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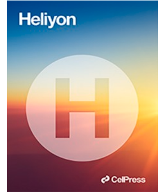

Research article

Exploring the therapeutic potential of silymarin-based herbal remedy (prebiotic) and probiotic blend in a mouse model of NAFLD: Insights into gut microbiota modulation and liver health

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a significant consequence of metabolic dysfunction, often associated with changes in the intestinal microbiota. Prebiotics and probiotics have shown promise in NAFLD management. This study evaluated a silymarin-based herbal remedy with piperine and fulvic acid, alongside a probiotic blend of *Bifidobacterium adolescentis*, *Bifidobacterium bifidum, Lactobacillus casei*, and *Lactobacillus rhamnosus*. Using a NAFLD mouse model induced by a high-fat and high-fructose diet, we assessed biochemical parameters, liver function, glucose levels, and conducted histological analysis. Stool samples underwent 16S rRNA metagenomic analysis to explore changes in microbiota composition. Mice on the high-fat diet exhibited elevated lipids, liver enzymes, and glucose, with reduced high-density lipoprotein levels (with p value < 0.001). Treatment, particularly with F3 (silymarin-piperine-fulvic acid herbal remedy and probiotic blend), significantly reduced hepatic fat accumulation and improved gut microbiota composition. This study highlights the potential of silymarin-based therapy combined with probiotics in attenuating NAFLD progression.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome. The primary reasons behind the development of NAFLD are obesity, hyperlipidaemia, diabetes, diet, etc. [1,2]. As NAFLD worsens, it can lead to more serious liver diseases as cirrhosis [3], fibrosis [4], hepatocellular carcinoma [5], and non-alcoholic steatohepatitis [6]. Beyond problems pertaining to the liver, NAFLD is linked to higher risks of type 2 diabetes, chronic renal disease, and cardiovascular disease [7,8]. Morbidity and mortality are increased overall due to these consequences, which are caused by inflammation and systemic metabolic dysfunction.

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Effective therapeutic solutions are desperately needed to minimize these harmful health effects, as seen by the rising prevalence of NAFLD worldwide. As observed, there is a high incidence and prevalence rate of NAFLD, but despite this, no treatment has been approved yet. The most probable reason behind this is the multiple factors which were involved in the disease development and progression [9].

Diet plays a crucial role in modulating the gut microbiota of the gastrointestinal tract, which in turn causes alteration in the physiological condition of the body [10,11]. Numerous studies have demonstrated the role of gut microbiota in the development and progression of NAFLD [12–14]. The most common explanation behind this is the disruption of the intestinal gut membrane when dysbiosis was observed in the intestine, which in turn led to increased translocation of pathogenic products to the hepatocytes, which finally started an inflammatory cascade in liver cells [15,16]. Therefore, in this paper, we have focused on the modulation of gut microbiota using prebiotics and probiotics.

Silymarin (SM), is widely used as a hepatoprotective agent, which is comprised of a mixture of flavonolignans [17,18]. Because it consists of a mixture of lignans, thus we have hypothesized that it might possess pre-biotic action. Therefore, we have used SM in our formulation, and we tried to predict changes in the gut microbiota after administration of SM. This would also help us in predicting the pre-biotic potential of SM [19].

Piperine (PIP), the bioactive compound found in black pepper, has been investigated for its potential role in the management of NAFLD [20]. While research in this area is still emerging, some studies suggest that piperine may offer benefits for individuals with NAFLD through its anti-inflammatory effects [21], antioxidant activity [22] and modulation of lipid metabolism [23].

Fulvic acid (FA), a natural organic compound derived from soil, has garnered attention for its potential health benefits, including its role in addressing NAFLD [24]. While research on fulvic acid's specific effects on NAFLD is still emerging, its antioxidant and anti-inflammatory properties suggest promise in mitigating liver damage and inflammation associated with the condition [25,26]. Additionally, its immunomodulatory effects may help regulate immune responses in the liver, contributing to a reduction in inflammation and oxidative stress, both hallmarks of NAFLD progression [27]. Fulvic acid has been investigated for its potential to improve digestion and nutrient absorption by promoting the growth of beneficial gut bacteria and enhancing the bioavailability of nutrients [28].

For the selection of the probiotic blend, we have selected two strains each from the widely used bacteria, i.e., *Lactobacillus* and *Bifidobacterium*. The selected bacterial combination includes *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Lactobacillus casei*, and *Lactobacillus rhamnosus* [29–32]. There are two reasons for choosing this combination. Firstly, these species were the most widely present in the gut. Secondly, many studies have used these bacteria either alone or in combination for the treatment of NAFLD, and they have shown good results [33,34].

Higher calorie intake causes both dysbiosis in the gut and deposition of fat in the liver [35]. Moreover, fructose and fructose-enriched drinks cause changes in the lipid profile due to their lipogenic characteristics as compared to glucose-rich beverages [36]. Hence, to mimic the modern dietary conditions, in this study, we have fed a high-fat diet (HFD) with 30 % fructose in water for 10 weeks to C57BL/6J mice. After the development of the NAFLD model, we administered three different formulations for four weeks and then compared their effectiveness in improving the NAFLD condition. We also studied how gut microbiota changed in the HFD model and how these formulations led to a change in gut microbiome composition, its diversity and microbial richness in stool samples.

This study presents a novel investigation into the synergistic effects of a silymarin-based herbal remedy combined with a specific probiotic blend on NAFLD. Moreover, we have utilized the phytopharmaceutical of silymarin which is not used in any study till yet.

By utilizing a NAFLD mouse model induced through a high-fat and high-fructose diet, the research uniquely integrates biochemical, histological, and metagenomic analyses to evaluate the therapeutic potential of this combination. The inclusion of piperine and fulvic acid with silymarin, alongside a probiotic mix of *Bifidobacterium adolescentis, Bifidobacterium bifidum, Lactobacillus casei,* and *Lactobacillus rhamnosus*, offers a comprehensive approach that not only targets hepatic fat accumulation but also enhances gut microbiota composition. This dual-action strategy represents a significant advancement in the management of NAFLD, providing a promising avenue for future therapeutic development.

2. Materials and methods

2.1. Materials

SM (80 % methanol extract) was provided as a gift sample by Nectar Phytochem, Uttarakhand, India. PIP and FA were obtained as a gift sample from Dabur India limited, Ghaziabad, India. Probiotic blend containing *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Lactobacillus casei*, *Lactobacillus rhamnosus* was obtained as a gift sample from Fermentis life sciences, Gurugram, India.

2.2. Preparation of formulations

Formulation 1 (F1): 140 mg of Silymarin phytopharmaceutical containing 14 mg of PIP and 28 mg of FA [37,38], Formulation 2 (F2): Probiotic blend (*Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Lactobacillus casei*, *Lactobacillus rhamnosus*). Formulation 3: Combination of F1 and F2 and SM suspension denoted as D.

2.3. Induction of NAFLD

36C57BL/6J male mice having weight of 20–25 g were approved by Jamia Hamdard Institutional animal ethic committee (1900)

for conducting pharmacodynamic study. All the mice were acclimatized for 1 week by providing them standard normal pellet diet (NPD) which contain 12 % calories as fat with access to water ad libitum and were kept in 12/12 h of light and dark cycle. The temperature was maintained to 20–21 °C and 40–45 % relative humidity. After 1 week of acclimatization, 6 mice were kept in control group which were fed with NPD and normal water and remaining 30 mice were given High fat diet (HFD containing 58 % calories as fat, 25 % protein and 17 % carbohydrate, as a percentage of total kcal) and 30 % fructose in water for the period of 10 weeks [39,40]. The procedure to prepare the HFD was mentioned in Table 1 [41,42]. The body weight of all the mice were evaluated two times in every week. The mice with at least 20 % more body weight than initial was chosen and divided into 5 groups of n = 6 mice each. The 5 groups were as follows: a) NAFLD mice treated with formulation 1, b) NAFLD mice treated with formulation 2, c) NAFLD mice treated with formulation 3, d) NAFLD mice treated with drug suspension containing (silymarin equivalent to the animal dose), and e) NAFLD toxic group treated with HFD and 30 % fructose in drinking water. The dosing of formulations was mentioned in Table 2. All the formulations and samples were dissolved in 200 μL of drinking water and were given orally once daily for a period of one month. All 14 weeks of duration all the mice were subjected to fasting conditions and further sacrificed. Liver, intestine, pancreas, and kidney were collected and their histological investigation were performed. Histological samples were also fixed in 10 % formaldehyde for further histological examination. Blood sample were collected for further examination.

2.4. Liver and body weight analysis

The initial weight before the start of the study was noted for each mouse and the body weight (BW) after 4 weeks, 8 weeks and 10 weeks of high fat diet was noted. The mice with more than 20 % increase in body weight [43] were chosen for the treatment. Further, the BW after 4 weeks of dosing was also noted.

2.5. Biochemical analysis

Blood samples of the mice were obtained using cardiac puncture and the samples were further centrifuged at 5000 rpm for 10 min to isolate the serum from blood. The serum samples were scored at −80 °C until used for assay. Aspartate aminotransferase (AST), Alanine transaminase (ALT) and Alkaline Alkaline phosphatase (ALP) were performed using an automatic analyzer. Triglycerides (TG), Total cholesterol (TC), Low-density lipoprotein (LDL), and High-density lipoprotein (HDL) of the serum samples were measured using a rapid blood lipid analyzer. Fasting (18 h of fasting) and random blood glucose levels were estimated using glucometer.

2.5.1. Random glucose assessment

For estimating the random glucose level in the mice IPTT test had been performed. In this test, after fasting of 15 h in mice, (2.5 g/kg) glucose was injected intraperitoneally into the mice [44]. Glucometer was used to assess the glucose levels after 0, 30, 60, 90 and 120 min.

2.5.2. Intraperitoneal glucose tolerance test (IGTT)

For testing the status of insulin resistance, IGTT test was performed after 14 weeks of starting the study i.e., 10 weeks of HFD and subsequent 4 weeks of dosing. After fasting of 15 h (2.5 g/kg) glucose was injected intraperitoneally into the mice [44]. Glucometer was used to assess the glucose levels after 0, 30, 60, 90 and 120 min.

2.5.3. Histological examination

Liver, kidney and small intestine were excised from the mice and were then fixed in 10 % of formalin solution. The samples were then embedded in the paraffin wax from which a slice of 4 μm tissue was cut and then strained with the help of hematoxylin and eosin dye [44]. These tissues were then examined using the optical microscope at 40× magnification.

2.6. Stool sample metagenomic examination

Stool samples from seven different groups in n = 3 was collected and placed in 7.4 pH phosphate buffer until the isolation of DNA from the sample. The seven groups were a) control, b) Toxic-1 i.e., induction of NAFLD for 10 weeks, c) Toxic-2 i.e., induction of NAFLD for14 weeks without treatment, d) F1 for 4 weeks, e) F2 for 4 weeks, f) F3 for 4 weeks and g) drug suspension for 4 weeks.

Table 1
Composition of 1 kg of High fat diet.

Component	Quantity (g/kg)
Powdered NPD	365
Oil	310
Casein	250
Cholesterol	10
Vitamin and mineral mix	60
DL-methionine	3
Yeast	1
NaCl	1

Table 2
Grouping of animals and their dosing in Pharmacodynamic study.

No. of animals	Group	Dosage through oral route	Parameters
6	Control	Normal saline and NPD	• Metagenomic studies
6	NAFLD model (toxic group)	HFD plus 30 % fructose in drinking water	• Histopathological studies
6	Silymarin containing formulation	50 mg/kg i.e., 1.5 mg/day	• Relevant biomarkers
6	Bacterial combination	7.14 mg/kg	
6	Silymarin plus bacterial combination	1.5 mg/day + 7.14 mg/kg	
6	Drug suspension	50 mg/kg i.e., 1.5 mg/day	

XploregeneTM gDNA Extraction kit was used for extracting the DNA from the stool sample. The amplicons from each sample were purified with Ampure beads to remove unused primers and an additional 8 cycles of PCR was performed using Illumina barcoded adapters to prepare the sequencing libraries. Libraries were purified using Ampure beads and quantitated using Qubit dsDNA High Sensitivity assay kit. Sequencing was performed using Illumina Miseq with 2 × 300PE v3-v4 sequencing kit. Taxonomic profiling, alpha diversity, beta diversity, refractive curve, heatmap and dendrograms were prepared for all the samples.

2.7. Statistical analysis

Statistical analysis was performed using SPSS version 11.5 for Windows (SPSS Inc., Chicago, IL, USA) and Graph pad prism. The mean of 2 continuous normally distributed variables was compared by independent sample Student's test. ANOVA was used to compare the means of more than 2 groups and then post hoc turkey test was applied for each pair. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Liver and body weight

The body and liver weight of mice in different groups were reported in Table 3 and Table 4. As depicted in Fig. 1, a drastic increase in weight had been observed in groups receiving HFD as compared to the control group. The weight of mice in different groups at the start of study have shown no statistically significant differences (Fig. 1A). After 10 weeks of HFD and 30 % fructose in drinking water, 70–80 % increase in weight have been observed (Fig. 1B). Moreover, the liver weight has also increased statistically in toxic group both after 10 weeks and 14 weeks of HFD +30 % fructose in drinking water as compared to control group (Fig. 1D). Therefore, combination of HFD with 30 % fructose in water had shown an increase body wight and liver weight as compared to control. The weight loss after taking 4 weeks of F3 formulation was statistically significant as compared to toxic group 1 and toxic group 2 (Fig. 1C). The weight change after 14 weeks was statistically insignificant only between F2 and F3, remaining all the groups have shown statistically significant result (Fig. 1C). The least weight was seen in F3 among F1, F2, F3 and drug suspension. Initial liver weight was statically insignificant among different groups whereas after 14 weeks of HFD, the LW was statistically insignificant among F1, F2 and F3, while other groups are statistically significant (Fig. 1D). An increase in two-fold of liver weight was observed after 14 weeks of HFD as compared to the control group. Consumption of formulations have yielded 21 % decrease in liver weight as compared to HFD group after 10 weeks of HFD.

3.2. Biochemical estimation

3.2.1. Liver profile

AST, ALT and ALP have been increased drastically in the group which were receiving HFD and 30 % fructose in water. After 10 weeks of HFD and fructose containing water, 4.65-, 3.09- and 1.70-fold increase have been observed in AST, ALT and ALP levels,

Table 3
The initial body weight, Body weight after 4 weeks, Body weight after 10 weeks and Body weight after 14 weeks for control, toxic 1, toxic 2, F1, F2, F3 and drug suspension groups (results were indicated as mean ± Standard deviation).

	Control	Toxic group after 10 weeks	Toxic group after 14 weeks	F1	F2	F3	Drug suspension
Initial BW	21.8 ± 0.3	22.4 ± 0.5	22.3 ± 0.6	21.5 ± 0.5	21.8 ± 0.8	22.0 ± 0.5	21.3 ± 0.3
BW after 4 weeks of HFD	22.2 ± 0.3	27.3 ± 0.7	27.6 ± 0.6	26.2 ± 0.3	27.0 ± 0.5	28.3 ± 0.3	26.8 ± 0.3
BW after 10 weeks of HFD	22.6 ± 0.2	37.7 ± 0.3	37.8 ± 0.8	36.2 ± 0.3	37.3 ± 0.3	38.2 ± 0.3	37.2 ± 0.4
Treatment started (4 weeks treatment)							
BW after 14 weeks of HFD	23 ± 0.5	–	42.5 ± 0.5	29.3 ± 0.3	28.2 ± 0.3	27.3 ± 0.3	33.5 ± 0.5

Table 4
The initial liver weight, and Liver weight after 14 weeks for control, toxic 1, toxic 2, F1, F2, F3 and drug suspension groups (results were indicated as mean ± Standard deviation).

	Control	Toxic group after 10 weeks	Toxic group after 14 weeks	F1	F2	F3	Drug suspension
Initial LW	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
LW after 14 weeks of HFD	1.2 ± 0.3	1.9 ± 0.2	2.2 ± 0.2	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.8 ± 0.1
LW/BW after 14 weeks	0.052	0.050	0.052	0.054	0.057	0.055	0.054

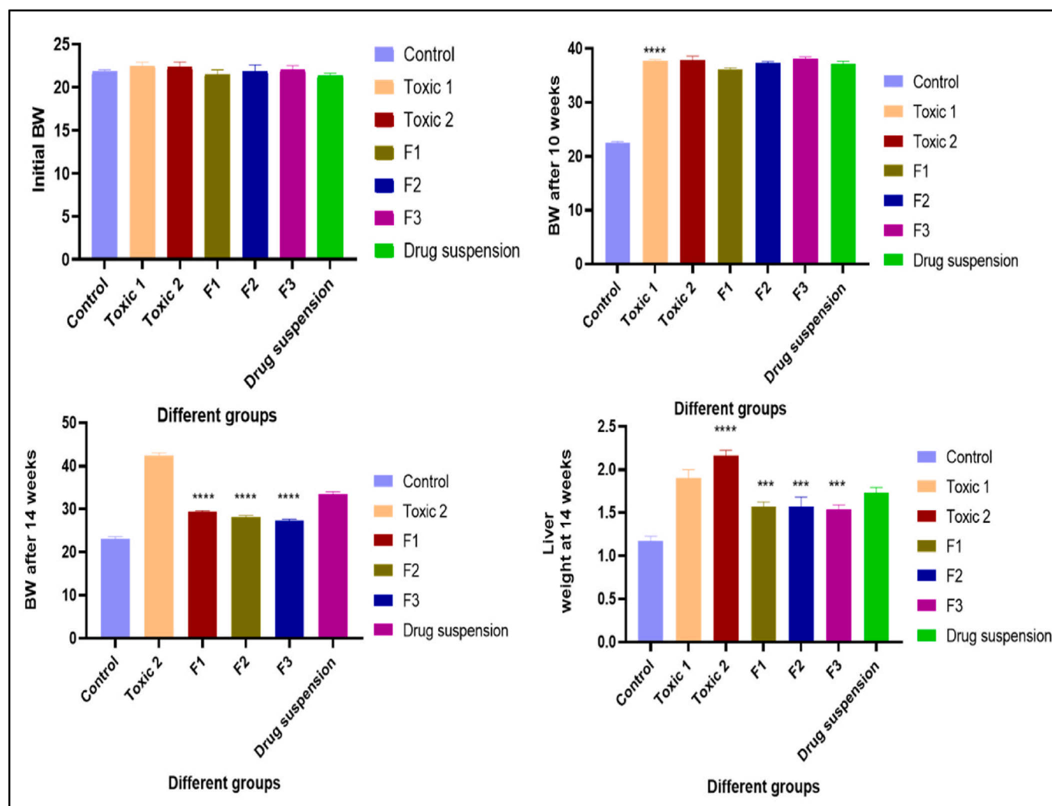

Fig. 1. The a) initial body weight, b) Body weight after 10 weeks, c) Body weight after 14 weeks and d) Liver weight after 14 weeks for control, toxic 1, toxic 2, F1, F2, F3 and drug suspension group using Graph pad prism. (F1, F2 and F3 compared with toxic 2 group and toxic 2 group compared with control) (**** signifies $p < 0.001$).

respectively (Fig. 2 A, 2B, & 2C, respectively). Furthermore, after 14 weeks of HFD and fructose containing water, 5.36-, 4.35- and 1.82-fold increase have been observed in AST, ALT and ALP levels, respectively. As compared to the toxic group, a statistically significant reduction in levels of AST, ALT and ALP have been observed in all the formulation groups i.e., F1, F2, F3 and drug suspension. The maximum reduction for all the parameters has been observed in F3 formulation (2.56-, 2.98- and 1.67-fold decrease in AST, ALT and ALP, respectively as compared to parameters observed in HFD and water containing fructose group after 14 weeks). As compared to control group, the levels of AST, ALT and ALP were found to be 2.08, 1.46 and 1.08 times in F3 group. These results demonstrated that using the combination of SM-PP with the bacterial combination can leads to amelioration of the biochemical parameters which were disturbed by NAFLD. All these biochemical estimations were shown in Fig. 2 and Table 5.

3.2.2. Lipid profile

TG, TC, and LDL have increased drastically in the group which were receiving HFD and 30 % fructose in water. After 10 weeks of HFD and fructose containing water, 1.46-, 2.88- and 1.78-fold increase have been observed in TG, TC and LDL levels, respectively (Fig. 2 D, 2E and 2F, respectively). Furthermore, after 14 weeks of HFD and fructose containing water, 1.94-, 3.47- and 2.04-fold increase have been observed in TG, TC and LDL levels, respectively. As compared to the toxic group, a statistically significant reduction in levels of triglycerides, total cholesterol and LDL have been observed in all the formulation groups i.e., F1, F2, F3 and drug suspension. The maximum reduction for all the parameters has been observed in F3 formulation (1.77-, 4.91- and 1.81-fold decrease in TG, TC and LDL, respectively as compared to parameters observed in HFD and water containing fructose group after 14 weeks). As

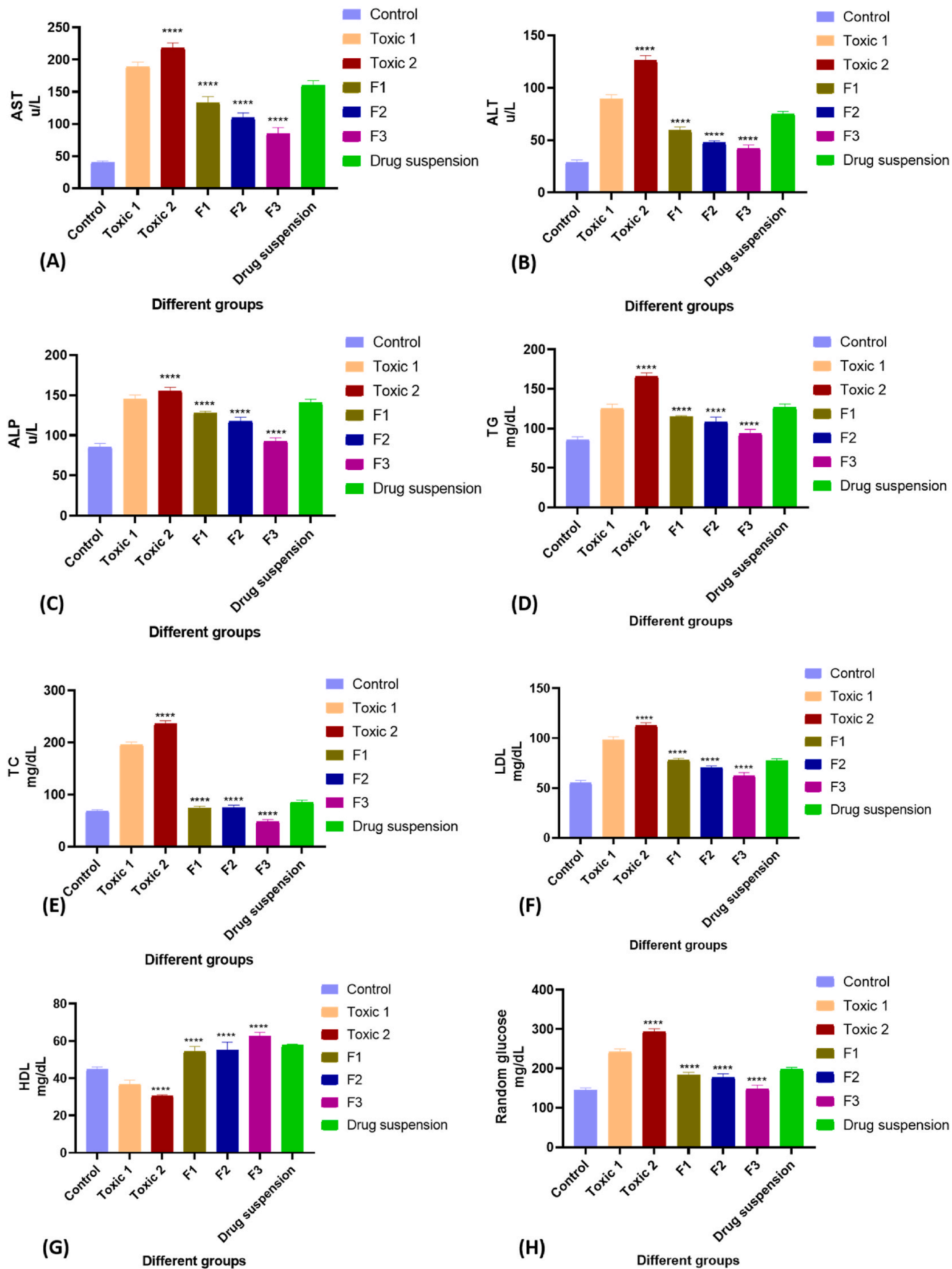

Fig. 2. Biochemical estimation of A) AST, B) ALT, C) ALP, D) TC, E) TG, F) HDL, G) LDL, and H) random glucose levels for control, toxic 1, toxic 2, F1, F2, F3 and drug suspension groups using Graph pad prism. (F1, F2 and F3 compared with toxic 2 group and toxic 2 group compared with control). (**** signifies $p < 0.001$).

compared to control group, the levels of TG, TC and LDL were found to be 1.08, 0.70 and 1.39 times in F3 group. In contrast, the level of HDL, good cholesterol has been increased in all the formulation groups which was decreased by the administration of HFD and the maximum increment was observed in F3 formulation (Fig. 2 G). These results demonstrated that using the combination of SM-PP with the bacterial combination can leads to amelioration of the biochemical parameters which were disturbed by NAFLD. All these biochemical estimations were shown in Fig. 2 and Table 5.

Table 5
Biochemical estimation of A) AST, B) ALT, C) ALP, D) TC, E) TG, F) HDL, G) LDL, and H) random glucose levels for control, toxic 1, toxic 2, F1, F2, F3 and drug suspension groups (results were indicated as mean ± Standard deviation).

Serum parameters	Control	Toxic group after 10 weeks	Toxic group after 14 weeks	F1	F2	F3	Drug suspension
Liver profile							
AST/SGOT	40.6 ± 1.8	189 ± 7	217.9 ± 7.9	132.4 ± 9.9	109.6 ± 7.5	84.8 ± 9.2	160.1 ± 7.3
ALT/SGPT	28.9 ± 2.3	89.4 ± 4	126 ± 4.9	59.1 ± 3.6	47.5 ± 1.8	42.2 ± 3.3	74.7 ± 2.9
ALP	85.1 ± 4.9	144.8 ± 5.2	155.3 ± 4.3	127.8 ± 2.2	117.5 ± 5.2	92.6 ± 4.2	140.3 ± 4.6
Lipid profile							
TC	85.6 ± 3.7	125.2 ± 5.5	165.7 ± 4.4	114.7 ± 1.3	108.2 ± 6.2	93.1 ± 5.7	126.2 ± 4.7
TG	68.3 ± 2.8	196.2 ± 5	236.4 ± 5.5	73.3 ± 4.3	74.8 ± 4.9	48.2 ± 4.1	85.1 ± 4.4
HDL	44.7 ± 1.4	36.8 ± 2.2	30.4 ± 0.7	54.3 ± 2.7	54.9 ± 4.5	62.5 ± 2.1	57.8 ± 0.6
LDL	55.1 ± 2.7	98.4 ± 3.1	112.8 ± 2.7	77.8 ± 2	70.5 ± 1.6	62 ± 3.3	77.3 ± 2
Sugar levels							
Random	144.7 ± 5.5	241.7 ± 7.6	293 ± 7.2	183.3 ± 6.7	177.3 ± 8.7	147.7 ± 9.3	197 ± 5.3

3.2.3. Random glucose level

The random glucose level in the control group was found to be statistically significant difference from the group containing HFD and fructose. After 10 and 14 weeks of HFD, the glucose levels were found to be 1.66 and 2.02 times, respectively as compared to the initial levels. The significant reduction in glucose level have been observed in all the formulation groups with maximum reduction in F3 (1.02 times as compared to control group). All these biochemical estimations were shown in Fig. 2H and Table 5.

3.2.4. IGTT

The glucose level in all the groups have been found to be elevated at 30 min of administration of glucose injection which after 30 min started falling. While comparing the levels of glucose of control with toxic groups, a statistically significant difference has been observed. The glucose level has returned to normal levels after 120 min of glucose administration in control group whereas it remained at higher levels in toxic group even after 120 min (288.7 and 330.3 mg/dL in HFD group after 10 weeks and 14 weeks, respectively). The levels of glucose in all the formulation groups were found to be statistically significant as compared to the toxic group but the maximum reduction in glucose levels have been observed in F3 group i.e., 142 mg/dL after 120 min. The following results have been demonstrated in Table 6 and Fig. 3.

3.3. Histological examination

3.3.1. Liver

The histological examination of liver tissue revealed an excessive accumulation of fat globules in the liver cells after administration of HFD and 30 % fructose in drinking water for 10 and 14 weeks (Fig. 5) as compared to control group (Fig. 4). As compared to drug suspension group, the histological examination of liver of F1, F2 and F3 revealed a better recovery of liver cells as the deposited lipid globules started reducing after administration of F1, F2 or F3 for 4 weeks. This finding has shown that all the three formulations were showing hepatoprotective action (Fig. 7). As compared to control group, the hepatocytes in NAFLD group were found to have demonstrated fatty degeneration with deranged lobular and vacuole boundaries. Further, treatment with F1, F2 or F3, these deranged hepatic cells have started showing recovery and reduction in lesion area was observed.

3.3.2. Intestine

The histological examination of intestine revealed that after administration of HFD and 30 % fructose in drinking water for 10 weeks i.e., Toxic-1 (Fig. 5), the intestinal cells started degrading and gaps were observed in the cells which leads to enhanced intestinal

Table 6
IGTT levels for control, toxic 1, toxic 2, F1, F2, F3 and drug suspension groups after 0, 30, 60, 90 and 120 min (results were indicated as mean ± Standard deviation).

IGTT	0 min	30 min	60 min	90 min	120 min
Control	101.7 ± 3.1	284.3 ± 5	191.3 ± 3.2	153 ± 3	130 ± 1
Toxic group after 10 weeks	165.7 ± 4	426.7 ± 5.9	351.7 ± 3.1	332 ± 2.6	288.7 ± 60.4
Toxic group after 14 weeks	186.7 ± 5.9	462.7 ± 6.5	393.3 ± 1.5	357.7 ± 8	330.3 ± 5
F1	122 ± 3.6	322.7 ± 6.8	205.3 ± 4.5	178 ± 3	162.3 ± 3.2
F2	119.3 ± 4.7	306.7 ± 4.9	198.3 ± 3.5	163 ± 2	155 ± 2
F3	110 ± 1	290.3 ± 4.2	181.3 ± 3.2	148.7 ± 2.5	142 ± 3.6
Drug suspension	138 ± 6.2	337 ± 2.6	232.7 ± 2.5	194.7 ± 3.2	187.3 ± 2.1

Drug suspension	138±6.2	337±2.6	232.7±2.5	194.7±3.2	187.3±2.1

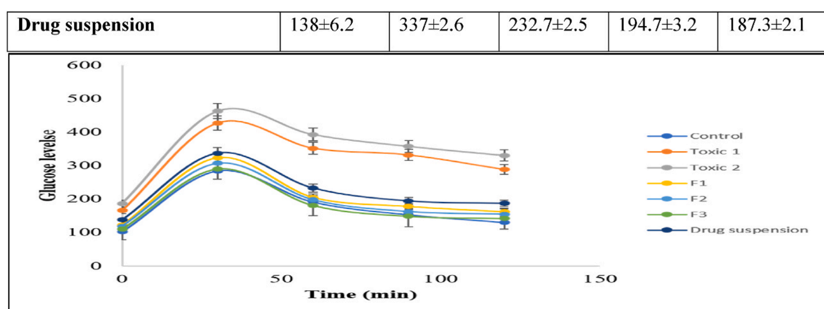

Fig. 3. IGTT levels for control, toxic1, toxic 2, F1, F2, F3 and drug suspension groups after 0, 30, 60, 90 and 120 min.

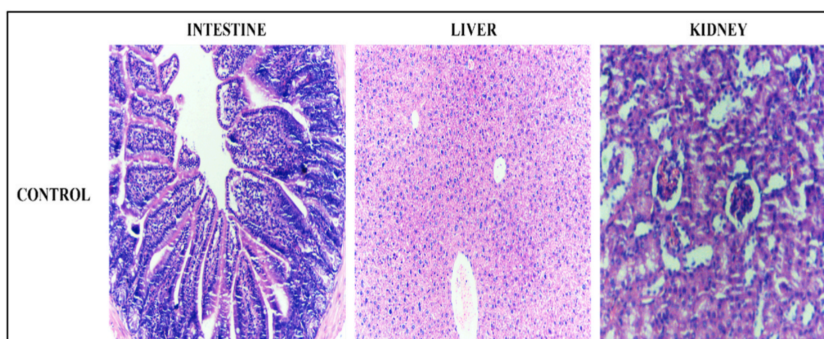

Fig. 4. Photomicrographs showing histopathological sections of normal C57BL/6×J mice intestine, liver and kidney (Olympus, 40 × magnification).

permeability and leaky intestine. These changes were seen more enhanced in Toxic-2 group in which HFD was given for 14 weeks. Moreover, as seen in Fig. 6, the intestinal cells started showing recovery after taking F1, F2, or F3 after 4 weeks as compared to drug suspension.

3.3.3. *Kidney*

The histology of kidney revealed that after administration of HFD and 30 % fructose in drinking water for 10 weeks i.e., Toxic-1 (Fig. 5), the tubular epithelial lining within kidney tissue started degrading and expanding. These changes were seen more enhanced in Toxic-2 group in which HFD was given for 14 weeks. Moreover, as seen in Fig. 6, the cells of kidney started showing recovery after taking F1, F2, or F3 for 4 weeks as compared to drug suspension.

3.4. *16S metagenomic estimation*

The gut microbiome mainly comprised of bacteria belonging to four phyla i.e., Firmicutes, Bacteroidetes, Proteobacteria and Actinomycetes. After feeding HFD and 30 % fructose in water for 10 weeks, the Firmicutes (F) and Proteobacteria levels found to be increased and the levels of Bacteroidetes (B) started decreasing as shown in Fig. 7. These results were found in accordance with a previous study conducted by Gang wang et al. [34]. Hence, the ratio of Firmicuts/Bacteroidetes (F/B) have been elevated significantly ($p < 0.05$) as shown in Fig. 8. Furthermore, the F/B ratio increased further when HFD diet was administered for 14 weeks (Toxic-2). These results further confirmed the correlation of F/B ratio with the development of NAFLD as confirmed in many other studies also [45]. Moreover, treatment with F1, F2 or F3 have shown that gut composition started reversing towards normal state by decreasing F/B ratio. As seen in Fig. 8, the ratio of F/B was found in the order of D > F2 > F1 > F3. The maximum improvement was observed with F3 which is the mixture of F1 and F2 i.e., 140 mg SM-PP + 14 mg PIP +28 mg FA + probiotic blend of four bacterial species. As it can be observed in Figs. 7 and 8, both F1 and drug suspension were improving the gut composition, thus they signify that SM have the high probability to act as a prebiotic agent for the beneficial bacteria already present in the gut. Further, the combination of F1 and F2, have shown best results which signifies that the SM not only increasing the concentration of already present bacteria in the gut but also helping the administered probiotic blend to proliferate after reaching the intestine. As can be seen from Figs. 7 and 8, administering the probiotic blend standalone i.e., F2, was found to show lesser results as compared to F1, which signifies that administration of probiotic alone cannot able to proliferate better in the gut. Thus, administration of probiotic with prebiotic acts as a better alternative for treatment. Relative abundance of different phylum for all the groups were shown in Fig. 9.

As observed in Shanon and Simpson index (Fig. 10A and B, respectively), the alpha diversity, i.e., homogeneity and species number started decreasing after feeding with HFD for 10 and 14 weeks. Whereas, the diversity of species number started increasing after the

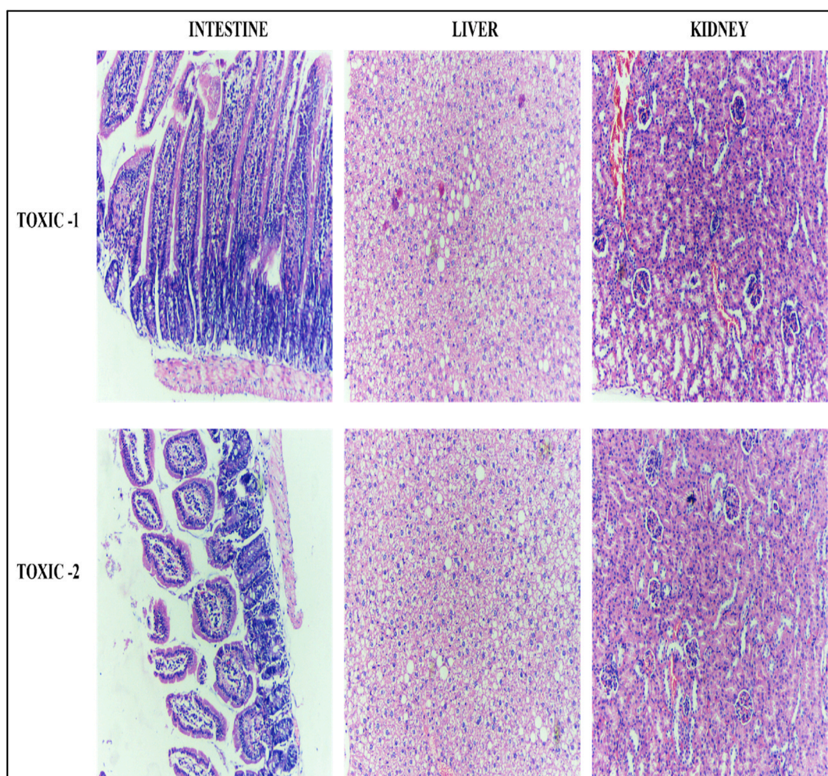

Fig. 5. Photomicrographs showing histopathological sections of diseased C57BL/6J mice intestine, liver and kidney after 10 weeks of HFD and fructose (Toxic 1) and after 14 weeks of HFD and fructose (Toxic 2×) (Olympus, 40 × magnification).

administration of F1 and F3. Like the above results, the maximum diversity was found to be seen with the F3 formulation and minimum diversity was observed with Toxic-2 group. The comparison of both the formulations F1and F3 were found to be statistically significant ($p < 0.05$) when compared with control and Toxic −1 and Toxic-2 group, whereas, the results for F2 and D were not found to be statistically significant with Toxic group. This signified that F1 and F3 were increasing the homogeneity and species number (microbial diversity) but not D and F2.

As observed in Fig. 11 (A&B), the bacterial composition of Toxic 1 and Toxic 2 group was significantly separated from control group and F1, F2, F3 and D group. It was observed that bacterial heterogeneity was not statically significant different between F1, F2, F3 and D, but they were found to be different from Toxic groups.

As seen in Fig. 12, the beneficial Lactobacillus concentration decreases drastically in Toxic 1 and Toxic 2 group as the lactobacillus concentration was not present in Top 10 genus. Furthermore, the concentration of Lactobacillus has started increasing in different formulation groups i.e., F1, F2, F3 and D. Moreover, notable differences have been observed in different genus like clostridium, Bifidobacterium, Acinetobacter, Bacteroides, and Flavobacterium. As compared to the NC mice, the HFD promoted a significant decrease in the abundance of Bacteroides, Bifidobacterium and Lactobacillus in the intestinal microbiota ($p < 0.05$). These results were found in accordance with a previous study conducted by Gang Wang et al. [34] and Yan et al., [46]. Different treatment groups drastically increased the concentration of Lactobacillus, Bacteroides and Parabacteroides in the intestine ($p < 0.05$). Moreover, the level of Bifidobacterium has reduced to zero in both the toxic groups which started increasing after taking different formulations and maximum increase in Bifidobacterium level was observed after taking F1 formulation. The relative abundance of all the genus in different groups have been shown in Fig. 13.

As seen in Fig. 14, the dendrogram of the groups confirmed that the bacterial composition of formulations F1 and F3 were found to be most similar to control group whereas, the composition of F2 and D were found in close proximity to the toxic groups. Fig. 15, demonstrated the refractive curves which revealed the species richness of the stool samples. The results confirmed that the species richness was decreased in toxic groups as compared to control group. Moreover, the species richness started increasing with the administration of different formulations with F1, F2 and F3 showed statistically insignificant differences between three groups. All these results confirmed that standalone probiotic blend is not giving better results when compared with its mixture with SM-PP.

4. Discussion

This study explored the therapeutic efficacy of a silymarin-based herbal remedy supplemented with piperine and fulvic acid, combined with a specific probiotic blend, in mitigating non-alcoholic fatty liver disease (NAFLD) induced in C57BL/6J mice through a

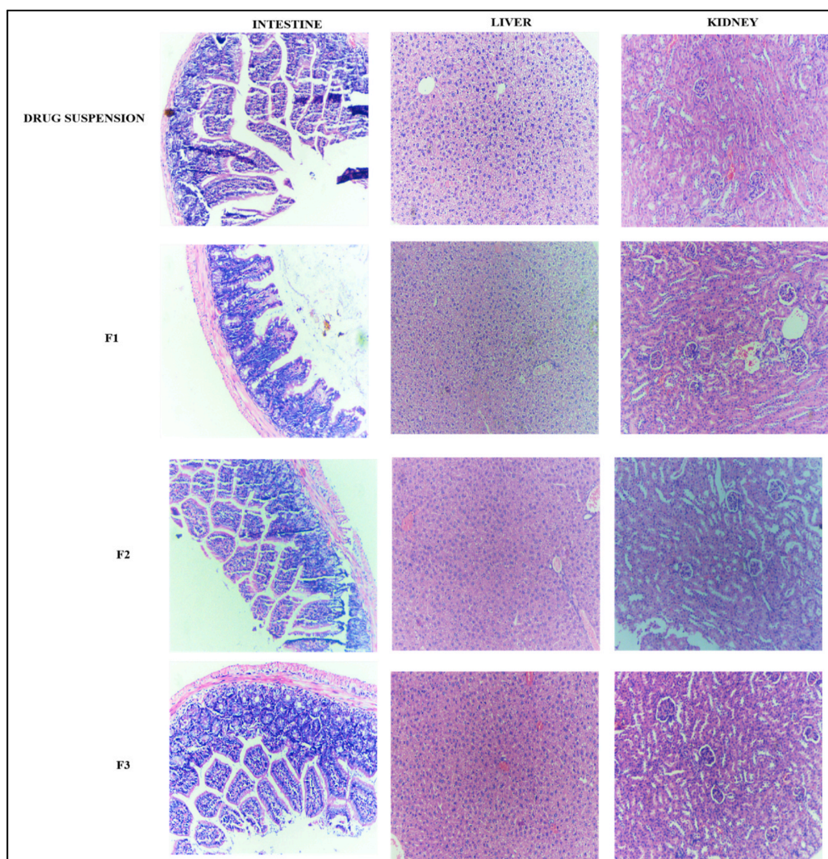

Fig. 6. Photomicrographs showing histopathological sections of the intestine, liver and kidney of C57BL/6J mice after 4 weeks of oral administration of API drug suspension, F1, F2 and F3 formulation (Olympus, 40 × magnification).

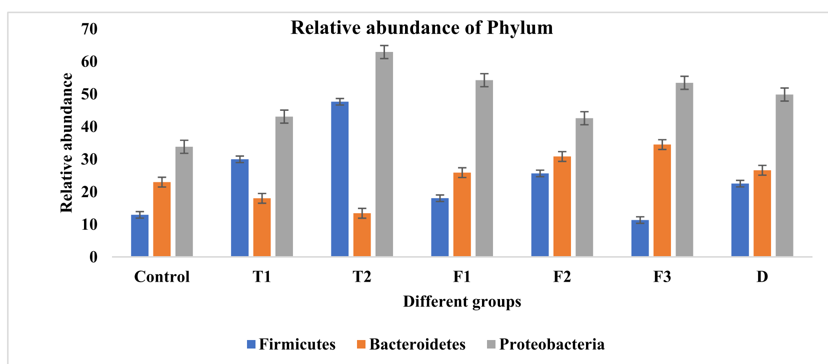

Fig. 7. Relative abundance of Firmicutes, Bacteroidetes and Proteobacteria in stool samples of different groups of mice.

high-fat diet (HFD) and 30 % fructose in drinking water. The results demonstrate that the combination treatment significantly ameliorated the pathological changes associated with NAFLD, including body weight gain, liver weight increase, dyslipidemia, liver enzyme elevation, and gut microbiota dysbiosis. The significant increase in body and liver weight observed in the HFD and fructose group underscores the severity of the NAFLD model. The combination treatment (F3) led to a substantial reduction in both body and liver weights, suggesting a potent anti-obesity and hepatoprotective effect. This weight reduction correlates with improved liver histology, where the F3 treatment significantly decreased lipid accumulation in liver cells, indicating enhanced lipid metabolism and reduced steatosis. Biochemical analysis further supported the therapeutic potential of the combination treatment. The HFD and fructose diet drastically elevated triglycerides (TG), total cholesterol (TC), and low-density lipoprotein (LDL) levels, while reducing high-density lipoprotein (HDL) levels. The F3 formulation notably normalized these lipid profiles, with the greatest improvements

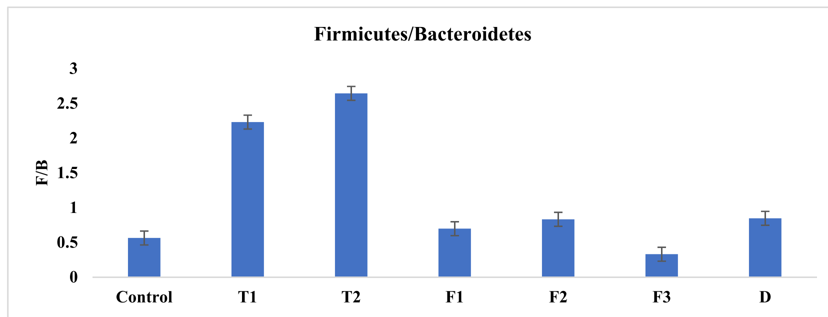

Fig. 8. Firmicutes/Bacteroidetes ratio in stool samples of different groups of mice.

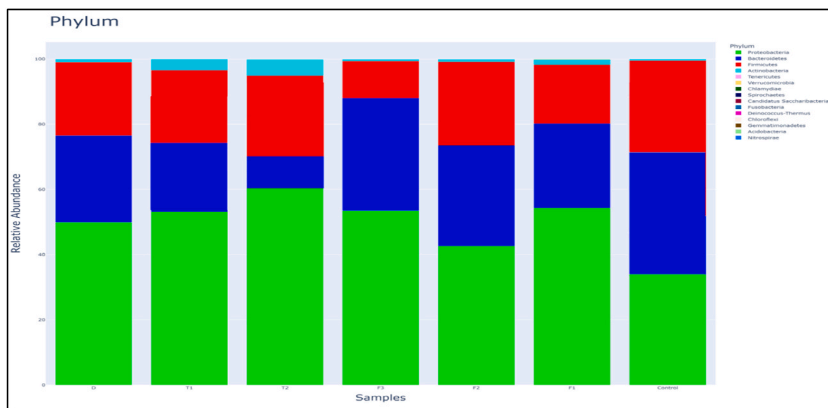

Fig. 9. Relative abundance of different Phylum in stool samples of different groups of mice.

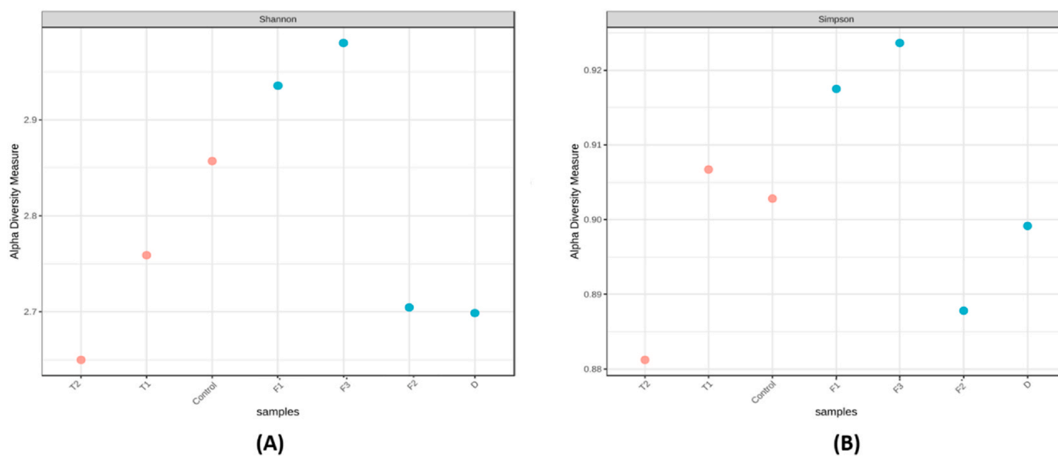

Fig. 10. Alpha diversity of different stool samples (A) By Shanon and (B) By Simpson.

observed in TG, TC, and LDL levels, aligning closely with control group values. This lipid-lowering effect is crucial for mitigating cardiovascular risks associated with NAFLD. Liver function tests revealed elevated aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) levels in the NAFLD model, indicative of liver damage. The F3 treatment markedly reduced these enzymes, reflecting improved liver health and reduced hepatic inflammation. Histological examination of the intestine and kidney showed that the combination treatment also ameliorated the structural damage induced by HFD and fructose, highlighting its broader protective effects beyond the liver. This comprehensive tissue protection is likely due to the synergistic effects of silymarin, piperine, fulvic acid, and probiotics. The study also delved into the gut microbiota, revealing that the F3 treatment significantly modulated the gut microbiome towards a healthier composition. The F3 treatment decreased the Firmicutes/

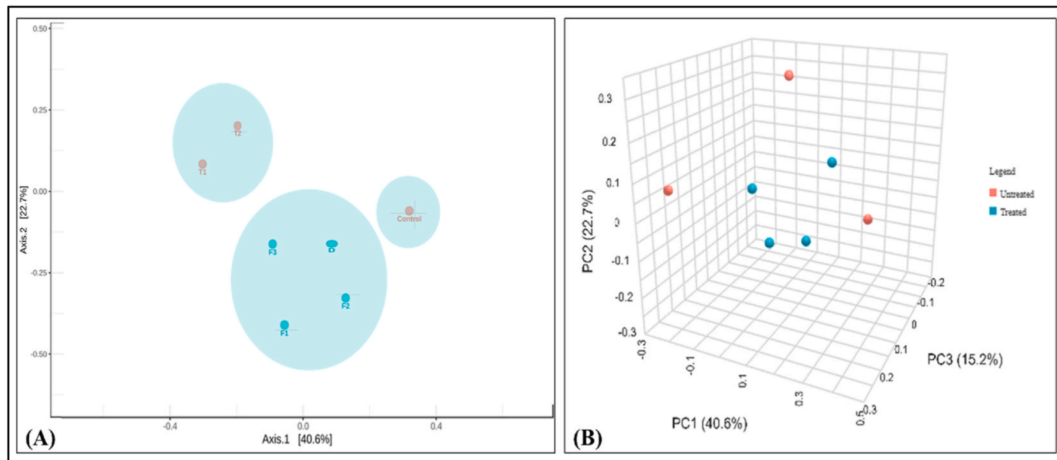

Fig. 11. (A) Beta diversity of different stool samples. (B) Principal component analysis of different stool samples.

Bacteroidetes (F/B) ratio, a marker of gut dysbiosis in NAFLD, and increased beneficial bacteria like Lactobacillus and Bifidobacterium. This suggests that silymarin might act as a prebiotic, enhancing the growth and activity of administered probiotics and native beneficial gut microbe. Based on our findings, the hepatoprotective effects of our formulations likely involve several mechanisms: 1) Anti-inflammatory Effects: Silymarin is known for its anti-inflammatory properties, which may contribute to reducing liver inflammation in NAFLD. The observed reduction in AST, A LT, and ALP levels supports this mechanism. 2) Modulation of Gut Microbiota: Our study showed that the combination of silymarin-piperine (SM-PP) with probiotics (F3) significantly modulated the gut microbiota, reducing the Firmicutes/Bacteroidetes (F/B) ratio, which is often associated with improved metabolic health. This modulation likely plays a critical role in mitigating NAFLD progression. 3) Metabolite Production by Microbiome: The probiotics included in our formulation might enhance the production of beneficial metabolites such as short-chain fatty acids (SCFAs), which are known to have anti-inflammatory and hepatoprotective effects. 4) Prebiotic Action of Silymarin: The prebiotic properties of silymarin could support the growth of beneficial gut bacteria, further contributing to the overall improvement in gut health and liver function.

In summary, the combination of silymarin, piperine, fulvic acid, and a probiotic blend shows promise as a therapeutic strategy for NAFLD by addressing both metabolic and microbial aspects of the disease. Future studies utilizing omics technologies are recommended to elucidate the precise molecular mechanisms underlying these beneficial effects and to validate these findings in human clinical trials. This integrated approach could pave the way for more effective treatments for NAFLD and related metabolic disorders.

5. Limitations of the study and research suggestions

The study explored the therapeutic potential of a silymarin-based herbal remedy supplemented with piperine and fulvic acid, alongside a probiotic blend, in addressing non-alcoholic fatty liver disease (NAFLD) in C57BL/6J mice. Significant limitations of the study include its focus on animal models rather than human subjects, potentially limiting direct extrapolation to clinical settings. Moreover, the mechanisms underlying the observed effects remain partially understood, necessitating further elucidation. Additionally, the study did not explore long-term effects or potential adverse reactions associated with the tested interventions, which are crucial considerations in clinical practice.

Research suggestions stemming from the study include the need for human clinical trials to validate the efficacy and safety of the tested formulations in NAFLD patients. Further investigations into the specific mechanisms by which the interventions modulate gut microbiota composition and attenuate NAFLD pathology are warranted. Longitudinal studies assessing the durability of treatment effects and potential interactions with other medications or interventions would provide valuable insights for clinical implementation. Moreover, comparative studies evaluating the effectiveness of the silymarin-based therapy alone versus in combination with probiotics could elucidate the synergistic effects observed in this study. Additionally, it is essential to highlight the need for future studies utilizing omics technologies to further investigate the molecular basis of the observed hepatoprotective effects. This addition will strengthen the discussion and provide a clear direction for future research.

6. Conclusions

We have taken three formulations for the pharmacodynamics study a) F1, containing SM-PP +20 % FA +10%PIP, b) probiotic blend (F2) and c) combination of F1 and F2 as F3. NAFLD model was prepared by administering high fat diet and 30 % fructose in drinking water for 10 weeks in C57BL/6J mice. The mice in HFD group have shown an elevation in TG, TC, LDL, AST, ALT, ALP, and glucose levels. Moreover, HDL levels have been reduced. Histological examination of liver revealed a drastic accumulation of lipid in the liver cells after 10 weeks. 16S metagenomics of stool samples shown drastic increase in F/B ratio and decrease in Bacteroidetes level and increase in firmicutes level. Moreover, the fat accumulation, body weight, liver weight AST, ALT, glucose levels, LDL, TC,

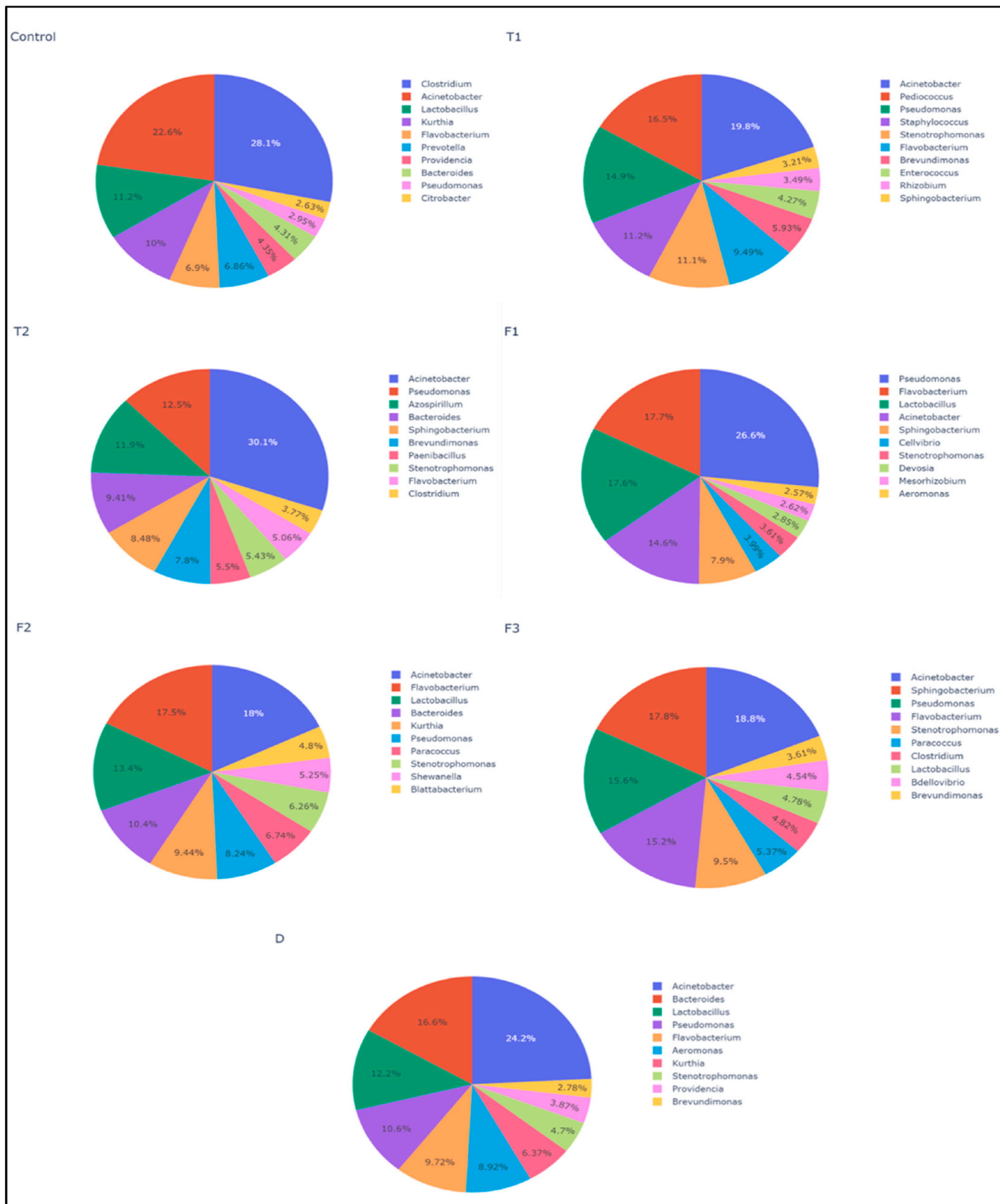

Fig. 12. Top 10 genus of different groups.

ALP, and TG were reduced drastically after 4 weeks of dosing with different formulations but maximum was observed with F3. Metagenomic profiling of stool samples of mice receiving different groups of formulations have shown almost same results with control group for F3 group then F1 and lastly F2. This indicated that SM-PP containing formulation is also modulating the gut microbiota towards normal side and combining SM with probiotic showing enhanced activity as compared to stand alone probiotic blend. This indicated that SM-PP when given in combination with probiotic is showing enhanced activity which might be due to the prebiotic action of SM. Based on all these results, we have stipulated that silymarin PP in combination with prebiotic blend can be used for the treatment of NAFLD by modulating the gut microbial composition.

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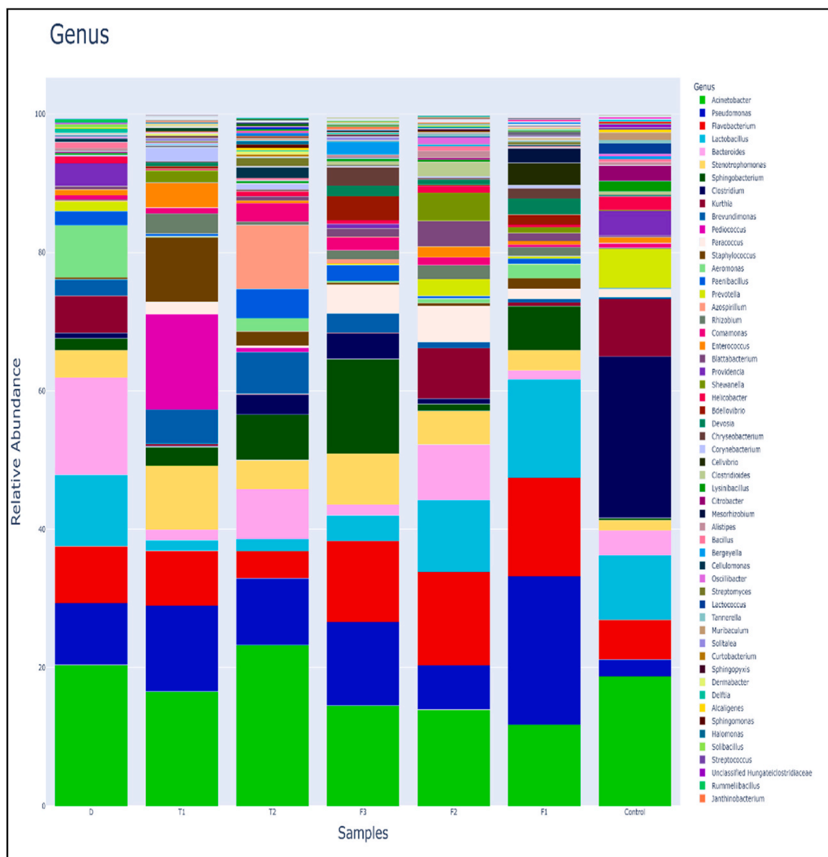

Fig. 13. Relative abundance of different genus in stool samples of different groups of mice.

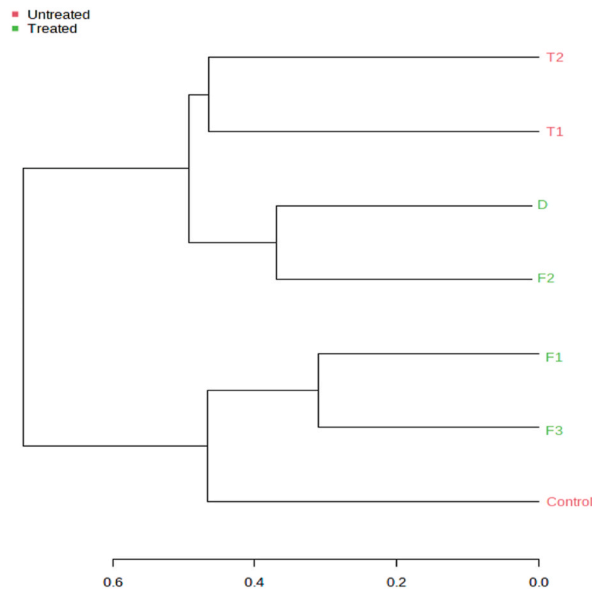

Fig. 14. Dendrogram of different group of mice.

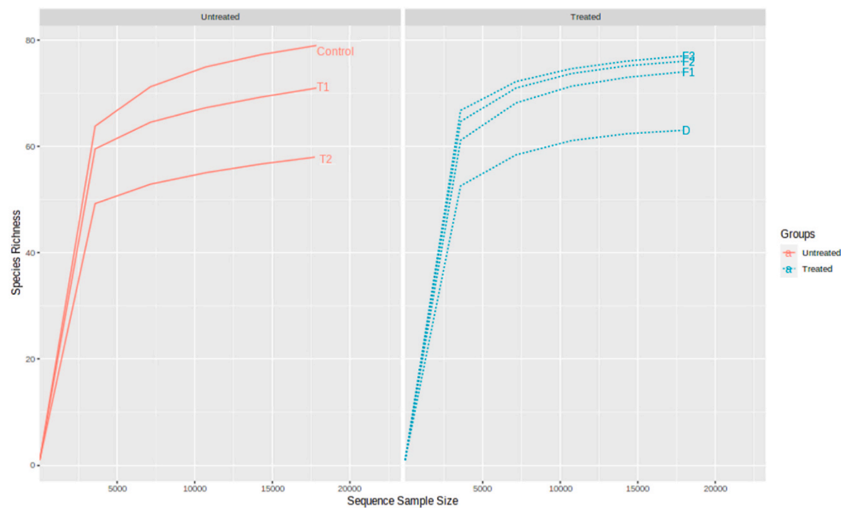

Fig. 15. Refractive curve showing species richness in the stool samples of mice in different groups of mice.

Ethics statement

66C57BL/6J male mice were approved by Jamia Hamdard Institutional animal ethic committee (1900), Jamia Hamdard, New Delhi, India, for conducting pharmacodynamic study.

Consent statement

Not applicable.

Data availability statement

The data of this article are provided upon request from corresponding author.

CRediT authorship contribution statement

Tanya Ralli: Writing – original draft, Visualization, Validation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Shahnawaz Ahmad:** Data curation. **Zoya Saifi:** Formal analysis. **Abdulsalam Alhalmi:** Writing – review & editing. **Vidhu Aeri:** Conceptualization. **Mohd Aqil:** Conceptualization. **Kanchan Kohli:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Tanya Ralli reports financial support was provided by India Ministry of Science & Technology Department of Science and Technology. If there are other authors, they 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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