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**Research Article**

# Effect of synbiotic fermented milk on serum cholesterol level in diet-induced hypercholesterolemic rats

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## Abstract

**The current research project was undertaken to evaluate the hypocholesterolemic effect of the prepared functional fermented milk using two selected strains of Lactobacilli and one prebiotic (i.e., *Lactobacillus helveticus* MTCC 5463 (V3) and *Lactobacillus fermentum* (M5) added with corn-starch) based on short-chain fatty acid production, by *in vivo* experiment in healthy adult Wistar rats. The fermented product with Lactobacilli and prebiotic showed significant effects on serum lipid profile by an efficient decrease of 67.21% in serum cholesterol level, 66.21% in triglycerides level and 63.25% of LDL cholesterol level comparing with the rats that received cholesterol enriched (High-Fat) diet (HFD Model) only. Additionally, the fermented milk showed comparable results with that of rats fed with atorvastatin drug (Model). Thus, the developed functional fermented milk is able to manage the bad cholesterol without any side effects.**

**Key words:** Fermented milk, Hypercholesterolemia, Lactobacilli, Short-chain fatty acids, Synbiotics

## Highlights

- Synbiotic fermented milk prepared with *Lactobacillus helveticus* MTCC 5463 (V3) and *Lactobacillus fermentum* (M5) offers health benefits including lowering the cholesterol.
- Rats on high fat diet (HFD) had the highest triglycerides level, which was reduced significantly in rats fed with HFD + atorvastatin (HFD+STD), HFD + unfermented milk + corn starch (HFD+C) and HFD + fermented milk + corn starch (HFD+S).
- There was significant increase in HDL cholesterol in all the three treatment groups, but nonsignificant differences were observed in mean values of the HFD+STD, HFD+S and HFD+C groups.
- Liver from the HFD group showed widespread lipid vacuoles deposited inside the parenchyma cells. Liver sections from rest of the groups showed lesser micro-vesicular fatty changes as compared to HFD group.

## INTRODUCTION

Elevated serum cholesterol level can result in numerous hypertension diseases such as hypertension, coronary heart diseases and atherosclerosis. As per a survey carried out by the World Health Organization (WHO), CVD, by 2030 is said to emerge as the chief reasons of death along with people around 23.6 million affected globally (WHO, 2009). Several investigators stated in their reports that even a 1% reduction in the serum cholesterol level could cause 2-3% of decrease in the coronary heart disease (Manson *et al*., 1992). Some drugs have been already tested and proven to benefit in hypercholesterolemia. Nowadays, several non-pharmacological tactics (which consist of dietary ones) are known to be utilised in decreasing the serum cholesterol. Prebiotics that are added to probiotics are well-proved preventive measure to lessen the serum cholesterol level. Numerous drugs have been

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developed including statin drugs (atorvastatin and rosuvastatin) as the most common drugs to treat hypercholesterolemia. Though, continued repetition of these medications can result in extremely harmful side effects (Anandharaj *et al*., 2020).

Administration of synbiotics are stated as probiotics and prebiotics used together in combination. This blend is thought to offer the symbiotic actions and encourages the viability, numbers, and colonization of probiotic and the pre-existing advantageous microorganisms in the colon. Probiotic bacteria have numerous health advantages in many health ailments such as inflammatory bowel disease, diarrhoea, gastroenteritis, irritable bowel syndrome, depressed immune function, *Helicobacter pylori* infections, cancer, infant allergies, hyperlipidemia, hepatic diseases, and a few others (Brown and Valiere, 2004). Probiotics or their cofactors are known to be a good substitute for prebiotics, which are low-digestible starchy materials of food components like dietary fibers, may help in encouraging the probiotic microorganisms during their growth in the colon of the hosting bacterium (Crittenden and Playne, 1996; Manning and Gibson, 2004). In the colon, oligofructose is fermented due to the existence of intestinal microorganisms, which increases the probiotic inhabitants in gut microflora, shortens the gastrointestinal transport time, elevates the calcium absorption and faecal bulk, and most importantly lowers the serum cholesterol level and thereby confers the advantageous effects to the humans.

The key end products of microbial metabolism are Short-chain fatty acids (SCFA) in the large human intestine. Chiefly, they are formed from oligosaccharide, polysaccharide, protein, glycoprotein and peptide precursors by anaerobic micro-organisms (Cummings and Macfarlane, 1991). Fukuda *et al*., 2011 reported that acetate has been proven as a significant part of the process in the capability of Bifidobacteria to hinder Enteropathogens. Furthermore, Jung *et al*. (2015) reported that butyrate fuels the epithelial cells in the intestine and elevates mucin production which may affect in variations of the microbial adhesion and enhanced tight-junctions integrity (Peng *et al*., 2009). Therefore, the SCFA production seems to display a significant part in the protection of the gut barrier function.

Short chain fatty acids reduce the formation of cholesterol and also capable to fuel the elimination of cholesterol by elevating the secretin production, which results in the hepatocytes excreting more cholesterol-containing bile (Shimizu *et al*., 1987). In several studies it has been reported that the feeding of SCFAs or dietary fibre to humans and rats both results in a decrease in the cholesterol concentrations of plasma (Hardie and Pan, 2002; Fushimi *et al*., 2006). Moreover, a dose-dependent, significant decrease in body weight in subjects with obesity was observed when short-chain fatty acids were administered daily throughout a month (Demigne *et al*., 1995).

## MATERIALS AND METHODS

*In vivo* study was conducted in order to evaluate the hypocholesterolemic properties of the prepared fermented milk using *Lactobacillus helveticus* MTCC 5463 (V3) and *Lactobacillus fermentum* (M5) added separately at 2% level and prebiotic corn-starch (CS) at 3% level using rats as animal model. Probiotic lassi was prepared as per the method of Patidar and Prajapati (1998) with slight modification. Five groups containing six animals in each group were created.

**Animals:** The study was permitted by the Institutional Animal Ethical Committee (IAEC) of Pioneer Pharmacy Degree College, Vadodara (OGECT/PPDC/IAEC/2019/17/1). Wistar rats (male albino) weighing 180-200 g were obtained from the registered breeder and were kept in cages (six animals per cage) in proper environmental conditions at 25°C, 12 h light/ dark cycle housed. The relative humidity was maintained between 50-55%. The animals were

allowed to have free access to water and standard laboratory pellet *ad libitum*.

**Grouping of animals:** The animals were alienated into five groups as given below:

Group 1 - Rats on normal saline (Control group) + basal diet (NC),

Group 2 - High-fat diet (HFD) fed rats + Normal saline (HFD),

Group 3 - HFD fed rats + Standard Drug - Atorvastatin (10 mg/kg) (HFD+STD),

Group 4 - HFD fed rats + (Unfermented milk + corn-starch, 1 mL/animal/day) (HFD+C),

Group 5 - HFD fed rats + (Fermented milk + corn-starch, 1 mL/animal/day) (HFD+S).

**Feeding and collection of samples:** The hyperlipidaemia was induced in all groups except group 1 (i.e. normal control (NC) group) by feeding HFD with 0.5% cholesterol, 1% cholic acid, 20% groundnut oil for 28 days. Basal diet was composed of crude protein (min. 14%), crude fibre (max. 4%), crude fat (min. 5%), Nitrogen-Free Extract (NFE -min. 60%), calcium (1%), phosphorous (0.5%) in pallet form (size 12 mm). The blood was withdrawn from retro-orbital plexus of animals on 14$^{th}$ day and 28$^{th}$ day for the estimation of glucose level. Simultaneously serum was parted and utilised for the estimation of total cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol. The serum samples were kept at -80°C till analysis. At the end of the study on 28$^{th}$ day, from each group, one rat was selected for liver tissues extraction. The extracted tissues were instantly placed in 10% formalin solution and it was used for histopathological evaluation.

**Biochemical and histopathological analysis:** The serum levels of total cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and total protein were estimated using the enzymatic colorimetric method of diagnostic kits by Beacon (Bucolo and David, 1973). Very low-density lipoprotein (VLDL) was calculated by using Freidewald's formula (VLDL = TG/5).

For histopathological analysis, carefully chosen animals were foregone and under ethyl ether anaesthesia, livers were hurriedly removed and transferred in 10% neutral buffered formalin for 72 h. Over nightly, the fixed specimens of liver tissue were kept for dehydration, cleaned and then permeated using an automatic tissue processor. The tissue samples were securely fixed into paraffin blocks. Implanting station and rotary microtome were used for cutting the slices of 4-micron thickness. Hematoxylin and Eosin staining for the sliced samples were carried out using an Autostainer. Then, under light microscopy the stained slices were tested and essential images were captured by digital microscopic camera (Nikon, Japan).

**Statistical analysis:** Data were analysed by Completely Randomized Design (CRD) and Factorial Completely Randomized Design (FCRD) as per the protocol prescribed by Steel and Torrie (1960). The influence of each parameter on the particular characteristic was determined at 5% level of significance. The microbial counts were transformed to log before evaluations.

## RESULTS

SCFAs (acetate, propionate and butyrate) production ability was checked during reconstituted skim milk (RSM) fermentation by Lactobacillus cultures. The average content of acetic acid was 1.20 µg/mL at 0 h, which increased to 1.53 and 1.93 µg/mL, respectively after 12 and 24 h of incubation. Cultures M5 and NK9 showed relatively higher amount of acetic acid after 24 h. Overall propionic acid production ranged from 1.44 µg/mL (M10 at 0 h) to 3.42 µg/ mL (V3 at 24 h). M5 and M31 produced significantly higher propionic acid than the rest of the cultures. It was observed that butyric acid produced by the cultures M5 (2.09 µg/mL) was highest followed by

09 (2.08 μg/mL) and M31 (2 μg/mL). Bile deconjugation ability of the Lactobacillus cultures was checked. Cultures V3 (376 μg/mL), NK9 (369 μg/mL) and M5 (368 μg/mL) showed much higher bile deconjugation ability as compared to rest of the cultures. The reduction in cholesterol (cholesterol assimilation ability) was significantly (P<0.05) higher in culture NK9 (17.84%) followed by V3 (14.33%), M31 (12.6%), 09 (10.48%), M5 (10.15%), NK10 (9.17%), M16 (8.82%), M10 (8.15%), NK2 (6.7%) and M38 (6.1%). It was observed that 09, NK2, NK9 had very good BSH activity. For determining survival of LAB in gastric juice, all the LAB cultures were exposed to different pH for different time intervals and Lactobacillus counts (log cfu/mL) for the all Lactobacillus cultures were measured at 0, 2 and 4 h of incubation at 37°C. It was observed that the Lactobacillus counts (log cfu/mL) were significantly (P<0.05) lower for pH 2, up to 4 h of incubation. It was also observed that the Lactobacillus counts (log cfu/mL) was lower for pH 3, up to 4 h of incubation. At pH 2, and after 2 h of incubation, the survival was 66%, while after 4 h, it reduced to 59%. At pH 3, the survival was 70.55% and after 4 h of exposure, it reduced to 67%. Cultures V3 (*L. helveticus*), M5 (*L. fermentum*), NK9 (*L. casei*), M31 (*L. rhamnosus*) and 09 (*L. acidophilus*) were selected for further study on the basis of highest SCFAs production, bile deconjugation ability, cholesterol assimilation ability, BSH activity and survival in gastric juices.

Four prebiotics i.e. inulin, corn-starch (CS), β-glucan and resistant starch (RS) (amylum) were considered for this study. Each of these prebiotics were added at the rate of 3% in reconstituted skim milk powder (RSMP) individually and then fermented with the selected LAB cultures and assessed for their SCFAs production ability. Overall acetic acid (μg/mL) production ranged from 1.1 to 4.7 μg/mL. The mean of acetic acid (2.91) was significantly (P<0.05) higher for corn-starch, compared to β-glucan (2.35), amylum (RS) (2.23) and inulin (2.0). The highest mean observed for cultures was for M5 and 09 (2.65), followed by V3 (2.50) and M31 (2.18) and NK9 (1.90). The amount of propionic acid produced by different cultures ranged from 1.2 to 2.9 μg/ mL. The amount also increased with the increase in period, while the effect of prebiotic was found to be non-significant. However, the cultures had variable effect on production of propionic acid. The highest production was by M5 (1.91 μg/ mL), followed by V3 (1.75 μg/ mL), while the rest three cultures were at par in terms of propionic acid production. Overall, butyric acid production ranged from 1.2 to 3.1 μg/mL. The mean of butyric acid was significantly (P<0.05) higher (2.12 μg/mL) for corn-starch followed by inulin (1.94), β-glucan (1.87) and amylum (1.74). The mean observed for cultures was significantly higher for M5 (2.13), followed by V3 (2.03), NK9 (1.83), M31 (1.81) and 09 (1.79). The SCFAs production was evaluated after fermenting reconstituted skim milk (RSM) with five chosen Lactobacillus cultures added with selected prebiotic cornstarch at different rates (1, 3 and 5%). LAB cultures and period showed significant (P<0.05) difference in acetic acid production, whereas the rate of addition did not show any significant (P>0.05) difference. The mean for acetic acid production was higher for V3 (4.70 μg/mL), followed by NK9 (4.40), M5 (4.37), 09 (4.30) and M31 (4.23). Culture V3 had highest production of acetic acid, which was significantly higher than all other cultures. The average acetic acid production by M5, M31 and 09 was at par. Addition of CS increased the production of acetic acid marginally and it ranged from 5.25 to 5.54 μg/mL after 24 h of fermentation. The production significantly increased at 24 h, as compared to 12 h of incubation. The effect of cultures, period and rate of CS addition on propionic acid production was found to be statistically significant (P<0.05). The mean value for propionic acid production was higher for the culture V3 (2.0 μg/mL), followed by M5 (1.84), M31 (1.73), NK9 (1.68) and 09 (1.29).

The mean for propionic acid production was found higher at 5% rate of addition (1.79), followed by 3% (1.72) and 1% (1.61). The propionic acid production has significantly increased (P<0.05) after 12 and 24 h regardless of all the cultures. The butyric acid production (μg/mL) differed significantly (P<0.05) by the cultures and increased upon period of 12 h and 24 h. The effect of level of CS also differ significantly (P<0.05) for the butyric acid production. The highest mean found for butyric acid production was at 5% rate of addition for CS (3.46 μg/mL), as compared to 1% rate (3.36), while 3 or 5 % addition rate were at par in terms of butyric acid production. The mean observed for butyric acid production was higher for V3 (3.56), followed by M5 (3.40), M31 (3.39), 09. SCFA aids in intestinal health, can invade pathogenic adherence and thus promotes overall health. From the above-mentioned results, cultures V3 and M5 along with the prebiotic corn-starch were selected for further product development.

The blood samples were obtained from retro-orbital plexus of the animals on 14th day and 28th day for the estimation of glucose level, simultaneously serum was separated and used for the estimation of total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) cholesterol and low density lipoprotein (LDL) cholesterol using the enzymatic colorimetric method of diagnostic kits.

**Effect of fermented milk on total cholesterol level:** Total cholesterol levels of rats fed with particular diets (NC, HFD, HFD+STD, HFD+S and HFD+C) was as shown in Fig. 1. HFD rats group showed significant (P<0.05) increase in serum TC in comparison with NC group. HFD+STD, HFD+S and HFD+C group exhibited significantly (P<0.05) reduced serum cholesterol level in comparison with HFD group. The effect of period of feeding on TC was significantly (P<0.05) different in all the groups. However, HFD+C group showed decrease in TC after 28 days, whereas the other groups showed increase in TC.

**Effect of fermented milk on triglycerides level:**

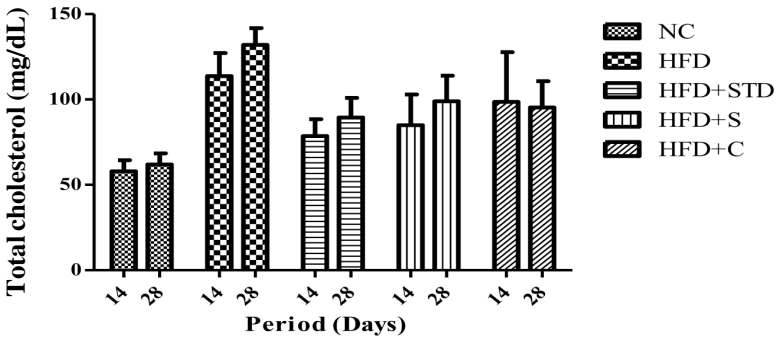



**Fig. 1. Changes in total cholesterol at 14 and 28 days**

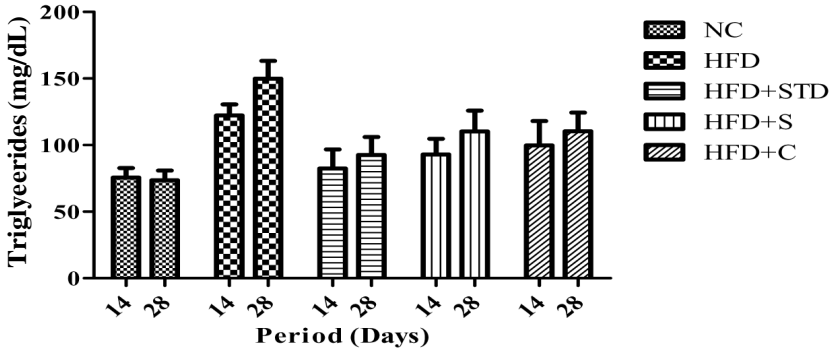



**Fig. 2. Changes in triglycerides at 14 and 28 days**

The changes in blood triglyceride content of rats fed with different diets (NC, HFD, HFD+STD, HFD+S and HFD+C) are shown in Fig. 2. The results as compared to the NC group, proved a significant (P<0.05) increase in the TG levels in the HFD group. The other three groups showed significant decrease (P<0.05) in serum TG levels as compared to levels in the HFD group. The lowest mean value was observed for HFD+STD group, which significantly differs (P<0.05) with HFD+S and HFD+C groups.

**Influence of fermented milk on HDL level:** Fig.3 illustrates HDL cholesterol concentrations in serum of rats fed different diets (NC, HFD, HFD+STD, HFD+S and HFD+C). The serum HDL cholesterol concentration in HFD group was significantly lower (P<0.05) as compared to the NC group. There was no significant difference observed in mean value for HDL level of the HFD+STD, HFD+S and HFD+C groups.

**Effect of fermented milk on LDL level:** As per Fig. 4, LDL cholesterol significantly (P<0.05) increased in the HFD group in comparison with the other four groups. HFD+STD, HFD+S and HFD+C groups exhibited significantly decreased (P<0.05) LDL level in rats of these groups as compared with the rats fed HFD; however, HFD+C was more effective followed by HFD+S and HFD+STD for reducing LDL.

**Effect of fermented milk on VLDL level:** The VLDL cholesterol levels of rats fed with different diets (NC, HFD, HFD+STD, HFD+S and HFD+C) is shown in Fig. 5. HFD group animals had significantly (P<0.05) elevated VLDL cholesterol levels as compared to animals in the NC group. In the HFD+STD, HFD+S and HFD+C groups compared with those in the HFD group, where the VLDL level was significantly declined (P<0.05).

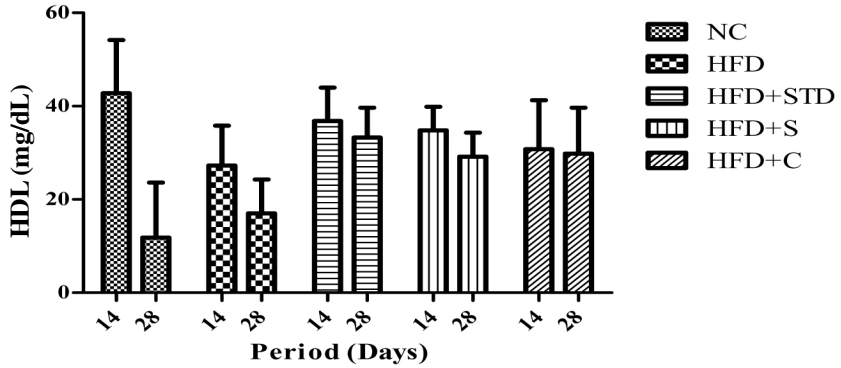



**Fig. 3. Changes in HDL at 14 and 28 days**

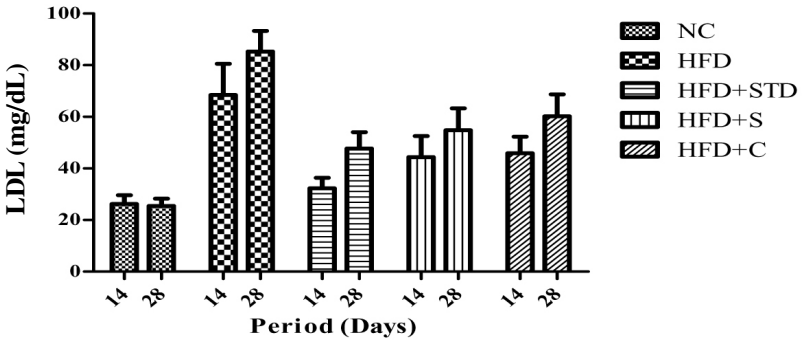



**Fig. 4. Changes in LDL at 14 and 28 days**

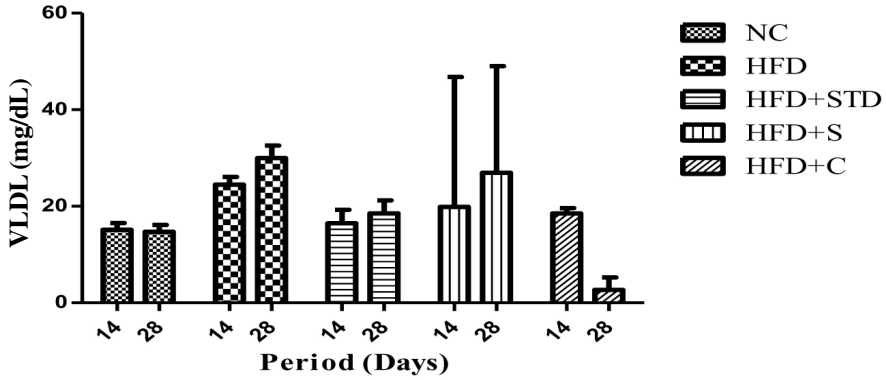



**Fig. 5. Changes in VLDL at 14 and 28 days**

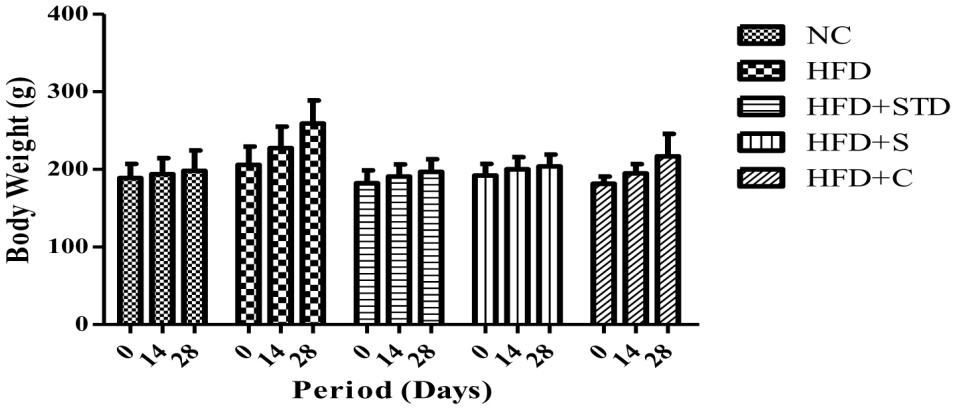


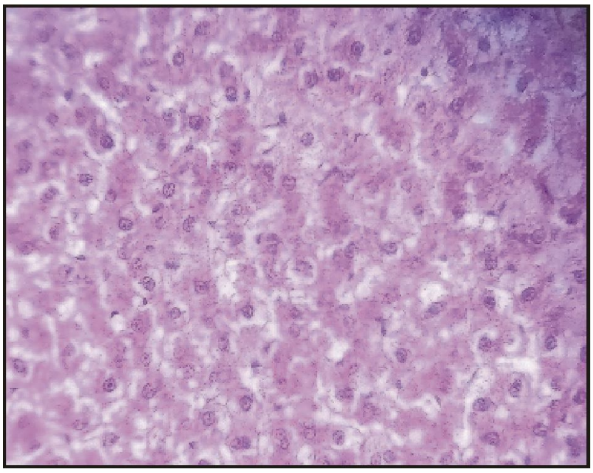

**Fig. 6. Changes in body weight at 14 and 28 days**

**Effect of fermented milk on body weight of animals:** The results as per Fig. 6 showed that the fermented as well as non-fermented products helped in controlling the body weight of HFD fed diet. In a study, where sour milk and SL (*S. thermophilus* + *Lactobacillus delbrueckii*) milk were fed to all groups, there was no significant (P>0.05) difference observed by Xiao *et al.* (2003) in body weight. In a Similar study by Chiu *et al.,* 2006, hamsters were fed fermented milk and high-cholesterol diet which resulted in no significant (P>0.05) difference in body weight.

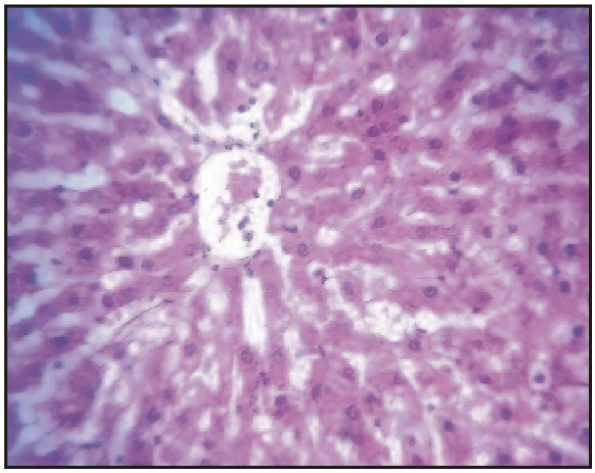
**Histopathological analysis of liver:** The effects of products on liver histopathology of rats were evaluated. Hematoxylin and Eosin (H&E) stained sections of livers are portrayed in Fig. 7 as 1) NC, 2) HFD, 3) HFD+STD, 4) HFD+S and 5) HFD+C. The histology of the liver seemed usual in animals of NC group. Liver of the HFD group displayed widespread lipid vacuoles deposited inside the parenchyma cells. Liver sections from rest of the groups showed lesser micro-vesicular fatty changes as compared to HFD group, and the liver tissues of HFD+S was looking better than HFD+STD and HFD+C groups.

## DISCUSSION

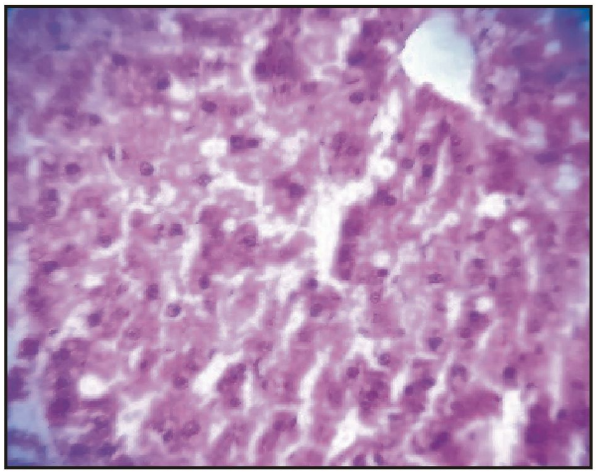
**Effect of fermented milk on total cholesterol level:** The results showed that animals in the HFD+STD group were lowest in serum total cholesterol after 28 days of study, which is almost at par with the groups HFD+S and



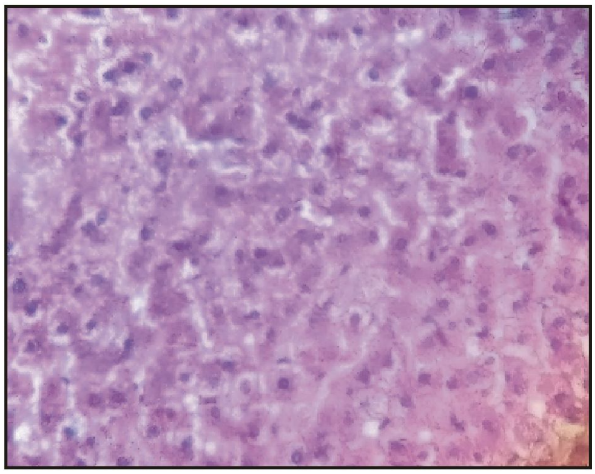
1) NC group



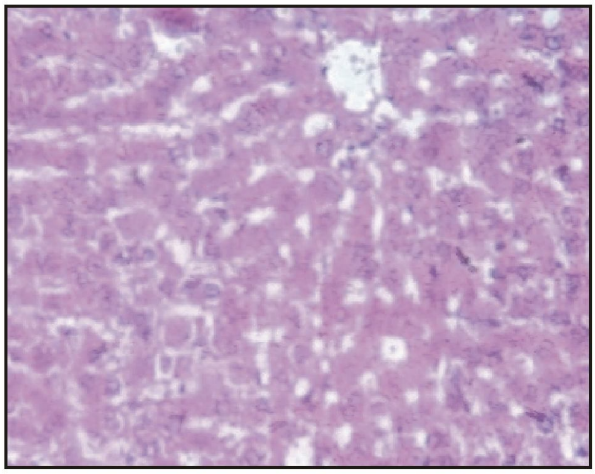
2) HFD group



3) HFD+STD group



4) HFD+S group



5) HFD+C group

Fig. 7. Hepatotoxicity in rats' liver tissue in various groups

HFD+C. Similar to our study, Guo and Li (2013) reported that when compared with levels in control group of rats that fed a high-cholesterol diet, rats fed high doses of *L. casei* F0822 had significantly ($P<0.05$) reduced serum TC concentration. Similarly, as per Hassanein *et al.* (2013), the TC levels in the plasma of rats fed yoghurt prepared using *L. lactis* K F 147 was lower as compared to the TC level of control group rats. Our results indicate that, our both the products help in controlling the cholesterol level. The reduction in cholesterol was statistically at par with our fermented milk sample and the standard drug atorvastatin. However, the effect shown by controlled product was not comparable to the standard drug.

**Effect of fermented milk on triglycerides level:** Levels of TG in all the groups except normal control increased upon time of storage on 14 and 28 days. On an average, the standard drug and both the products reduced serum TG. However, the effect of standard drug was significantly superior to both the products. A study (Kaur *et al.*, 2002) on the decrease of cholesterol demonstrated that total cholesterol was decreased by 38% when *Lactobacillus reuteri* CRL 1098 is fed to mice at the rate of $10^4$ cells per day for 7 days, which resulted in reduction of 40% in triglycerides. Causey *et al.* (2000) carried out a crossover, randomized and double-blind study using hypercholesterolemic subjects to evaluate the effects of inulin (extracted from chicory root) on blood cholesterol levels of subjects which comprised of twelve men, who were randomly allocated to two groups, viz. the control group (which spent one pint of vanilla ice-cream without inulin) and the inulin group (which spent one pint of vanilla ice-cream containing 20 g of inulin) and after study of 3 week, they stated that the significant ($P<0.05$) decrease in serum triglycerides were achieved by everyday ingestion of 20 g of inulin. In another study, the average plasma TG levels were shown reducing by 40% using dual probiotic supplementation (*L. plantarum* KY1032 and *L. curvatus* HY7601) when compared with rats' placebo treatment (Park *et al.*, 2013). As per Ahn *et al.* (2015) there was reduction in TGs and increase in the LDL particle size in hypertriglyceridemic subjects through 12 weeks ingestion of two probiotic strains.

**Influence of fermented milk on HDL level:** A significant increase in HDL level was noticed in other groups as compared to HFD group. This indicated that our products can increase HDL cholesterol as par with standard drug. Similar to our results, Al-Sheraji *et al.* (2012) found that rats which fed yogurt cultured with *B. pseudocatenulatum* G4 and *B. longum* BB536 had elevated HDL cholesterol levels. Liong *et al.* (2007) also reported similar discoveries in hypercholesterolemic pigs which were ingested with a synbiotic preparation for 8 weeks consisting of *L. acidophilus*, inulin, fructooligosaccharide and mannitol.

**Effect of fermented milk on LDL level:** With respect to LDL cholesterol, our both the products showed better control over HFD diet. However, the effect was not as good as the standard drug given along with HFD diet. These results are similar with results from Chiu *et al.* (2006) who also have reported that for reduction of LDL in blood serum of hamsters, *Lactobacillus* containing fermented milk proved to be high-efficiency. As per Rodriguez-Figueroa *et al.* (2013), spontaneously hypertensive rats (SHR), when consumed with fermented milk consisting of *L. lactis* NRRL B-50572 and *L. lactis* NRRLB-50571, resulted in a decreased level of LDL by 8.4 mg/dL and 21.9 mg/dL, respectively, when compared with that of LDL given water to SHR rats.

**Effect of fermented milk on VLDL level:** Compared with NC animals, these group animals exhibited no significant differences in the levels of VLDL. Mortensen *et al.* (2002), in their study, fed forty male mice with a purified diet for 16 weeks which also consisted of 10%

of long-chained fructan. The researchers observed a significant reduction in LDL-cholesterol level by 25.9% (PÂ0.01), blood cholesterol by 29.7% (5ØCÜ<0.001), and VLDL-cholesterol level by 37.3% (PÂ0.05) by fructan when compared with that of the mice of control group.

**Histopathological analysis of liver:** The effects of fermented skim milk compared to the unfermented skim milk of camel on the cholesterol levels in blood were examined in rats. Our results as shown in Fig. 7 are comparable with Yahya *et al.* (2018), who reported that concentrations of serum cholesterol and LDL-C/HDL-C ratio were decreased significantly in Wistar rats fed with fermented skim camel milk along with cholesterol-enriched diet when compared with rats ingested with unfermented milk (P<0.05). As well, there was a significant (at (P<0.05)) reduction was observed in inflammation, apoptosis/necrosis, liver tissue degeneration, and fatty changes (fibrosis and steatosis) by histopathological evaluation in the rats that were fed with fermented skim camel milk when compared with that of the rats which were ingested with unfermented skim camel milk and the histopathological diagnosis of liver tissues of rats fed fermented skim camel milk added along with high cholesterol diet showed mild hepatotoxicity.

**Conflict of interest:** Authors have no conflict of interest in this study.

**Author's contribution:** MM: Involved in conceptualization, methodology, formal analysis, investigation, writing of original draft, reviewing and editing; JBP, SV and SH: Involved in supervision, writing, reviewing and editing.

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