

VALUATION OF CO-CULTURE SOYMILK AS A PRAGMATIC APPROACH ON HYPERGLYCEMIA AND HYPERCHOLESTEROLEMIA IN SPRAGUE - DAWLEY RATS

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ABSTRACT

This study investigated the effect of co-culture soy milk against hyperglycemia and hypercholesterolemia on *Sprague Dawley* rats. Soy milk fermented by co-culture (*L. acidophilus* and *L. casei*) and analyzed for certain physicochemical properties to compare it with non-fermented soy milk. Then bio-efficacy study was done designed as study I (Normal rats) study II (Hyperglycemic rats) and study III (Hypercholesterolemic rats). Serum lipid profile, glucose, insulin level, liver (AST, ALT, ALP), renal (creatinine, BUN) functioning and hematological (RBC, WBC) analyses were determined. The results showed that fermentation causes proteolysis and lipolysis that significantly enhances the acidity, ash contents and antioxidant potential of soy milk. Compared to non-fermented soy milk (NFSM) the co-culture fermented soy milk (CFSM) resulted in reduction of glucose level from 3.61 to 17.11% and inclining insulin level from 1.8 to 8.3% in diabetic rats. It was most effective in lowering cholesterol level from 2.62 to 13.51%, the LDL reduction from 6 to 12.47% and elevation of HDL from 2.78 to 9.52% in hypercholesterolemic rats. The safety test on liver, kidney and blood biochemistry suggested it as a safe and non-toxic product. The current research proposed that soymilk fermented by *L. acidophilus* and *L. casei* has a valuable effect against hyperglycemia and hypercholesterolemia in comparison to non-fermented soy milk in managing diabetes complications and cardio vascular diseases.

Key words: Co-culture, Hyperglycemia, Hypercholesterolemia.

INTRODUCTION

Foods encompassing bioactive components are getting great attention due to their functionality in deterrence and treatment of various diseases. Soybean and its products comprehend surplus bioactive phytochemicals such as isoflavones, phytic acids, phytosterols, saponins and inhibitors of trypsin. Soybean's phytochemicals are helpful as protective effect against breast cancer, prostate cancer and bone loss in postmenopausal women, also in contraction of cholesterol and ultimate abatement of cardiovascular diseases and diabetes manifestation (Júnior and Ida, 2015). Diabetes mellitus is metabolic disorder categorized as Type 1 (T1DM) that is insulin dependent and Type 2 diabetes mellitus (T2DM) which is non-insulin dependent due to impaired insulin secretion (Butler *et al.*, 2003). Globally majority of people are suffering from T2DM which is an austere metabolic syndrome. The Type 2 diabetes is one of the world's most predominant, expensive, and lethal chronic conditions and alarming situation is proliferation of number of cases every year (Mattei *et al.*, 2015). T2DM is an increment for onset of numerous ailments such as cancer, myocardial infarction, atherosclerosis and neurological infection. Intake of soybean and fermented soybean in Asians may be associated with antecedently

lower percentage of Type 2 diabetes (Li *et al.*, 2018). Momentous decline of plasma glucose has been observed in diabetic rats after consumption of soybean milk kefir (Tyas and Kristian, 2015).

Dyslipidemia, hyperlipidemia and hypercholesterolemia are well-known cardiovascular risk factors that may cause death (Schnabel *et al.*, 2015). Integrative treatments with soybean that is low in saturated fat and can be helpful in reducing coronary heart diseases by reducing total and saturated fat level in the body (Acharjee *et al.*, 2015). Soy proteins are also well-known for lipid lowering action by increasing bile acid synthesis, apolipoprotein B receptor activity and reducing hepatic lipoprotein secretion associated with clearance of cholesterol from the blood (Cicero and Colletti, 2016). The soy protein appear to reach beyond its putative cholesterol lowering effect by offering protection against renal dysfunction, oxidative stress, and by improving markers of endothelial function (Ramdath *et al.*, 2017). Soy isoflavones like estrogen act as cardio protector due to oxidation of low density lipoprotein (LDL) cholesterol whose existence in blood vessel walls confer development of atherosclerotic plaques. Epidemiological investigations suggested that endothelial function and relaxation of arteries can boost up by soy isoflavones (Bahmani *et al.*, 2015).

Fermentation releases proteases to convert protein into bioactive peptides. Bioactive peptides act as

an antioxidant, antihypertensive, anti-tumor, antidiabetic and also recognized to preclude atherosclerosis. So, they can act as an alternate to synthetic drugs. During fermentation the microbial activity converts glycine (non-absorbable) form of isoflavones into aglycone that can be easily absorb into large intestine (Nemitz *et al.*, 2015; Hirose *et al.*, 2016).

Here, we will use lactic acid bacteria, for the fermentation of the soy milk. LAB positively affects human health by improving the natural gut micro-flora, shield from gastroenteritis, deterrence of coronary heart disease, colon cancer, irritable bowel syndrome, helps in digestion and increase mineral bioavailability (Lara *et al.*, 2014). There are many other health benefits such as treatment of gastrointestinal diseases (constipation and diarrhea), reduction of cholesterol biosynthesis, inhibition of intestinal bacterial enzymes that convert pre-carcinogens into active carcinogens, pre-digestion of lactose as well as competitiveness against pathogenic *Helicobacter pylori*, *Candida albicans* and different species of molds (Lili *et al.*, 2017). It was found that β -glucosidase-producing LAB strains exhibited a higher degree of β -glucosidase activity and significantly higher aglycone isoflavone content. Fermented foods are well-known for their benefits to human gastro intestinal tract and metabolic fitness (Ouweland and R oyti , 2015). A research was planned to check the effect of *Lactobacillus acidophilus* and *Lactobacillus casei* as a co-culture to ferment soy milk (CFSM) in comparison to non-fermented soy milk (NFSM) and to check its effect on reducing cholesterol and glucose level in rats.

MATERIALS AND METHODS

Preparation of inocula and fermented soy milk: The present research was conducted at Food Microbiology

$$\text{Nitrogen (\%)} = \frac{\text{Vol. of 0.1N H}_2\text{SO}_4 \times 0.0014 \times \text{Vol. of dilution (250mL)}}{\text{Vol. of distillate taken} \times \text{Weight of sample}} \times 100$$

$$\text{Crude Protein (\%)} = \text{Nitrogen (\%)} \times 5.80$$

Fat: The fat contents in soy milk were analyzed by following the Gerber method (AOAC, 2016).

Ash: Ash content in milk was analyzed by following reference method stated earlier (AOAC, 2016).

Total solids: The total solids in soy milk samples were calculated by following the method as described in (AOAC, 2016) protocols. According to which 5 g soy milk sample in china dish was first kept in water bath at 65°C for about 15 min. Then in hot air oven at 100°C until the sample completely dried. Dried sample was cooled in desiccator and then weighed.

Solid Not Fat: The total solids-not-fat was determined as described previously (AOAC, 2016). It was obtained by

and Biotechnology Lab National Institute of Food Science and Technology, Department of Biochemistry and Biotechnology, University of Agriculture, Faisalabad. The culture of *Lactobacillus acidophilus* (ATCC® 4356™) and *Lactobacillus casei* (ATCC® 393™) were procured from American type culture collection (ATCC). The cultures were inoculated into MRS broth for 72 h at 37°C. Then, it was centrifuged at 2286×g for 15 min at 24°C. The supernatant was removed and more MRS broth was added in it and kept under same incubation conditions for second time growth of culture. After two consecutive transfers of the activated cultures they were kept in 1mL of buffer peptone water. The soy milk was prepared by blending soaked soybeans with distilled water ten times of their weight for 3 min. The resultant slurry was filtered through double-layered cheese cloth to obtain soymilk and then sterilized by heating for 15 min at 121°C in an autoclave. Afterwards 100 mL of sterile soymilk was inoculated with 0.1 mL of inocula. Inoculated soymilk was incubated without shaking at 37°C for 12h. After that it was store at refrigeration temperature 4°C for further analysis (Rui *et al.*, 2016).

Determination of pH and Acidity: The pH of milk was measured through electronic digital pH meter (Schott Lab-150) by immersing electrodes of pH meter in soy milk after calibrating the instrument (AOAC, 2016). Acidity of samples was determined by titration method as given in (AOAC, 2016).

Protein: Total protein in the soy milk was determined by the method as described in (AOAC, 2016) protocols and using these formulas,

taking the difference between % total solids and % fat content.

$$\text{Solid Not Fat (\%)} = \% \text{ Total Solids} - \% \text{ Fat content}$$

Anti-oxidative activities of fermented soy milk: Soybean varieties were investigated for antioxidant activity including Diphenyl picryl hydrazyl (DPPH) and 2,2-azinobis-3-ethylbenzothiazoline-6-sulphonate (ABTS) assay. The fermented soy milk was centrifuged (5000×g for 10 min) and the clear supernatant obtained was assayed for antioxidant potential (Dai *et al.*, 2017).

Efficacy trials

Experimental Design: The co-culture fermented soy milk (CFSM) was evaluated on *Sprague Dawley* rats as

experimental model. For this purpose, 36 male *Sprague Dawley* rats (weighing 200-250 g) were purchased from National Institute of Health (NIH), Islamabad followed by acclimatizing in the animal room of National Institute of Food Science and Technology (NIFSAT), University of Agriculture, Faisalabad. The rats were acclimatized to the laboratory conditions by giving them normal chow diet for one week. The environmental conditions of room like temperature (21-25°C) and relative humidity (50±5%) along with 12 h light-dark cycle were controlled throughout the trial period. The rats were divided into three parallel studies (normal, hyperglycemic and hypercholesterolemic) each comprising of two groups receiving respective dietary treatment (T₀ and T₁) with six rats in each group as illustrated in Table 1. At the start of bioefficacy trial representative sample from each group was sacrificed to get the baseline data. The restorative potential of co-culture fermented soy milk (CFSM) and non-fermented soy milk (NFSM) was

$$\text{Percent Change} = \frac{\text{Effect of Non fermented soy milk} - \text{Effect of co - culture fermented soy milk}}{\text{Effect of Non fermented soy milk}} \times 100$$

Study I: Normal rats: In study I, rats were divided homogeneously into two groups fed on normal diet along with provision of fermented soy milk or non-fermented soy milk as a drink [8 mL/(kg.d)].

Study II: Hyperglycemic rats: In study II, hyperglycemia was induced intraperitoneally (IP) in overnight fasted rats by using fresh prepared streptozotocin (STZ) injection. The glucometer was used to check glucose level in the blood and if the blood glucose level is above 150 mg/dL then it is indication of diabetes. The rats become diabetic within seven days of injection. The drink CFSM and NFSM soy milk was given via oral gavage simultaneously to synchronize their effect in respective group along with chow diet.

Study III: Hypercholesterolemic rats: In study III, cholesterol was given along with normal diet in order to induce hypercholesterolemia in rats. In addition to this CFSM and NFSM were also given to check their impact concurrently against HDL, LDL, cholesterol and triglycerides.

Serum lipid profile analysis: Serum lipid profile of rats including total cholesterol (Stockbridge *et al.*, 1989), high density lipoproteins (HDL) (Alshatwi *et al.*, 2010), low density lipoproteins (LDL) and triglycerides were measured by using respective commercial kits (Kim *et al.*, 2011). All values are expressed in mg/dL.

Serum glucose and insulin levels: Determination of glucose in plasma (Nurliyani, 2015) and Insulin concentration were measured using commercially available kits.

evaluated during eight-week trial in all groups. During this trial period the feed and drink intakes were recorded on daily basis however, change in body weight was monitored on weekly basis. Overnight fasted rats were decapitated at the end of the efficacy trials. Blood samples were collected and centrifuged at 1463×g for 6 min to collect the sera and stored at -80°C in microcentrifuge tubes. Sera samples were evaluated for numerous biochemical assays via Microlab 300, Merck, Germany (Sartang *et al.*, 2015; Abdel-Salam *et al.*, 2018).

This study was approved by the Director of Graduate Studies, University of Agriculture, Faisalabad vide letter number 2067-70 dated 15 January 2016 after approval from the departmental scrutiny and ethical committee. The resultant data has expressed as mean ± standard error however, graphical representation is on the percent change for each variable calculated by following the formula;

Liver function probability: Liver functioning or hepatoprotective tests like ALT (alanine aminotransferase), ALP (alkaline phosphatase) and AST (aspartate aminotransferase) were determined according to their respective protocols (Basuny, 2009).

Renal function tests: The renal functioning test like creatinine and blood urea nitrogen (BUN) concentration in serum was measured using the commercial kit. The results were expressed in mg/dL (Jacobs *et al.*, 1996).

Hematological analysis: Hematological parameters including red and white blood cells (RBC and WBC) and platelets count was assessed by adopting the protocols described earlier (Al Haj *et al.*, 2011).

Statistical analysis: The data of each parameter was statistically analyzed using completely randomized design for each study, separately using Statistix 8.1. Furthermore, Tukey's Honest Significant Difference (HSD) was applied for posthoc comparison between the groups (Montgomery, 2008).

RESULTS AND DISCUSSION

The effect of fermentation on nutritional composition of soy milk was mentioned in Table 2. The comparison of fermented and co-culture fermented soy milk showed a clear picture that fermentation augments the quality and nutritional contents of soy milk. The LAB decreases the pH and ultimately acidity was increased that was helpful in flavor enhancer and shelf life modification. The fermentation in soy milk showed the dramatic increase in titratable acidity and decrease in pH values as compared to non-fermented (Lee *et al.*, 2015;

Kuda *et al.*, 2016) that can be due to exopolysaccharide secreted by LAB. However, ash, fat and protein contents were slightly lesser than non-fermented soy milk that might be due to some anabolic processes leading to polymer build-up or due to microbial cell proliferation (Lee *et al.*, 2015; Mühlhansová *et al.*, 2015). Several studies have reported that activities of lactic acid bacteria increase the production of free fatty acids by lipolytic enzymes which hydrolyse fat components (triacylglycerol) into fatty acid and glycerol (Vieira *et al.*, 2015). These fatty acids used as sources of energy by LAB resulting in lower fat content in fermented soymilk at the end of fermentation (Lee *et al.*, 2015). Likewise antioxidant status checked by DPPH and ABTS showed their enhanced values. The LAB also enhanced antioxidant capacity in fermented soy milk as compared to non-fermented soy milk due to the significant bioconversion of the glucosidic form of isoflavones into their bioactive aglyconic form of isoflavones (Xiao *et al.*, 2015; Embirikah *et al.*, 2016).

The statistical results regarding the blood glucose level showed that treatments (fermentation) have non-substantial ($P>0.05$) effect on glucose in normal rats, while the substantial ($P<0.05$) effect was noticed in diabetic and cholesterolemic group. Mean values shown in Table 2 relating to glucose level in study I (normal group) feeding NFSM was 88.61 ± 1.90 mg/dL and in CFMSM was 85.44 ± 1.83 mg/dL. Whereas in study II (hyperglycemic) glucose level was momentarily decline by using fermented soy milk T₁ (258.57 ± 5.55 mg/dL) in comparison to non-fermented soy milk T₀ (311.97 ± 6.70 mg/dL). Glucose level in study III (hypercholesterolemia) showed a significant effect in reducing glucose level by feeding fermented soy milk. The recorded glucose level in NFSM feeding group was 271.79 ± 5.84 mg/dL and in CFMSM it was 241.49 ± 5.19 mg/dL. The study I (Normal rats) elucidated 3.61% decrease in glucose level. In study II (hyperglycemic rats) perceived highest decrease in glucose as 17.11% while in study III of hypercholesterolemic rats substantial decrease of 7.46% glucose in blood was noticed. It showed that fermented soy milk is valuable to control the diabetes due to reduction of plasma glucose level (Cai *et al.*, 2017) and other factor is augmenting the secretion of insulin.

The analysis revealed the non-significant ($P>0.05$) effect of treatment on insulin level whilst study II presented the highly significant ($P<0.01$) effect and study III depicted the significant effect ($P>0.05$) of fermented soy milk on insulin level in blood of rats. The mean value for insulin given in Table 3 in the study I (Normal rats) was reported lower (8.73 ± 0.18 μ U/mL) in group feeding NFSM soy milk as compared to T₁ group fed on CFMSM it was 8.89 ± 0.19 μ U/mL. The dramatic change was noticed in hyperglycemic rats in which insulin level in NFSM group was 7.28 ± 0.35 μ U/mL and in CFMSM feeding group was 7.89 ± 0.38 μ U/mL. In

hypercholesterolemic rats (study III), least level of insulin was detected as 6.57 ± 0.32 μ U/mL in T₀ (NFSM) group and higher as 6.93 ± 0.34 μ U/mL was in T₁ (CFMSM) group. It was elucidated that fermented soy milk was effective in inclining insulin level, while vibrant effect documented in diabetic rats was 8.3% increase in insulin level trailed by 5.4% in high cholesterol rats and 1.8% in normal rats. The insulin production is directly related with blood sugar because higher level of glucose in blood activates pancreas to secrete more insulin into the bloodstream. There are array of components that may support the benefits of soy milk consumption for Type 2 diabetes management (Ding *et al.*, 2016). The LAB fermentation yields organic acid (lactic and acetic acid) due to breakdown of carbohydrates and these acids helps to delay the gastric emptying rate and led to drops in glucose absorption and plasma insulin in a glucose tolerance test in rats (Mikelsaar *et al.*, 2016). Isoflavonoids in soybean are associated in dropping insulin resistance and modifying glycaemic index. The literature has indicated tight correlation between soybean consumption and the occurrence of metabolic disorders, that the effects of soybean consumption on glucose metabolism showed predominantly effect due to the high bioavailability of isoflavones (genistein and daidzein). Genistein and daidzein potentiates glucose-induced insulin secretion (GIIS) and it was suggested that these isoflavones directly targets pancreatic beta cells (Dall'Asta *et al.*, 2015).

The statistical analysis of the results regarding the cholesterol level showed non-momentous ($P>0.05$) effect of soy milk in study I (Normal rats) however, substantial ($P<0.05$) variations were observed in study II (Diabetic rats) and most effective results were recorded in high cholesterol rats. The cholesterol level for normal rats showed a slight change (2.14 mg/dL) of cholesterol level after feeding with NFSM was 81.25 ± 1.74 mg/dL and in CFMSM feeding group was 79.11 ± 1.69 mg/dL. The diabetic rats group showed an obvious change (10.2 mg/dL) in cholesterol level as 108.84 ± 2.34 mg/dL was in NFSM feeding group and 98.64 ± 2.12 mg/dL in CFMSM group. The maximum cholesterol lowering effect (20.75 mg/dL) was observed in high cholesterolemic rats (153.55 ± 3.30 mg/dL to 132.80 ± 2.85 mg/dL). It is obvious from results that value added drink (CFMSM) was most effective in lowering cholesterol level as 13.51% decrease was recorded in high cholesterol rat group. Similarly, in diabetic rats 9.37% reduction was recorded in diabetic group while least reduction was reported in normal rats 2.62%. On the whole the present study showed the reduction of cholesterol in rats by feeding co-culture fermented soy milk. Higher level of cholesterol in blood causes formation of plaque that narrows down the blood arteries and becomes a primary factor of heart diseases. Soy isoflavones can help to manage cholesterol levels and lower coronary heart disease (CHD) risk in

certain individuals and animals. Other than isoflavones soybean contains “lunasin” that works in two ways to reduce the serum LDL cholesterol levels. First, it lowers the HMG-CoA reductase gene expression and makes it unavailable for the liver to conduct synthesis of cholesterol. Second, it enhances expression of the LDL-receptor gene, which increases quantity of receptors to clear LDL cholesterol from bloodstream (Lule *et al.* 2015). The probiotics or LAB used to ferment the soy milk can play a fundamental role in the significant reduction of total cholesterol level of rat's blood by feeding fermented soy milk (Anandharaj *et al.*, 2015). The fermented soy milk has positive effect on the lowering cholesterol level of blood when feeding fermented soy milk. Soy milk has many chain sugars (stachyose 4% and raffinose 1%) which can be used by lactic acid bacteria as carbon source (Švejtil *et al.*, 2015). Moreover, the lactic acid bacteria have the potential to absorb and bind cholesterol as well as bile acids with their bacterial cells and inhibit its absorption in the intestine. The other reason is undigested pepsin fraction of soybean protein that may effects fecal excretion of steroids or bile acids, which may influence the cholesterol metabolism (Tomat *et al.*, 2011).

The results for the consequence of value added soy milk on LDL (mg/dL) showed non-substantial effect ($P>0.05$) of LDL level on normal rats but diabetic rats showed significant ($P<0.05$), however the most obvious effect was recorded in study III hypercholesterolemic rats whereas LDL level varied highly significant ($P<0.01$). The mean values for normal rat group showed a diminishing effect on LDL level as 12.50 ± 0.27 mg/dL in NFSM feeding rat group and 11.75 ± 0.25 mg/dL in CFSM feeding group. However, in study II the LDL level was changed significantly as in NFSM group, LDL recorded was 24.42 ± 0.52 mg/dL and in CFSM rat group was 22.15 ± 0.48 mg/dL. Whilst, in high cholesterolemic rat group LDL for NFSM was 39.21 ± 0.84 mg/dL and in CFSM rat group was 34.32 ± 0.73 mg/dL. The highest LDL reduction was recorded in hypercholesterolemic rat group as 12.47% which supported that CFSM is effective in lowering LDL in hypercholesterolemia. However, LDL reduction was also observed during study II in diabetic rats was 9.29% and 6% in study I (normal rats). The modern medicines developed with the aim to decrease plasma LDL cholesterol because LDL cholesterol may causes formation of fatty deposits in arterial walls, which developed into plaques that enlarge, break, and arouse the production of blood clots that may causes blockage in arteries and eventually heart related disorders. Fermentation helps to improve heart related disorders by decreasing the level of LDL in blood which is considered as biomarker of cardio vascular diseases but the use of soybean products and specially fermented soybean helps to improve human health. Likewise, results of current findings showed reduction in LDL level by

feeding CFSM and explicated that consumption of fermented soy significantly decreases the LDL level (Babashahi *et al.*, 2015).

The statistical analysis of the results regarding HDL level in blood of rats showed the non-substantially ($P>0.05$) effect in study I (Normal rats), while significant ($P<0.05$) in study II (diabetic rats group) and highly significant ($P<0.01$) in study III (Hypercholesterolemic rats). The mean values in study I (Normal rats) increased gradually from 44.85 ± 0.96 mg/dL in NFSM (T_0) group versus 46.10 ± 0.93 mg/dL in CFSM feeding group, whilst in diabetic rats the HDL level increased from 38.05 ± 0.82 (NFSM) to 39.61 ± 0.85 mg/dL (CFSM). Similarly a greater increase was observed in study III from 36.73 ± 0.79 (T_0) to 40.23 ± 0.86 (T_1) mg/dL. CFSM was more helpful in increasing HDL level (9.52 %) in study III (hypercholesterolemic rats) and 4.02 in study II (diabetic rats) and 2.78% in (normal rats). In comparison to LDL cholesterol, HDL cholesterol is considered as good cholesterol due to its high density it does not form plaque in arteries instead of it pass out and excreted through the body. HDL is associated with reverse cholesterol transport from tissues and arteries and sends it back to the liver for its decomposition and ultimately excretion that decreases chances of atherosclerosis (hardening of arteries). The consumption of fermented soy milk significantly augments the HDL-C level ((Babashahi *et al.*, 2015; Shin *et al.*, 2016).

Triglycerides are type of fat in the bloodstream whose higher level in body is associated with heart diseases. The results regarding the triglyceride showed the non-significant ($P>0.05$) effect of treatments (T_0 and T_1) on normal rats, while their response was significant ($P<0.01$) in hypercholesterolemic rats and hyperglycemic rats. The mean value of triglyceride in study I was higher (66.64 ± 1.43 mg/dL) in T_0 (NFSM) than T_1 (CFSM) was 64.59 ± 1.39 mg/dL. In (diabetic rats), the triglyceride contents was also higher in T_0 (79.60 ± 1.71 mg/dL) than T_1 (72.48 ± 1.56 mg/dL). The prominent variations were observed in study III (hypercholesterolemic rats) as non-fermented soy milk group T_0 exhibited the higher 98.94 ± 2.12 mg/dL triglycerides than fermented soy milk (T_1) which had 86.73 ± 1.86 mg/dL of triglycerides. It is noticeable from results that value added soy milk showed maximum triglycerides reduction in study III (12.34%) lagged by 8.94% in diabetic rats and 3% in normal rats group. The fermented soy milk reduced the level of triglycerides as compared to the non-fermented control samples (Sartang *et al.*, 2015; Niamah *et al.*, 2017). Fermented soy milk play a major role in reducing heart diseases as higher level of triglycerides are associated with metabolic syndrome that may increase the risk of heart disease and diabetes. Likewise, Lin *et al.* (2005) reported that consumption of fermented soy milk powder was effective in reducing the accumulation of triglycerides in the liver.

The enzymes AST, ALT and ALP helps to process proteins and its higher level in the body is the indication that liver is injured or swollen. The results depicted the effect of value added soy milk on AST, ALT and ALP that treatment's effect were non-substantial ($P>0.05$) in study I and study II whilst, substantial ($P<0.05$) effect was recorded in study III. It was obvious from the findings that AST values were lower in all studies in co-culture fermented soy milk group as compared to NFSM group. The highest reduction in AST level was 12.74% recorded in high cholesterolemic rat group followed by 7.12% in diabetic rats and 2.54% in normal rats (Table 5). The data depicted the (58.78-51.22 IU/L) maximum ALT level decreased as 12.86% in study III trailed by (53.42-49.16 IU/L) study II as 7.97% and minimum ALT level (45.54-43.59 IU/L) reduced as 4.26% in study I. The mean values revealed that fermented soy milk (T_1) was more helpful in reduction of ALP level in comparison to non-fermented soy milk (T_0). The decrease in ALP level was recorded in study III (267.25 - 232.25 IU/L) was 15.06 %, lagged by study II (220.84-201.38 IU/L) as 9.66% and in study I (220.84 to 201.38 IU/L) was 4%. The fermented soy milk or soy extract administered rat group showed lower concentration of AST, ALP and ALT level. The findings were also supported by Shin *et al.* (2009) and Hong *et al.* (2012). They reported that fermentation helps to improve liver related disorders as use of fermented soybean products helps to improve liver health. These results recommend that fermented soy milk may play a fundamental role in hepatic related disorders and ultimately helpful for maintaining better health in humans as well. It is deduced from findings of current research that soy milk fermented by *L. acidophilus* and *L. casei* was positive in averting hepatocellular damage.

The effect of treatments on renal functioning test showed non-significant ($P>0.05$) variations on the level of BUN and creatinine in (study I) whilst, highest significant ($P<0.01$) effect was noticed in study II and substantial ($P<0.05$) variations in hypercholesterolemia rats (study III). The effect of treatment on the BUN level indicated that fermented soy milk (T_1) was more effective in the reducing of BUN and creatinine level in blood as compared to non-fermented soy milk (T_0). The maximum BUN reduction was noticed in study II as 3.45% followed by 2.87% in study I and 2.24% in study III. It has been revealed from study that fermented soy milk showed a profound effect in the reduction of BUN in blood. The maximum level of creatinine reduction was 7.22% noticed in diabetic rats, lagged by 4.59% in hypercholesterolemic rat and 2.43% in normal rats. Co-culture fermented soy milk's effect was also checked for the safety status of kidney (renal) through biological urea nitrogen (BUN) and creatinine level. During metabolism of protein in the liver the final product is formed as urea which is excreted via kidneys from the body. If the

kidney is not functioning properly then ultimately BUN level will be increased in the blood from normal range. The results of current findings showed pronounced reduction of BUN level by feeding CFMS was relatively higher than NFSM. It can be suggested that soy milk play an important role in renal functioning disorders and may also be valuable for maintaining health in human beings. Creatinine level in the blood is proportional to the glomerular filtration rate. Kidney damage is mostly associated with higher level of creatinine so, it is the more efficient as compared to BUN. However, the administration of fermented soy milk showed pronounced reduction of creatinine level in all study groups. The statistical analysis for RBC in Table 6 showed that consequence of treatment was non-significant ($P>0.05$) in study I but a significant variations ($P<0.05$) were recorded for study II and highly significant ($P<0.01$) in study III. The maximum increase in RBC was reported as 6.99% in study III, 4.81% in study II and 2.17% in study I. The haematological analysis showed increase on RBC in case of fermented soy milk group as compared to non-fermented soy milk group. The percentage of RBC was higher in fermented soy milk feeding group (Sartang *et al.*, 2015; Niamah *et al.*, 2017). White blood cells and platelets count showed non- momentous ($P>0.05$) effect in all studies. The statistical results for WBCs and platelets count inferred that WBCs were not affected by the treatments in all the studies. The statistical results indicated non- momentous ($P>0.05$) effect of treatments in study I, II and III. The results were not as higher that significant variation could be noticed but overall it is noticeable that value added soy milk showed maximum white blood cells reduction in study III as 9.41% lagged by 6.48% in study II and only 3.58% in study I. The co-culture fermented soy milk showed a minor increase in platelets count as 2.65% was recorded in high cholesterol rat group, 2.53% was recorded in diabetic group while least increase was reported as 1.53% in normal rats.

Table 1. Treatment plan for efficacy trials.

| Study | Group | Dietary Treatments |
|--------------------------------------------------|-------|-----------------------------------------|
| Study I (Normal Rats) | I | Non-fermented soy milk (T_0) |
| | II | Co-culture Fermented soy milk (T_1) |
| Study II (Hyperglycemic Rats) | I | Non fermented soy milk (T_0) |
| | II | Co-culture Fermented soy milk (T_1) |
| Study III (Hypercholesterolemic Rats) | I | Non fermented soy milk (T_0) |
| | II | Co-culture Fermented soy milk (T_1) |

Table 2. Comparison of nutritional composition in non-fermented soy milk (NFSM) and co-culture fermented soy milk (CFSM).

| Parameters | Non-fermented soy milk (NFSM) | Co-culture fermented soy milk (CFSM) |
|------------|-------------------------------|--------------------------------------|
| pH | 6.89±0.19 | 5.31±0.14 |
| Acidity | 0.42±0.01 | 0.88±0.02 |
| Protein | 3.02±0.08 | 2.65±0.07 |
| Fat | 1.92±0.05 | 1.43±0.04 |
| Ash | 0.85±0.02 | 0.49±0.01 |
| DPPH (%) | 26.23±0.66 | 60.23±1.59 |
| ABTS (%) | 59.81±1.51 | 87.69±2.32 |

Results are expressed as mean ± standard error of means; n = 3 sets

Table 3. Effects of non-fermented soy milk (NFSM) and co-culture fermented soy milk (CFSM) consumption on plasma glucose and insulin level in rats.

| Studies | Glucose level (mg/dL) | | Insulin level (µU/mL) | |
|-----------|--------------------------|--------------------------|------------------------|------------------------|
| | T ₀ | T ₁ | T ₀ | T ₁ |
| Study I | 88.61±1.90 ^a | 85.44±1.83 ^a | 8.73±0.18 ^a | 8.89±0.19 ^a |
| Study II | 311.97±6.70 ^a | 258.57±5.55 ^b | 7.28±0.35 ^b | 7.89±0.38 ^a |
| Study III | 271.79±5.84 ^a | 241.49±5.19 ^b | 6.57±0.32 ^b | 6.93±0.34 ^a |

Means in rows with similar superscripts do not differ ($P>0.05$), Results are expressed as mean ± standard error of means; n=5

Table 4.1 Effects of non-fermented soy milk (NFSM) and co-culture fermented soy milk (CFSM) consumption on serum level of cholesterol, low-density lipoprotein cholesterol (LDL-C), High-density lipoprotein cholesterol (HDL-C) and Triglycerides.

| Studies | Cholesterol level (mg/dL) | | Low density lipoprotein (LDL) level (mg/dL) | | High density lipoprotein (HDL) level (mg/dL) | | Triglycerides level (mg/dL) | |
|-----------|---------------------------|--------------------------|---------------------------------------------|-------------------------|----------------------------------------------|-------------------------|-----------------------------|-------------------------|
| | T ₀ | T ₁ | T ₀ | T ₁ | T ₀ | T ₁ | T ₀ | T ₁ |
| Study I | 81.25±1.74 ^a | 79.11±1.69 ^a | 12.50±0.27 ^a | 11.75±0.25 ^a | 44.85±0.96 ^a | 46.10±0.93 ^a | 66.64±1.43 ^a | 64.59±1.39 ^a |
| Study II | 108.84±2.34 ^a | 98.64±2.12 ^b | 24.42±0.52 ^a | 22.15±0.48 ^b | 38.05±0.82 ^b | 39.61±0.85 ^a | 79.60±1.71 ^b | 72.48±1.56 ^a |
| Study III | 153.55±3.30 ^a | 132.80±2.85 ^a | 39.21±0.84 ^a | 34.32±0.73 ^b | 36.73±0.79 ^b | 40.23±0.86 ^a | 98.94±2.12 ^b | 86.73±1.86 ^a |

^{ab}Means in rows with similar superscripts do not differ ($P>0.05$), Results are expressed as mean ± standard error of means; n=5

Table 5. Effects of non-fermented soy milk (NFSM) and co-culture fermented soy milk (CFSM) consumption on liver and renal functioning of rats.

| Studies | Liver Functioning Tests | | | | Renal Functioning Tests | | | | | |
|-----------|-----------------------------------------------|--------------------------|-----------------------------------------|-------------------------|----------------------------------------|--------------------------|----------------------------------------------|-------------------------|--------------------------|------------------------|
| | Aspartate Aminotransferase (AST) level (IU/L) | | Alanine Transaminase (ALT) level (IU/L) | | Alanine Phosphatase (ALP) level (IU/L) | | Biological urea nitrogen (BUN) level (mg/dL) | | Creatinine level (mg/dL) | |
| | T ₀ | T ₁ | T ₀ | T ₁ | T ₀ | T ₁ | T ₀ | T ₁ | T ₀ | T ₁ |
| Study I | 104.25±2.24 ^a | 101.60±2.18 ^a | 45.54±0.97 ^a | 43.59±0.94 ^a | 139.39±2.99 ^a | 134.02±2.87 ^a | 22.51±0.48 ^a | 21.88±0.47 ^a | 0.82±0.02 ^a | 0.80±0.01 ^a |
| Study II | 121.18±2.60 ^a | 112.54±2.41 ^a | 53.42±1.15 ^a | 49.16±1.05 ^a | 220.84±4.74 ^a | 201.38±4.32 ^a | 40.06±0.86 ^a | 38.72±0.83 ^b | 0.97±0.02 ^a | 0.90±0.01 ^b |
| Study III | 138.28±2.97 ^a | 121.03±2.59 ^b | 58.78±1.26 ^a | 51.22±1.87 ^b | 267.25±5.74 ^a | 232.25±4.98 ^b | 36.02±0.77 ^a | 35.23±0.75 ^b | 0.87±0.01 ^a | 0.83±0.02 ^b |

^{ab}Means in rows with similar superscripts do not differ ($P>0.05$), Results are expressed as mean ± standard error of means; n=5

Table 6. Effects of non-fermented soy milk (NFSM) and co-culture fermented soy milk (CFSM) consumption on hematological analysis in rats.

| Studies | Red Blood Cell (RBC) cells/pL | | White Blood Cells (WBCs) cells/nL | | Platelets Count (PLC) x 10 ⁹ /L | |
|------------------|-------------------------------|------------------------|-----------------------------------|-------------------------|--------------------------------------------|------------------------|
| | T ₀ | T ₁ | T ₀ | T ₁ | T ₀ | T ₁ |
| Study I | 7.35±0.15 ^a | 7.51±0.16 ^a | 6.99±0.15 ^a | 6.53±0.14 ^{!a} | 7.15±0.15 ^a | 7.26±0.13 ^a |
| Study II | 7.49±0.16 ^b | 7.85±0.17 ^a | 7.86±0.17 ^a | 7.35±0.16 ^a | 6.65±0.14 ^a | 6.74±0.13 ^a |
| Study III | 7.15±0.15 ^b | 7.65±0.16 ^a | 7.65±0.16 ^a | 6.93±0.15 ^a | 6.17±0.12 ^a | 6.27±0.13 ^a |

^{ab}Means in rows with similar superscripts do not differ ($P>0.05$), Results are expressed as mean ± standard error of means; n=5.

Conclusion: It can be concluded from present research that soy milk fermented by using (*L. acidophilus* and *L. casei*) can be targeted as a part of complementary dietary strategies for hyperglycemia and hypercholesterolemia and related diseases combined with traditional drug and nutritional therapies, including fruits, vegetables and other legumes. Fermented soy milk prevented hypercholesterolemia by modulating cholesterol metabolism and hyperglycemia by decreasing glucose level and increment in insulin production. Dieticians should recommend fermented soy milk based in routine diet to mitigate many metabolic ailments.

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