



A study to evaluate the hepatoprotective activity of prebiotics, probiotics, and synbiotic in CCl₄ induced hepatotoxicity in rats

Aswad B. Devi^{1,2}, Anand B. Rahigude², Pradeep B. Parab², Anupama S. Engineer², Prashant K. Dhakephalkar³, Kishori G. Apte^{1,2*}

¹Symbiosis International University SIU, Gram Lavale, Pune, India.

²APT Research Foundation, Pune, India.

³Agharkar Research Institute, Pune, India.

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ABSTRACT

The current study was evaluated for the hepatoprotective activity of prebiotics, probiotics alone, and synbiotic of them. Animals were grouped into 12 groups ($n = 6$), i.e. normal control (NC), disease control (DC), treatment with prebiotics, probiotics alone, and synbiotic of them, and standard marketed hepatoprotective Silymarin along with Synbiotic 2000. All treatments were orally administered daily for 28 days. On the 28th day, all animals except the NC group received carbon tetrachloride (CCl₄) (1 ml/kg, i.p.). Levels of serum glutamic pyruvic transaminase (SGPT), serum glutamic-oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP), total bilirubin, total protein, lactate dehydrogenase, and urea were evaluated. The tissues were evaluated for oxidative markers levels of catalase (CAT), lipid peroxidation (LPO), and glutathione (GSH), and total protein. Histopathology of tissues was carried out. Compositional analysis of intestinal microbiota was done by 16S amplicon sequencing. CCl₄ caused elevation in SGPT, SGOT, ALP, total bilirubin, urea, and lactate dehydrogenase in the DC group which was prevented with pretreatment of prebiotics, probiotics, and synbiotics. Total protein was decreased in serum and tissues after administration of CCl₄ which was restored with pretreatment of test substances. Oxidative marker LPO was increased in the DC group and catalase (CAT) and GSH were also depleted in liver tissue. These changes were prevented in pretreated test groups. The histological change in hepatic parenchyma and hepatocytes was minimal in test groups compared to DC. The 16S amplicon sequencing indicated that *Lactobacillus acidophilus* and prebiotic treatment effectively displaced *Staphylococcus*. Results suggested that the use of prebiotics, probiotics alone, and synbiotic of them has a protective effect against CCl₄-induced hepatotoxicity in rats.

INTRODUCTION

The liver is the largest vital organ that protects the body from various metabolic diseases and toxic substances. Exposure to xenobiotics and therapeutic reagents results in liver inflammation, necrosis, cirrhosis, fibrosis, and functional weakening of the liver (Ashoush *et al.*, 2013; Eidi *et al.*, 2012; Sun and Karin, 2008). Liver cirrhosis is the 11th most common cause of death and liver cancer is the 16th major cause of death worldwide (Asrani *et al.*, 2019). Therefore, there is a need to prevent hepatic fibrosis which

is a basic pathological factor involved in cirrhosis and liver cancer (Jiao *et al.*, 2009).

Increased oxidative stress in the liver is responsible for hepatic fibrosis. Management of oxidative stress is useful in protection against hepatic fibrosis (Nieto *et al.*, 1999). Carbon tetrachloride (CCl₄) is responsible for increasing oxidative stress in liver tissue and causes injury to the liver, and, therefore, is used as an animal model for evaluation of the effectiveness of drugs against liver fibrosis (Hernández-Muñoz *et al.*, 1990; Neubauer *et al.*, 1998; Poli, 2000; Reeves and Friedman, 2002).

Probiotics have been considered to play an important role in the reduction in risk of diseases by restoring gut flora and improving liver enzymes, inhibition of harmful bacteria by alteration of the intestinal environment, synthesis of vitamins, and depleting cholesterol levels (Bang *et al.*, 2014; Kirpich *et al.*, 2008). Probiotics are also being considered as helpful

*Corresponding Author

Kishori G. Apte, Symbiosis International University SIU,
Gram Lavale, Pune, India; APT Research Foundation, Pune, India.
E-mail: aptresearch@gmail.com

adjuvant therapy in the treatment of liver disease caused due to different factors such as alcohol, viral infections, and disorders of metabolism (Cesaro *et al.*, 2011; Loguercio *et al.*, 2005; Rishi *et al.*, 2009; Wang *et al.*, 2011).

Prebiotics are nondigestible oligosaccharides that help to stimulate the growth of beneficial bacteria in gut flora. Among these, inulin, fructooligosaccharide (FOS), and lactulose are well-known prebiotics (Lu *et al.*, 2018). Inulin is evaluated against CCl₄-induced hepatotoxicity (Liu *et al.*, 2015).

Synbiotics are a mixture of prebiotics and probiotics that help in the overall maintenance of beneficial gut flora along with better attachment and higher growth rate of the same. Synbiotics contribute to hepatic protection and better health management (Gibson and Roberfroid, 1995; Rishi *et al.*, 2009).

The presented study was designed, conducted, and evaluated for the hepatoprotective activity of probiotic strains of lactic acid bacteria *Lactococcus lactis* subspecies *cremoris* and *Pediococcus pentosaceus* isolated from curd and fermented wheat, respectively, as well as some procured strains of lactic acid bacteria like *Lactobacillus acidophilus* and *Lactobacillus plantarum* and prebiotics such as inulin, FOS, lactulose alone, and their combination, that is, synbiotic.

This study attempted to make effective synbiotic formulation as hepatoprotective nutraceutical by combinations of different prebiotics with probiotic organisms in an optimum proportion.

MATERIALS AND METHODS

Chemicals

We used MRS (deMan, Rogosa, and Sharpe broth; HiMedia, India); inulin (HiMedia, India); FOS (HiMedia, India); lactulose (TIS, Japan); potassium phosphate; disodium phosphate; triton X-100; CCl₄ (SRL, India); olive oil (SRL, India).

Microbial strains

The lactic acid bacteria *Lactococcus lactis* sp. *cremoris* (Ll) and *Pediococcus pentosaceus* (Pp) which were isolated from curd and fermented wheat, respectively, and isolated strains were identified at the Department of Microbiology, KEM Hospital, Pune, India.

The other two lactic acid bacteria used were *Lactobacillus plantarum* (Lp) National Centre for Industrial Microorganisms (NCIM) 2912, *Lactobacillus acidophilus* (La) NCIM 2285 which were procured from NCIM, National Chemical Laboratory, Pune, India.

Preparation of the microbial suspension

All the strains of lactic acid bacteria were grown in MRS broth (37°C, 18–24 hours). After incubation, cells were harvested by centrifugation (2,500 rpm, 15 minutes) with a serial wash of sterile saline and resuspended in 10 ml of sterile saline. The bacterial suspensions, for all the organisms, were freshly prepared.

Experimental animals

Adult Wistar rats (200–250 g) of both sexes were obtained from the National Toxicology Centre, Pune, in January

2016. Rats were randomized into different groups ($n = 6$) as follows. Animals were housed under standard environmental conditions of temperature $23 \pm 2^\circ\text{C}$, relative humidity $55\% \pm 10\%$, and 12 hours of light and dark cycle. The animals had access to food and water ad libitum. This study protocol was approved by the Institutional Animal Ethical Committee through research project number RP10/1415 and conducted according to the guidelines of The Committee for the Purpose of Control and Supervision of Experiments on Animals (Ministry of Environment and Forests, Government of India).

Experimental design

Animals were divided into the following groups:

Group 1: normal control (NC).

Group 2: disease control (DC).

Group 3: Test 1 (1×10^9 cfu/ml of *Lactobacillus acidophilus*).

Group 4: Test 2 (1×10^9 cfu/ml of *Lactobacillus plantarum*).

Group 5: Test 3 (1×10^9 cfu/ml of *Lactococcus lactis* subspecies *cremoris*).

Group 6: Test 4 (1×10^9 cfu/ml of *Pediococcus pentosaceus*).

Group 7: Test 5 inulin (10 mg/ml).

Group 8: Test 6 FOS (10 mg/ml).

Group 9: Test 7 lactulose (10 mg/ml).

Group 10: Test 8 synbiotic (1×10^9 cfu/ml of Test 1 to Test 4; 10 mg/ml of Test 5–Test 7).

Group 11: standard 1, Silymarin (100 mg/kg).

Group 12: standard 2, Synbiotic 2000 (1×10^9 cfu/ml).

In all treatments, microbial suspensions were orally fed for 28 days daily. On the 28th day, animals received a dose of CCl₄ (1 ml/kg, i.p.) (Park *et al.*, 2015). Animals were sacrificed after 24 hours of CCl₄ injection.

Biochemical estimation from serum

Approximately 3 ml of blood samples were collected at the terminal sacrifice of animals (Parasuraman *et al.*, 2010). The blood was allowed to clot at 37°C. The serum was separated by centrifugation at 2,500 Xg for 15 minutes. The serum was then subjected to measurement of serum glutamic pyruvic transaminase (SGPT), serum glutamic-oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP), total bilirubin, total protein, lactate dehydrogenase, and urea using commercially available kits (Delta, India) provided by the manufacturer's protocol.

Changes in organ weights

After sacrificing, animals were dissected and organs were collected. The liver, kidneys, and spleen were dissected and rinsed with ice-cold normal saline solution. The organs were weighed for their wet weights and stored in 10% formalin solution. The part of liver tissue was weighed and homogenized for evaluation of the oxidative markers. The relative organ weights were also recorded and calculated.

Biochemical estimation from tissues

Levels of catalase (CAT) and lipid peroxidation (LPO) were evaluated to determine the oxidative stress in various organs.

Preparation of tissue homogenate

Tissue samples were homogenized in 50 mM phosphate buffer (pH-7) in presence of 1% Triton X-100. The tissue homogenate prepared was 10% and used immediately for the evaluation of biochemical analysis.

Estimation of CAT

CAT levels were evaluated as described earlier (Aebi, 1984). Briefly, to the 2 ml of tissue homogenate, 1 ml of hydrogen peroxide was added to the homogenate. The change in absorbance of the resulting solution was measured at 240 nm for 30 seconds. The levels of CAT were calculated using the following formula:

mMol of H2O2 decomposed/min/gm

$$= \frac{\text{Abs at 0 Seconds} - \text{Abs at 30 seconds}}{\text{As of 30 mM H2O2}} \times \text{factor of tissue weight}$$

Estimation of LPO

LPO was evaluated by the method described by earlier (Ohkawa *et al.*, 1979). Briefly, to the 1 ml of tissue homogenate, 2 ml of trichloroacetic acid (TCA)-thiobarbituric acid-HCl reagent was added. Phosphate buffer served as blank instead of tissue homogenate. All tubes were then vortexed and kept in a water bath for 1 hour. Tubes were then allowed to cool down and centrifuged for 15 minutes. Supernatants were collected and absorbance was measured at 535 nm against the blank. LPO/gm of wet tissue was then calculated using the following formula:

$$\text{nmoles of LPO / gm of tissue} = \text{Absorbance} \times 1.56 \text{ M}^{-1} \text{ cm}^{-1}$$

Estimation of reduced glutathione (GSH)

GSH levels were estimated as described by Ellman (Ellman, 1959; Moron *et al.*, 1979). Briefly, the homogenate was prepared in TCA reagent. This homogenate was then centrifuged and the supernatant was collected. The supernatant was diluted with 2 ml of freshly prepared Ellman's reagent (5'5 dithiobis[2-nitrobenzoic acid] 10 mM, NaHCO₃ 15 mM) which was added. The absorbance of the resulting solution was measured at 412 nm. Standard GSH was run similarly. The standard calibration curve was plotted and the concentration of GSH from tissue was calculated based on the slope of the standard curve.

Estimation of total protein

Protein estimation was carried out by the earlier described method (Lowry *et al.*, 1951). Tissue homogenates were centrifuged and the supernatant was collected. To these supernatants, 5 ml of an alkaline sodium carbonate solution was added. The resulting solution was then mixed with 0.5 ml of Folin's reagent. The absorbance was recorded 30 minutes after the addition of Folin's reagent at 680 nm. The concentration of protein was evaluated from the standard calibration curve obtained from different concentrations of bovine serum albumin (50–250 µg/ml).

Intestinal microflora identification by RTPCR

Microbial analysis was carried out using real-time polymerase chain reaction (RT-PCR). The intestine was collected in a sterile Petri plate containing 15 ml sterile normal saline and washed gently to remove the blood part and other possible contaminants. Again, the same organ was taken in another sterile Petri plate containing fresh 15 ml sterile saline and softly scrapped the upper layer with a sterile scalpel. The intestines of the same group were collected and washed with sterile saline as above and all the washed solution of the same group was pooled. This solution was collected in a sterile centrifuge tube and pellet down at 1,500 rpm for 10 minutes and stored at –20°C until further analysis. Compositional analysis of the intestinal microbiota was done by EzBioCloud's Microbiome Taxonomic Profiling at Bioenergy group, at Agharkar Research Institute, Pune, India.

Histopathology

After completion of the dosing, schedule animals were sacrificed and dissected to harvest major organs such as the liver, kidneys, and intestine. The obtained tissues were fixed in formalin solutions and kept for fixing. After fixation, tissues were dehydrated and embedded in paraffin wax. The embedded tissues were then sectioned with a microtome (Ragavan and Krishnakumari, 2009). The sectioned tissues were stained by hematoxylin and eosin. The histological changes in tissues were then examined under a light microscope (Nikon). Liver tissues were observed for degeneration and necrosis of hepatocytes, infiltration of inflammatory cells, derangement of hepatic cords, and granular and vacuolar cytoplasmic changes. The case of intestine histopathological changes in mucosa and submucosa was observed along with necrotic and degenerative changes in enteric villi.

Statistical analysis

Results were expressed as mean ± SD. The data were analyzed by one-way followed by Tukey's multiple comparisons or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests. The results were considered as statistical significance at $p < 0.05$ in all the cases. The statistical test was performed by using GraphPad Prism 7.0.

RESULTS

Effect of treatments on change in body weight

Changes in body weight were recorded weekly. Two-way ANOVA revealed that the change in body weights was not significant on day 0 and day 7 of treatments. On day 14 onwards, there was a significant increase in body weight in group 4 ($p < 0.01$) and group 5 ($p < 0.05$) as compared to DC. A similar trend was observed on day 21 (group 4, $p < 0.001$; group 5, $p < 0.001$) and day 28 (group 4, $p < 0.001$; group 5, $p < 0.01$) of the experiment. Other groups showed increased body weight compared with the DC group (Fig. 1).

Effect of treatment on relative organ weight of liver, kidneys, and spleen

The relative organ weight of the liver, kidneys, and spleen was evaluated at the end of the study. Changes in relative

organ weights were not statistically significant in comparison with DC animals and also with NC group animals (Fig. 2).

Effect of treatments on levels of SGPT

The levels of SGPT were estimated to determine the extent of liver damage after exposure of CCl₄ administration in prebiotic, probiotic, and synbiotic pretreated rats. It was observed that the intraperitoneal injection of CCl₄ leads to an increase in levels of SGPT significantly in DC ($p < 0.001$) in comparison with the NC group. Tukey’s multiple comparison test revealed that all test treatments have statistically significantly decreased SGPT levels as compared to DC ($p < 0.001$) (Table 1 and Fig. 3A).

Effect of treatments on levels of SGOT

One-way ANOVA revealed that there was a significant ($p < 0.001$) increase in SGOT levels after CCl₄ administration in DC

group animals as compared to the NC group, thereby confirming the induction of liver damage. SGOT levels in treatment groups, Test 1 ($p < 0.001$); Test 2 ($p < 0.001$); Test 3 ($p < 0.05$); Test 4 ($p < 0.01$); Test 5 ($p < 0.001$); Test 8 ($p < 0.001$); Silymarin ($p < 0.001$); Synbiotic 2000 ($p < 0.01$) were decreased significantly in comparison with DC animals (Table 1 and Fig. 3B).

Effect of treatments on levels of ALP

The levels of ALP were found to be significantly elevated in the DC group in comparison with the NC group ($p < 0.001$), whereas the ALP levels were significantly decreased in all test groups in comparison with the DC group ($p < 0.001$) (Table 1 and Fig. 4A).

Effect of treatments on levels of total bilirubin

Total bilirubin levels were increased significantly ($p < 0.001$) in CCl₄-injected groups as compared to the NC group. The bilirubin levels were decreased in all test groups in comparison with DC group (Test 1, $p < 0.01$; Test 2, $p < 0.001$; Test 3, $p < 0.01$; Test 4, $p < 0.01$; Test 5, $p < 0.001$; Test 6, $p < 0.01$; Test 7, $p < 0.01$; Test 8, $p < 0.001$; silymarin, $p < 0.001$, and Synbiotic 2000, $p < 0.001$) (Table 1 and Fig. 4B).

Effect of treatments on levels of serum and liver total protein

Total protein levels were significantly decreased in the DC group in comparison with the NC group ($p < 0.001$), whereas in all test groups, the total protein content was significantly increased when compared with the DC group ($p < 0.001$) (Table 1 and Fig. 5A and B).

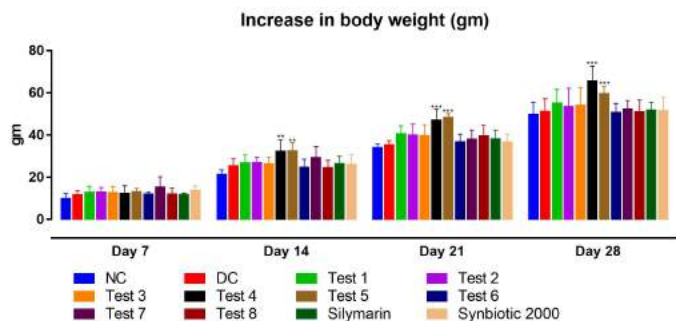


Figure 1. Effect of treatments on change in body weight.

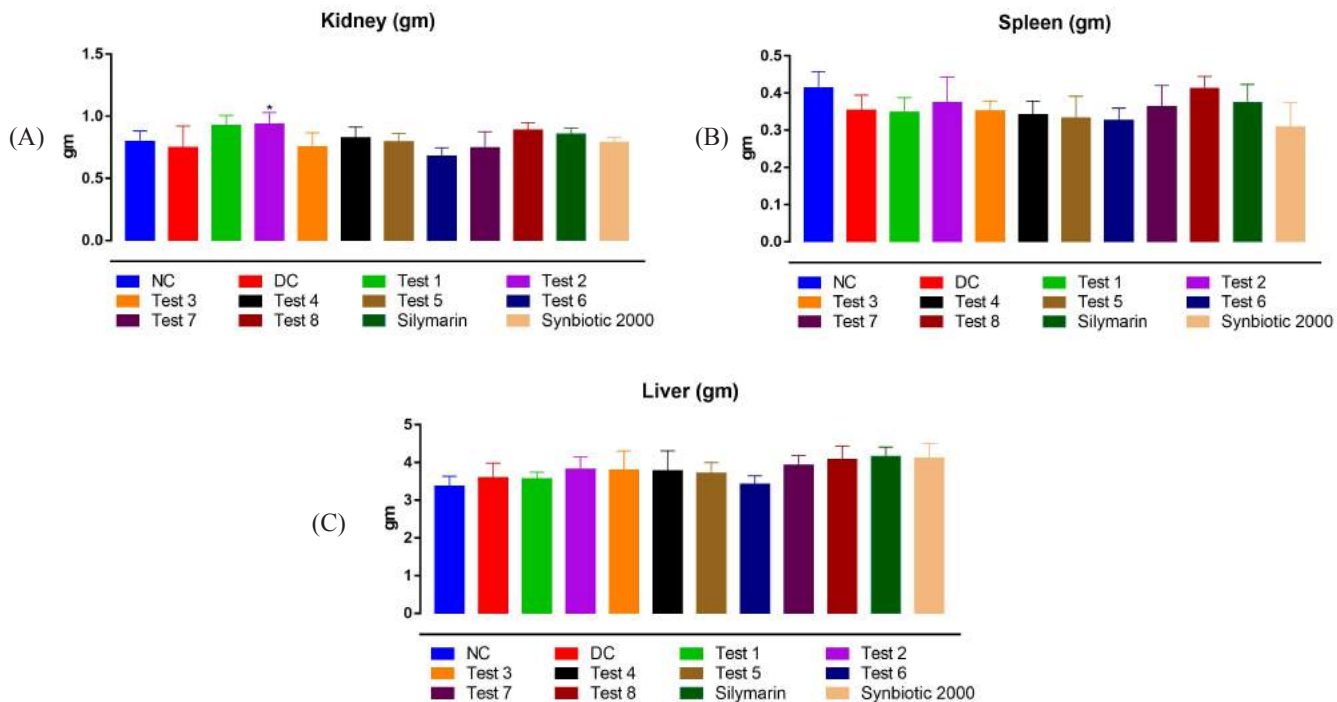


Figure 2. Effect of treatment on relative organ weight of liver, kidney, and spleen. Each bar represents the mean ± SEM of six observations (one-way ANOVA followed by Tukey’s post hoc test). NC = normal control; DC = disease control, T1–T4 = probiotics, T5–T7 = prebiotics; T8 = synbiotic.

Table 1. Effect of treatments on serum biochemical parameters.

	SGPT (U/l)	SGOT (U/l)	ALP (U/l)	Total bilirubin (mg/dl)	Serum total protein (mg/dl)	LDH (mg/dl)	Urea (mg/dl)
NC	54 ± 3.16***	97.17 ± 5.35***	150.7 ± 9.35***	0.093 ± 0.012***	6.78 ± 0.12***	215.5 ± 7.06***	19.55 ± 1.75***
DC	97.83 ± 4.45###	184.5 ± 8.55###	213.7 ± 5.5###	0.245 ± 0.044###	3.52 ± 0.23###	407.5 ± 13.32###	34.88 ± 4.54###
Test 1 (La)	68.83 ± 6.91***	132.5 ± 5.47***	164.2 ± 4.88***	0.133 ± 0.052**	7.1 ± 0.32***	320.7 ± 15.06***	26.5 ± 3.21**
Test 2 (Lp)	68.5 ± 4.72***	112.2 ± 4.07***	155.3 ± 4.32***	0.117 ± 0.041***	7.05 ± 0.25***	321.2 ± 11.13***	30.5 ± 6.19
Test 3 (Li)	71.5 ± 4.09***	116.7 ± 5.57***	161.3 ± 4.68***	0.133 ± 0.052**	7.17 ± 0.25***	335.5 ± 11.74***	26.17 ± 3.13**
Test 4 (Pp)	74.5 ± 2.88***	147.5 ± 4.23***	164.5 ± 7.4***	0.133 ± 0.052**	7.28 ± 0.23***	335.5 ± 8.29***	28.17 ± 4.67
Test 5 (Inulin)	63.17 ± 1.72***	127.5 ± 6.16***	158 ± 5.73***	0.117 ± 0.041***	7.02 ± 0.17***	306 ± 8.34***	22 ± 2.76***
Test 6 (FOS)	64.33 ± 3.2***	140.7 ± 5.65***	158.7 ± 4.8***	0.133 ± 0.052**	7.07 ± 0.19***	315.3 ± 10.75***	30.67 ± 3.08
Test 7 (Lactulose)	66.83 ± 3.06***	147.8 ± 5.98***	162.3 ± 3.78***	0.133 ± 0.052**	7.3 ± 0.24***	325 ± 9.38***	28.5 ± 2.81
Test 8 (synbiotic)	67.17 ± 3.97***	93.5 ± 6.44***	148.8 ± 5.98***	0.117 ± 0.041***	6.9 ± 0.14***	301.3 ± 1.75***	24.17 ± 1.47***
Silymarin	59.33 ± 2.66***	114.5 ± 6.09***	173.7 ± 6.22***	0.12 ± 0.032***	8.25 ± 0.22***	300.8 ± 8.18***	27.33 ± 2.98*
Synbiotic 2000	63.17 ± 5.04***	91.17 ± 3.97***	146.8 ± 3.66***	0.1 ± 0***	6.95 ± 0.1***	308 ± 9.08***	24 ± 2.61***

Values are expressed as Mean ± SD. Data were analyzed by one-way ANOVA followed by Tukey test.

NC = Normal control; DC = Disease control.

###*p* < 0.001 when compared to NC. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 when compared to DC.

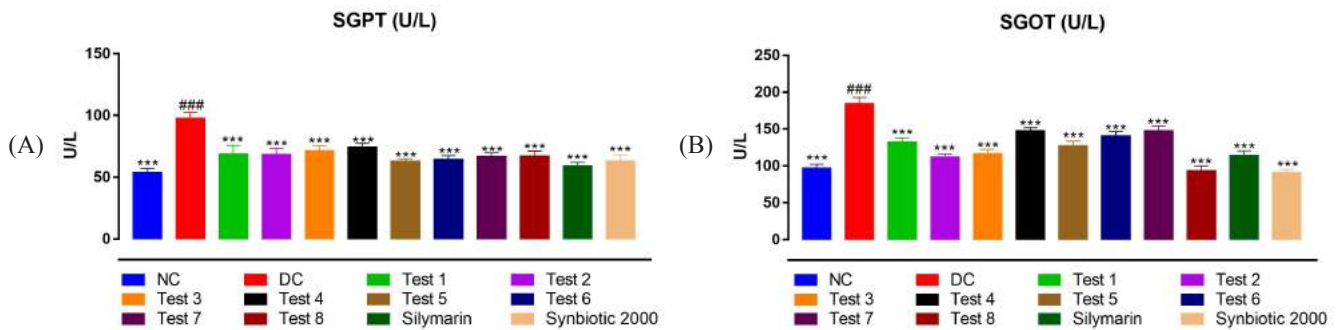


Figure 3. Effect of treatments on levels of SGPT and SGOT. Each bar represents the mean ± SEM of six observations. (A) SGPT levels in serum and (B) SGOT levels in serum. #*p* < 0.001 versus NC group; ****p* < 0.001 versus DC group (one-way ANOVA followed by Tukey’s post hoc test). NC = normal control; DC = disease control; T1–T4 = probiotics; T5–T7 = prebiotics; T8 = synbiotic.

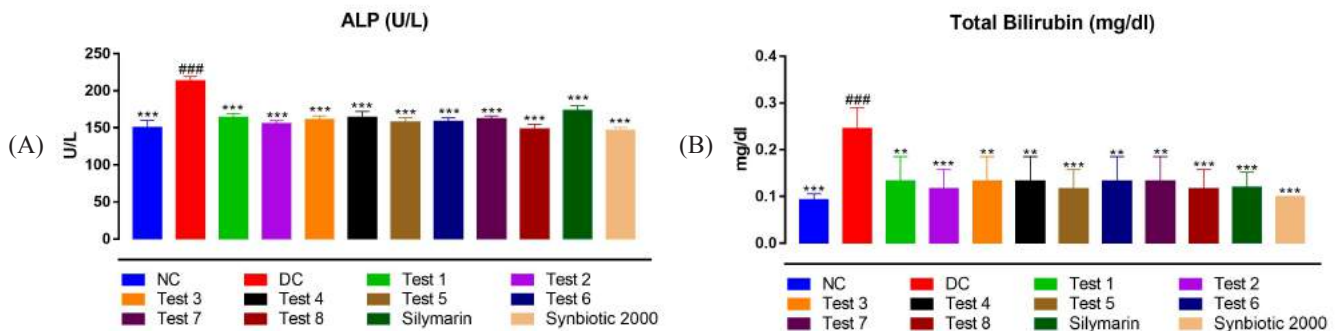


Figure 4. Effect of treatments on levels of ALP and total bilirubin. Each bar represents the mean ± SEM of six observations. (A) SGPT levels in serum and (B) SGOT levels in serum. #*p* < 0.001 versus NC group; ***p* < 0.01 versus DC group; ****p* < 0.001 versus DC group (one-way ANOVA followed by Tukey’s post hoc test). NC = normal control; DC = disease control; T1–T4 = probiotics; T5–T7 = prebiotics; T8 = synbiotic.

Effect of treatments on levels of lactate dehydrogenase

Lactate dehydrogenase (LDH) levels were significantly elevated in the DC group compared to the NC group (*p* < 0.001), whereas in all test groups, the lactate dehydrogenase levels were significantly decreased in comparison with the DC group (*p* < 0.001) (Table 1 and Fig. 6A).

Effect of treatments on levels of urea

Levels of urea were elevated significantly in DC (*p* < 0.001) as compared to NC. Test 1 (*p* < 0.01), Test 3 (*p* < 0.01), Test 5 (*p* < 0.001), Test 8 (*p* < 0.001), Silymarin (*p* < 0.05), and Synbiotic 2000 (*p* < 0.001) have shown a statistically significant decrease in the urea levels as compared to DC (Table 1 and Fig. 6B).

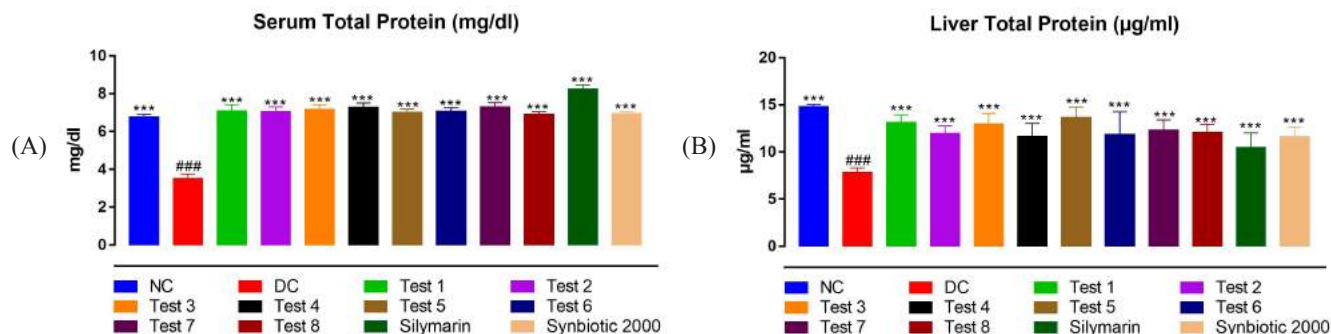


Figure 5. Effect of treatments on levels of serum and liver total protein. Each bar represents the mean \pm SEM of six observations. (A) SGPT levels in serum and (B) SGOT levels in serum. # $p < 0.001$ versus NC group; *** $p < 0.001$ versus DC group (one-way ANOVA followed by Tukey's post hoc test). NC = normal control; DC = disease control; T1–T4 = probiotics; T5–T7 = prebiotics; T8 = synbiotic.

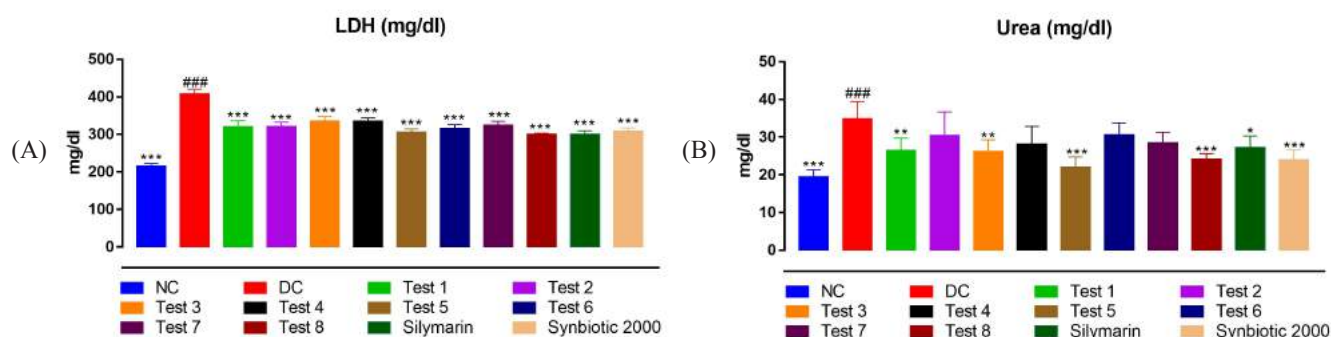


Figure 6. Effect of treatments on levels of lactate dehydrogenase and urea. Each bar represents the mean \pm SEM of six observations. (A) SGPT levels in serum and (B) SGOT levels in serum. # $p < 0.001$ versus NC group; * $p < 0.05$ versus DC group; ** $p < 0.01$ versus DC group; *** $p < 0.001$ versus DC group (one-way ANOVA followed by Tukey's post hoc test). NC = normal control; DC = disease control; T1–T4 = probiotics; T5–T7 = prebiotics; T8 = synbiotic.

Effect of treatments on levels of CAT

The CAT levels were significantly decreased in the DC group as compared to the NC ($p < 0.001$). In test groups, there was significant increase in CAT as compared to DC groups (Test 1, $p < 0.01$; Test 2, $p < 0.01$; Test 3, $p < 0.001$; Test 4, $p < 0.001$; Test 5, $p < 0.001$; Test 6, $p < 0.001$; Test 7, $p < 0.05$; Test 8, $p < 0.001$; silymarin, $p < 0.01$, and Synbiotic 2000, $p < 0.001$) (Fig. 7A).

Effect of treatments on levels of GSH

The GSH levels were significantly reduced in the DC group in comparison with NC ($p < 0.05$). In test groups, there was a significant elevation in GSH as compared to DC groups in Test 1 ($p < 0.05$), Test 2 ($p < 0.05$), Test 3 ($p < 0.05$), Test 4 ($p < 0.001$), Test 5 ($p < 0.001$), Test 7 ($p < 0.001$), Test 8 ($p < 0.01$), Silymarin ($p < 0.01$), and Synbiotic 2000 ($p < 0.001$) (Fig. 7B).

Effect of treatments on levels of LPO

The LPO levels were significantly increased in the DC group as compared to the NC ($p < 0.001$). In test groups, there was a significant reduction in LPO as compared to DC groups in Test 1 ($p < 0.001$), Test 2 ($p < 0.001$), Test 3 ($p < 0.001$), Test 4 ($p < 0.001$), Test 5 ($p < 0.001$), Test 7 ($p < 0.001$), Test 8 ($p < 0.05$), Silymarin ($p < 0.001$), and Synbiotic 2000 ($p < 0.001$) (Fig. 8).

Effect of treatment on histopathology of liver

Liver histopathology of NC group rats showed normal liver parenchyma comprised of hepatocytes arranged in a cord-

like pattern. Hepatocytes were in normal and uniform shape and size with normal morphology of the nucleus and cytoplasm. There was absence of any inflammatory changes throughout the section. After the administration of CCl_4 in rats, it was observed that there were moderate-to-severe pathological changes in hepatic tissue hepatocytes which showed degeneration and necrosis with cellular swelling and enlarged nucleus. Hepatic degeneration was with granular and vacuolar cytoplasmic changes and derangement of hepatic cords. All test treatment groups showed minimal pathological changes in hepatocytes. The focal basophilic areas of hepatocytes showed regeneration of histomorphological features of hepatic tissue. Hepatocytes showed focal areas of centrilobular degeneration with cellular swelling and enlarged nucleus (Fig. 9).

Effect of treatment on histopathology of kidney

Histopathology of kidney of normal rats showed intact renal parenchyma with renal tubules and glomeruli. The vascular tissue in the renal interstitial space, cortex, and medullary area was normal. The inflammatory or metabolic changes were absent throughout the section. After administration of CCl_4 , there were histopathological changes observed in renal tissues. Renal tubules showed the areas of degeneration and necrosis. Along with that cellular swelling and enlarged nucleus and formation of eosinophilic mass in tubular lumen was observed. The section showed focal areas of interstitial hemorrhages. All the treatment groups showed minimal histopathological changes in renal tissue, i.e. cortex and medulla. The mild degeneration of renal

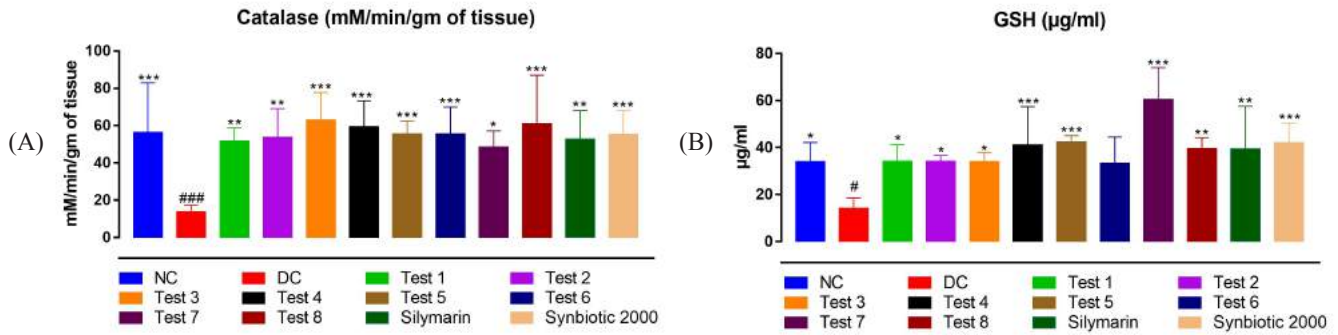


Figure 7. Effect of treatments on levels of CAT and GSH. Each bar represents the mean \pm SEM of six observations. (A) SGPT levels in serum and (B) SGOT levels in serum. $\#p < 0.001$ versus NC group; $*p < 0.05$ versus DC group; $**p < 0.01$ versus DC group; $***p < 0.001$ versus DC group (one-way ANOVA followed by Tukey's post hoc test). NC = normal control; DC = disease control; T1–T4 = probiotics; T5–T7 = prebiotics; T8 = synbiotic.

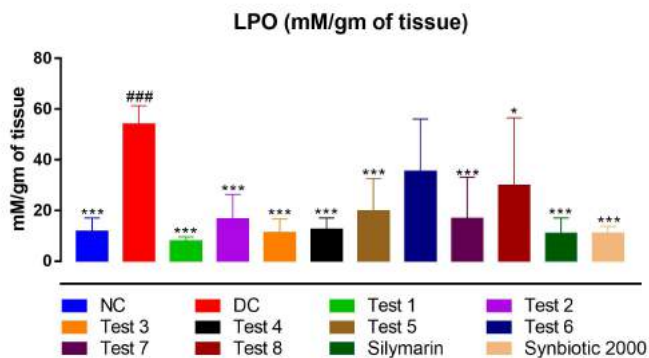


Figure 8. Effect of treatments on levels of LPO. Each bar represents the mean \pm SEM of six observations. (A) SGPT levels in serum and (B) SGOT levels in serum. $\#p < 0.001$ versus NC group; $*p < 0.05$ versus DC group; $***p < 0.001$ versus DC group (one-way ANOVA followed by Tukey's post hoc test). NC = normal control; DC = disease control; T1–T4 = probiotics; T5–T7 = prebiotics; T8 = synbiotic.

tubules in focal areas was observed. The focal areas of interstitial hemorrhages were observed (Fig. 10).

Effect of treatment on histopathology of intestine

Histopathology of intestine of normal rats showed normal histology of intestine with normal mucosa and submucosa. The mucosal epithelial tissue was intact and comprised of enterocytes and villi arrangement. The section was absent of any inflammatory and metabolic changes. The histopathology of DC rat showed severe pathological and metabolic changes in mucosa and submucosa in all the sections. There were diffused areas of degeneration and necrosis with the loss of enterocytes. The section was having multifocal areas of necrosis and degeneration with disarranged villi. The focal infiltration of inflammatory cells in mucosa and submucosa was observed.

All the treatment groups showed minimal histology changes of intestinal mucosa with occasional areas of degeneration of villi and necrosis with loss of villi. Hydropic changes of villi with increased goblet activity were observed minimally (Fig. 11).

Intestinal microflora identification by RTPCR

The highest number of amplicon sequence reads were obtained from the animal treated with inulin (1,25,833

amplicon reads), followed by animals treated with *Lactobacillus acidophilus* (1,04,069 amplicon reads). The least number of reads was obtained from the NC group which was not treated with any probiotic or prebiotic (5,783 amplicon reads). Taxonomic analysis of the V3 16S rRNA gene amplicon reads yielded a total of twelve major classifiable genera, two of which were dominant in the entire sample, namely, *Enterococcus* (1.81%–89.16%) and *Staphylococcus* (1.68–94.03). In the case of DC, the intestinal mucus harbored almost 94% of *Staphylococcus*, which was reduced to merely 4.6% by administration of *Lactococcus lactis*. This could be explained by the effective competitive displacement of *Staphylococcus* cells by *Lactobacillus* cells. The administration of prebiotic and FOSs also resulted in controlling the growth of *Staphylococcus*. Alpha diversities were calculated based on the formula suggested by Jost (2006), for Shannon diversity (Fig. 12).

DISCUSSION

This study was planned and conducted to evaluate the hepatoprotective activity of probiotics, prebiotics, and synbiotic isolated and identified at our lab. CCl_4 -induced acute liver injury in the rat is a widely used animal model to evaluate the hepatoprotective effect (Neubauer et al., 1998; Park et al., 2015). In the present study, we have evaluated the efficacy of probiotics *Lactococcus lactis*, *Pediococcus pentosaceus*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* (the first two were isolated from curd and fermented wheat, resp.) and prebiotics such as inulin, FOS, and lactulose alone as well as the synbiotic, that is, association of all above.

SGOT and SGPT are the hepatic metabolic enzymes that are released in the serum during the hepatic damage. These enzymes are considered to reflect the hepatic damage after the administration of hepatotoxic agents. It is widely accepted that CCl_4 gets accumulated into hepatic parenchyma cells where it gets converted into CCl_3 radical through cytochrome P450-dependent monooxygenase. This CCl_3 radical attacks polyunsaturated fatty acids and produces lipid peroxides which ultimately leads to alteration of hepatic enzyme levels such as SGPT, SGOT, ALP, LDH, and total bilirubin (Bishayee et al., 1995; Braide, 1991; Dwivedi et al., 1991; Taïeb et al., 2005; Yadav et al., 2008). These enzyme markers are leaked from hepatocytes due to disturbance in transport function which is a result of hepatic injury and altered membrane permeability (Paul et al., 2018; Zimmerman, 1970).

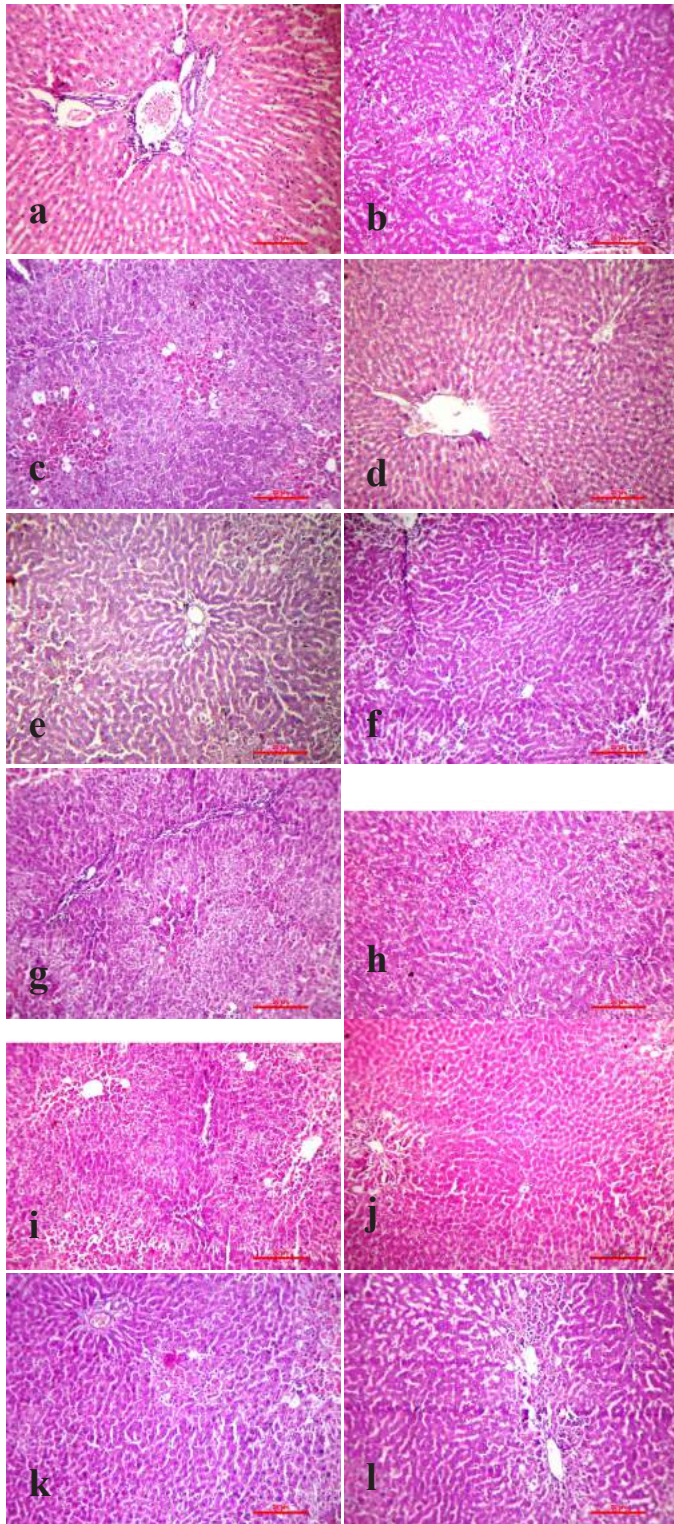


Figure 9. Effect of treatment on histopathology (liver). Each image is histopathology of the liver of an animal from the respective group. (A) NC, (B) DC, (C) T1, (D) T2, (E) T3, (F) T4, (G) T5, (H) T6, (I) T7; (J) T8, (K) Silymarin, (L) Synbiotic 2000. NC = normal control; DC = disease control; T1–T4 = probiotics; T5–T7 = prebiotics; T8 = synbiotic.

In agreement with this, we have observed similar results in our study that, after the administration of CCl_4 intraperitoneally, level of hepatic enzymes was elevated. This increase in levels of

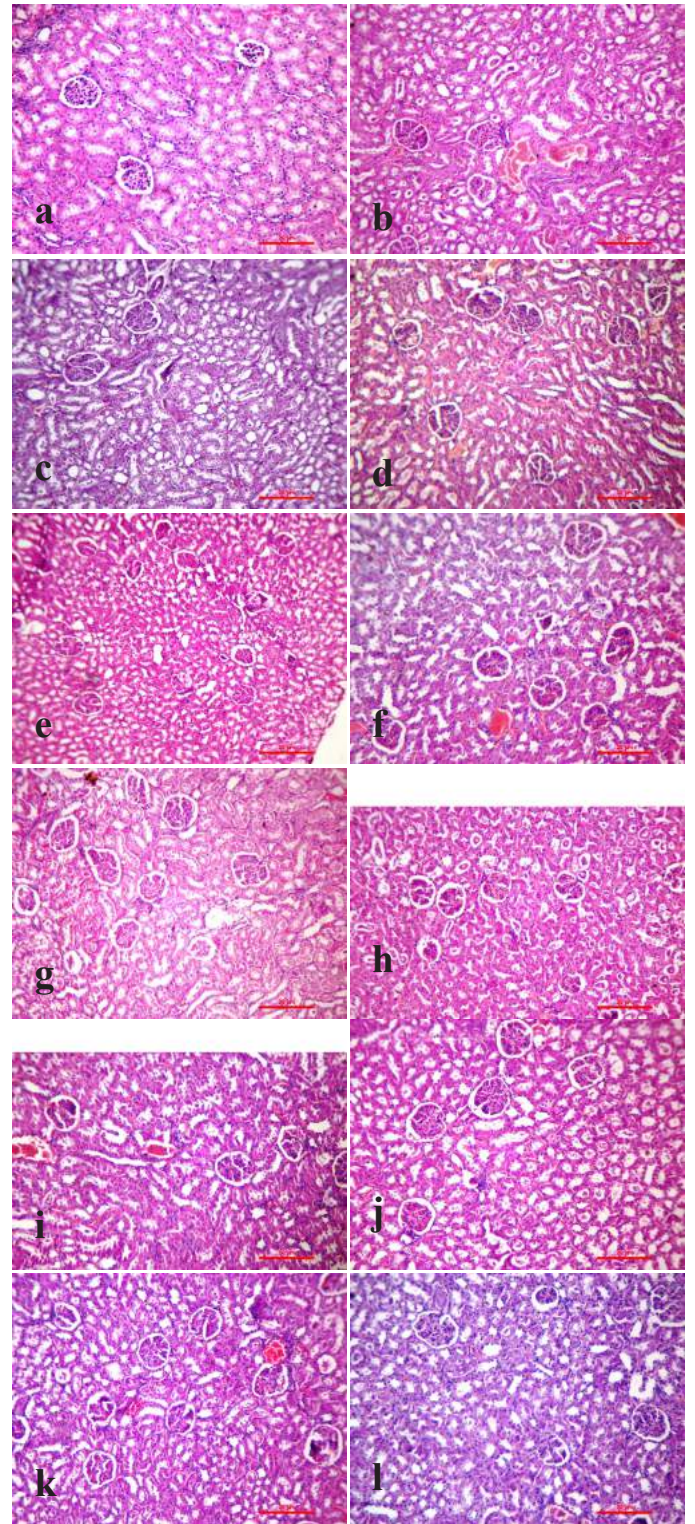


Figure 10. Effect of treatment on histopathology (kidney). Each image is histopathology of the kidney of an animal from the respective group. (A) NC, (B) DC, (C) T1, (D) T2, (E) T3, (F) T4, (G) T5, (H) T6, (I) T7; (J) T8, (K) Silymarin, (L) Synbiotic 2000. NC = normal control; DC = disease control; T1–T4 = probiotics; T5–T7 = prebiotics; T8 = synbiotic.

hepatic enzymes was prevented with the treatment of all the strains of probiotics used in this study, that is, *Lactococcus lactis*, *Pediococcus pentosaceus*, *Lactobacillus acidophilus*, and *Lactobacillus*

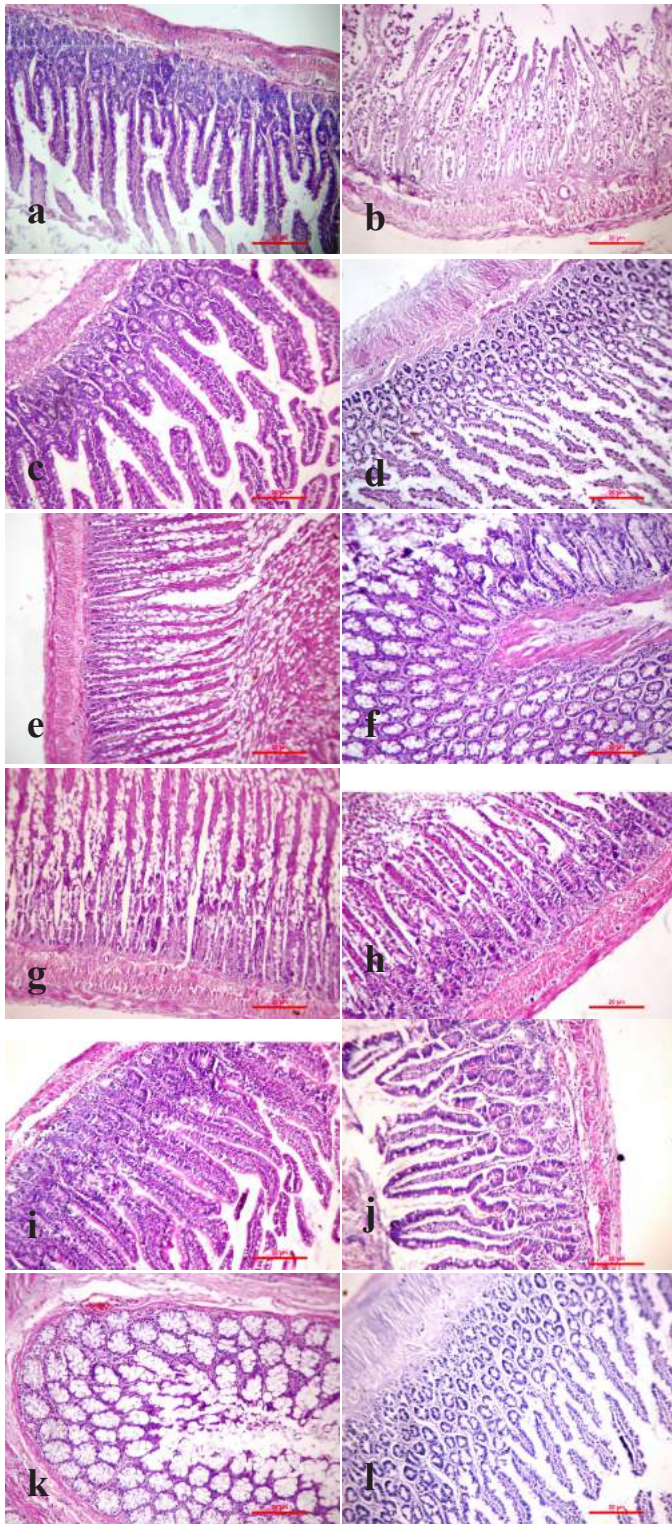


Figure 11. Effect of treatment on histopathology (intestine). Each image is histopathology of the intestine of an animal from the respective group. (A) NC, (B) DC, (C) T1, (D) T2, (E) T3, (F) T4, (G) T5, (H) T6, (I) T7; (J) T8, (K) Silymarin, (L) Synbiotic 2000. NC = normal control; DC = disease control; T1–T4 = probiotics; T5–T7 = prebiotics; T8 = synbiotic.

plantarum. The same phenomenon was observed in prebiotics, inulin, FOS, and lactulose and also in the synbiotic (the combination of all four probiotics and three prebiotics). This indicates that these

treatments are helpful in the prevention of changes in hepatic enzyme levels and the protection of the liver. Also, administration of these probiotics in the experimental animals did not show any adverse events during the study. The preliminary experiments carried for toxicity have not shown any signs of toxicity.

As mentioned above, CCl_4 radical generation alkylates hepatic cellular proteins and other macromolecules and contributes to the production of lipid peroxides through damaging polyunsaturated fatty acids and therefore causing hepatocellular necrosis (Bishayee et al., 1995; Brattin et al., 1985; Kouam et al., 2020; Taieb et al., 2005). Also, the overproduction of reactive oxygen species causes necrosis of hepatocyte through damage to DNA, proteins, lipids, and carbohydrates (Ahmad et al., 2009). Increased levels of liver LPO are indicative of hepatic damage and unmanaged antioxidant defense mechanism against the generated free radicals (Silveira et al., 2016). Thus, inhibition of free radical generation or increasing the levels of cellular antioxidants plays an important role in hepatoprotection against CCl_4 -induced liver toxicity. In the present work, we have observed similar results; namely, administration of CCl_4 caused increased levels of LPO and depleted levels of cellular antioxidants like CAT and GSH indicating increased oxidative stress at the cellular level and therefore hepatic necrosis pointing towards the induction of disease, that is, hepatotoxicity. The levels of cellular antioxidants were preserved due to the preadministration of probiotics, prebiotics, and synbiotics. Also, the levels of LPO were not much elevated in the preventive treatment indicating the management of free radicals. Thus, pretreatment with probiotics, prebiotics, and synbiotics is helpful in the management of oxidative stress produced due to free radical generation after induction of CCl_4 -induced hepatotoxicity.

Previously, *Lactobacillus Plantarum* isolated from Chinese yogurt has shown the upregulated expressions of SOD, GSH, and CAT and prevented the d-galactose induced oxidative aging. In agreement with this, *Lactobacillus plantarum*, *Lactococcus lactis*, *Pediococcus pentosaceus*, and *Lactobacillus acidophilus* have also shown upregulated levels of GSH and CAT with downregulated levels of LPO. This indicates that the administration of these probiotics and prebiotics as well as the symbiotic association of both prevents the oxidative stress caused by free radicals produced after CCl_4 administration and therefore prevents hepatic injury.

Also, in a similar context, it was demonstrated that probiotic (*Lactobacillus*, *Bifidobacterium*, *Clostridium*, and *Saccharomyces* species) administration improved the symptoms of CCl_4 -induced hepatic injury through the change in gut microbiota (Li et al., 2015; Liu et al., 2017). Our studies are in agreement with the above studies. Administration of probiotics and prebiotics causes displacement of *Staphylococcus* cells by *Lactobacillus* cells which was indicated through taxonomic analysis of the V3 16S rRNA gene amplicon reads.

Due to the failure of the gastric acid barrier, the gram-positive bacterial flora which mainly includes *Streptococcus* spp., *Staphylococcus* spp., *Micrococcus* spp., *Lactobacillus* spp., *Neisseria* spp., *Veillonella* spp., *Streptococcus* spp., *Gemella* spp., *Corynebacterium* spp., and *Actinomyces* spp. increases in the gastrointestinal tract. Along with this, the concentration of colonic flora (*Enterobacteriaceae*, *Enterococcus*, *Pseudomonas*,

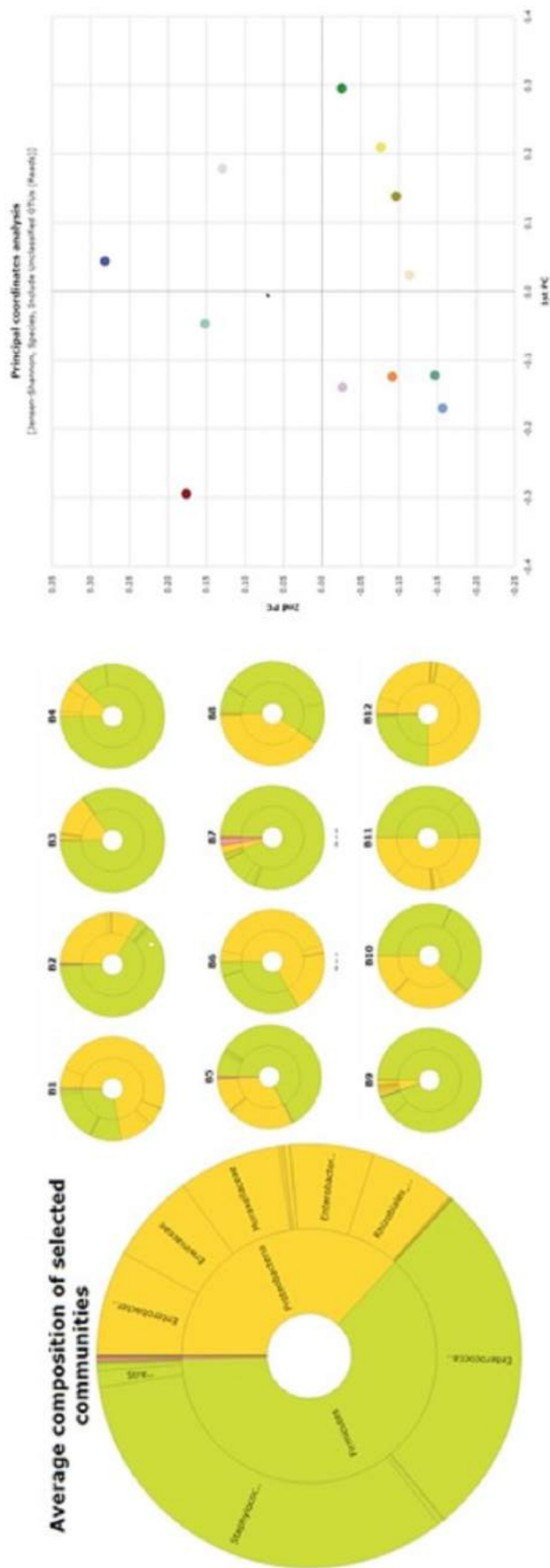


Figure 12. Identification of intestinal microflora by RTPCR.

and *Bacteroides*) also increases due to lower small intestinal motility (Bauer *et al.*, 2001). Furthermore, Bauer *et al.* (2002) demonstrated that the decreased small intestinal bacterial overgrowth and associated systemic endotoxemia are most common in liver cirrhosis. In a similar line, we have observed that *Staphylococcus*, one of the major parts of colonic flora responsible for endotoxemia, has been reduced and replaced by *Lactobacillus* and therefore helpful in attenuation of liver damage triggered due to CCl₄.

CONCLUSION

Our results suggest that the use of *Lactococcus lactis* subspecies *cremoris*, *Pediococcus pentosaceus*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* and prebiotics inulin, FOS, lactulose, and test synbiotic has a protective effect against CCl₄-induced hepatotoxicity in rats. The hepatoprotective activity is demonstrated through the management of levels of hepatic enzymes, total bilirubin, urea, and total protein levels. This hepatoprotection may be due to the administration of all organisms that caused the prevention of hepatic damage that occurred due to increased oxidative stress in hepatic cells after CCl₄ administration in rats as well as replacement of *Staphylococcus* by *Lactobacillus*.

As there were no adverse effects observed and no decrease in body weights and organ weights, blood chemistry parameters, oxidative stress parameters, and histologically showed improvement in the toxicity or damage induced by CCl₄ when compared with the DC group. All observations have shown a protective effect after administration of test substance for 28 days. It can also be concluded that the prebiotics, probiotics, and synbiotics are safe for use.

The data of the study will aid in formulating the better and effective combination of prebiotics with probiotic organisms having maximum hepatoprotective activity.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study protocol was approved by the Institutional Animal Ethical Committee through research project number

RP10/1415 and conducted according to the guidelines of The Committee for the Purpose of Control and Supervision of Experiments on Animals (Ministry of Environment and Forests, Government of India).

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