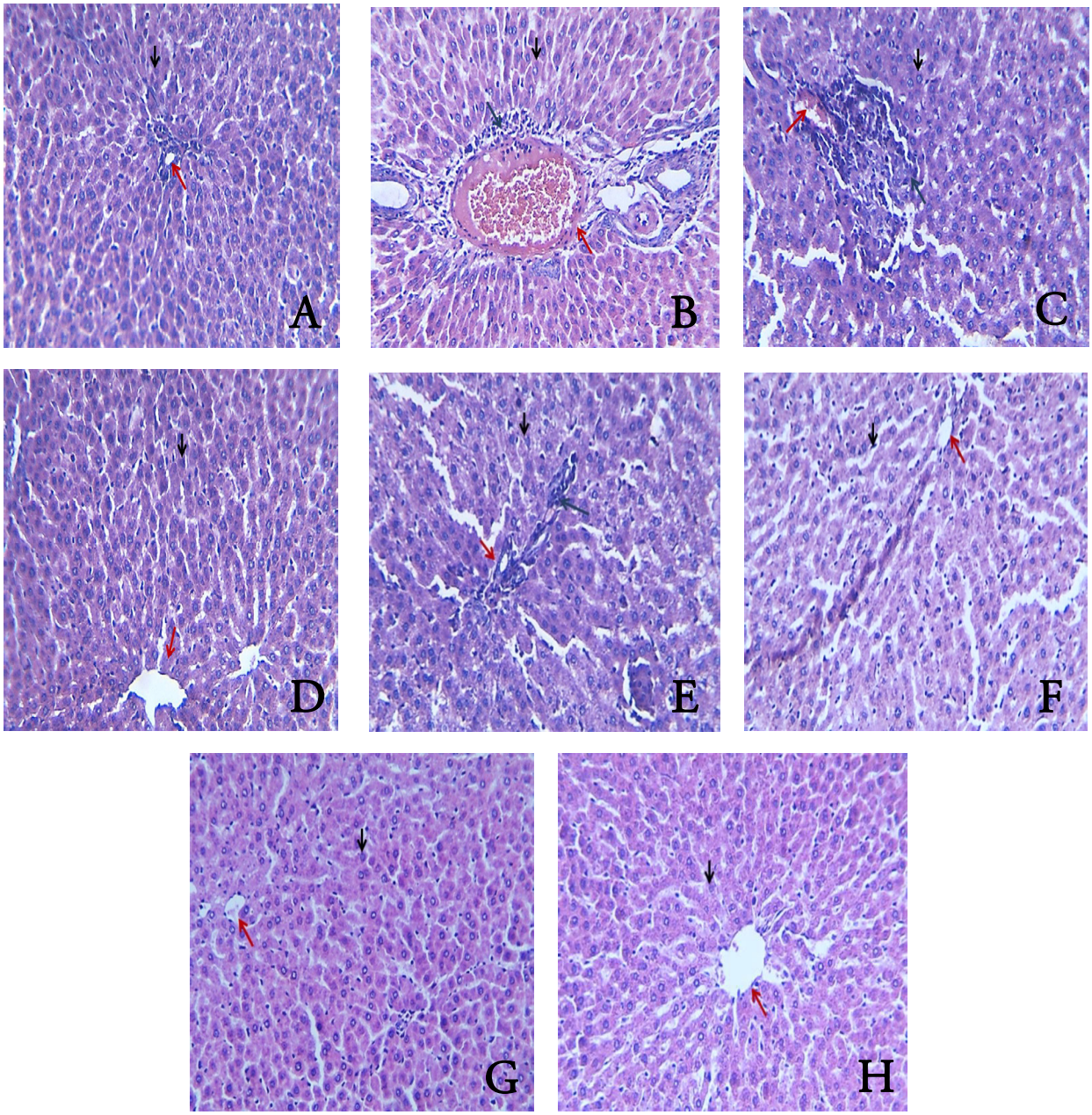

Figure 2. Effect of Probiotic Administration on Hepatic Histology

compromise intestinal barrier structural integrity, resulting in hepatic parenchymal inflammation (Briskey *et al.*, 2016; Jiang *et al.*, 2015). Prolonged exposure to high dietary fat intake therefore drives the development of obesity and gut microbiota-mediated inflammation , both of which are recognized as central and interrelated drivers in the pathogenesis and progression of MASLD (Laugerette *et al.*, 2020).

Histopathological examination confirmed reduced steatosis in probiotic-treated rats, consistent with the observations of Rishi *et al.* (2017). However, in contrast to certain prior investigations that reported only modest improvements in hepatic morphology following probiotic intervention, the multi-strain formulation employed in the present study achieved a near-complete normalization of liver weights to values approaching those of the negative control group. This finding suggests that probiotic synergy within a multi-strain formulation may confer stronger hepatoprotective effects than those previously recognized for

individual strains, and warrants further investigation across diverse animal models and ultimately in human clinical trials.

Consistent with the observations of Lee *et al.* (2020) and Hussain *et al.* (2021), rats maintained on an HFDs exhibited increased serum TG and TC concentrations, particularly in Group II, which also demonstrated reduced HDL-C and elevated LDL-C levels. Continuous exposure to HFD is regimen is well documented to promote lipid metabolic dysregulation and progressive hepatic lipid accumulation (Briseño-Bass *et al.*, 2019; DeFilippis *et al.*, 2013). In contrast, probiotic supplementation in Group VIII produced a substantial and statistically significant improvement in serum lipid profiles, characterized by reductions in TG and TC and restoration of a physiologically favorable HDL-C/LDL-C ratio. Whilst mechanistic pathways including bile salt de-conjugation and LDL receptor modulations have been proposed as potential explanatory mechanisms in the broader probiotic literature (Begley *et al.*, 2006; Song *et al.*, 2015), these specific molecular pathways were not directly assessed in the present study. The reported findings are therefore interpreted as consistent with, rather than confirmatory of, these proposed mechanisms, and cautious attribution of specific molecular pathways is warranted in the absence of direct mechanistic measurement.

With respect to hepatic function, the reductions in serum ALT and improvements in plasma albumin levels observed in probiotic-treated groups align with prior reports by Adesiji *et al.* (2019) and Kawaguchi *et al.* (2021); however, the degree of hepatic functional recovery observed in the present study was greater than that reported in several earlier investigations. This observation raises the possibility that the incorporation of a pre-feeding stabilization phase — designed to allow establishment of probiotic colonization and gut microbiota stabilization prior to HFD introduction — may have enhanced the overall hepatoprotective efficacy of the probiotic intervention. Such methodological distinctions should be carefully considered when undertaking cross-study comparisons. The enhanced albumin recovery in probiotic-treated animals is indicative of improved hepatic protein synthetic capacity and partial restoration of hepatocellular function (Ayyat *et al.*, 2018), Variability in outcomes across published studies in this area may be attributable to differences in probiotic strain composition, treatment duration, dosing regimens, and inter-individual variation in host metabolic responses (Alkhalf *et al.*, 2010).

Fasting blood glucose and serum insulin concentrations — both recognized as clinically significant indicators of MASLD-associated metabolic dysfunction — also reflected the differential effects of dietary and probiotic interventions. Consistent with the earlier observations of Helal *et al.* (2012) and Cho *et al.* (2014), HFD-fed rats in Group II demonstrated markedly elevated fasting blood glucose concentrations relative to the negative control. Conversely, administration of the multi-strain probiotic yogurt resulted in a significant decline in both blood glucose and insulin concentrations. These improvements may be attributable to enhanced insulin sensitivity, improved hepatic glucose uptake and glycogen storage, or modulation of incretin signalling pathways, as proposed in prior studies (Andreasen *et al.*, 2010; Duan *et al.*, 2015). It should be noted, however, that these specific mechanisms were not directly evaluated in the present investigation, and further experimental work — including assessment of pancreatic β-cell activity and hepatic glucose metabolism — will be required to elucidate the precise pathways through which multi-strain probiotic supplementation influences glycemic regulation in this model.

In summation, chronic HFD administration in the present study resulted in the progressive development of a metabolic phenotype consistent with MASLD, characterized by significant increases in liver mass, serum lipid parameters, fasting blood glucose concentrations, insulin resistance indices, and overall body weight. In contrast, supplementation with the multi-strain probiotic yogurt formulation alleviated these metabolic disturbances, restoring physiological balance. findings are consistent with the observations of Bloemendaal *et al.* (2021) and Liu *et al.* (2022), who demonstrated that probiotic supplementation facilitates the re-establishment of gut microbial homeostasis disrupted by high-fat feeding. Collectively, the present findings indicate that a combination of *Lactobacillus* and *Bifidobacterium* strains can reinforce gut–liver axis integrity, diminish hepatic lipid accumulation, and suppressing pro-inflammatory signaling pathways implicated in MASLD pathogenesis, consistent with the broader body of evidence supporting the therapeutic potential of probiotic formulations in the management of metabolic liver disease (Ashaolu and Fernandez-Tome, 2021; da Silva *et al.*, 2021; Mazloom *et al.*, 2019).

Several limitations of the present study must be acknowledged. First, the specific molecular mechanisms underlying the observed metabolic improvements — including bile salt de-conjugation, LDL receptor expression modulation, and insulin signaling pathways — were not directly measured. Second, the exclusive utilization of single rodent animal model limits generalizability of the findings across species and physiological contexts. Third, the inflammatory mediators and cytokine profile of the experimental animals were not assessed, representing an important gap in the mechanistic characterization of the probiotic intervention. Fourth, with respect to sensory evaluation, the relatively limited panel size (n = 20) constraints the strength of conclusions that can be drawn

regarding broader consumer acceptance and preference. Future studies should incorporate larger sensory panels, extended evaluation periods, diverse animal models, and ultimately rigorously designed human clinical trials to address these methodological limitations and to clarify the precise mechanisms by which multi-strain probiotic yoghurt formulations exert their hepatoprotective and metabolic regulatory effects.

5 Conclusions

The present study provides evidence that supplementation with a multi-strain probiotic yogurt formulation — comprising *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *Bifidobacterium lactis* — exert protective effects against hepatic lipid accumulation and metabolic dysregulation in rats subjected to high-fat diet induction, through mechanisms consistent with the modulation of gut–liver axis integrity and the restoration of intestinal microbial homeostasis. In animals exposed to chronic HFD administration, the probiotic intervention was associated with statistically significant and clinically meaningful improvements across a broad spectrum of biochemical and physiological parameters, including serum lipid fractions, fasting serum glucose and insulin concentrations, liver mass, and overall body weight, with values in the multi-strain treatment group approaching those recorded in the negative control group. Probiotic supplementation additionally contributed to favorable remodeling of the intestinal microbiota, characterized by significant suppression of pathogenic microorganisms — including total coliforms and *Staphylococcus aureus* — and enhancement of total viable bacterial counts

Collectively, these findings indicate that multi-strain probiotic supplementation holds considerable potential as a cost-effective, accessible, and palatable functional food intervention for the prevention and early management of MASLD, operating through the dual mechanisms of gut microbiota modulation and attenuation of hepatic metabolic dysfunction. However, given the exploratory nature of the present investigation and its restriction to a rodent animal model, the findings must be interpreted with appropriate caution. Validation through rigorously designed prospective clinical trials in human populations, incorporating mechanistic biomarker assessment and longer-term follow-up, is required before definitive conclusions regarding clinical applicability can be drawn.

Ethics Statement

All experimental procedures involving animals were conducted in strict accordance with applicable guidelines and regulatory requirements for the ethical treatment of laboratory animals. The study protocol received formal ethical approval from the Institutional Animal Care and Use Committee of Alexandria University, Egypt (ALEXU-IACUC), under protocol number AU: 19/22/10/20/3/24.

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