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Basic nutritional investigation

Hepatoprotective effects of synbiotic soy yogurt on mice fed a high-cholesterol diet

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Objectives: The role of probiotic yogurt in the prevention and treatment of hypercholesterolemia has attracted global attention. Mounting evidence has indicated that probiotics and prebiotics improve lipid metabolism by lowering low-density lipoprotein cholesterol concentration in plasma of hypercholesterolemic patients. The present study aimed to develop synbiotic soy yogurt that had a greater cholesterol-lowering effect in hypercholesterolemic mice compared with control soy yogurt.

Methods: Synbiotic soy yogurt was prepared using soy milk and synbiotic capsule containing *LactoBacil Plus* (SCLBP) probiotic cultures and fructo-oligosaccharide. Synbiotic soy yogurt was analyzed for proximate composition and microbiological and antioxidative properties during storage periods of 28 d. To study hypocholesterolemic effect, hypercholesterolemia was induced in mice with administration of 1.25% cholesterol and 0.5% cholic acid for 4 wk. After that 24 male Balb/c mice were randomly divided into four groups and fed basic, high-cholesterol, high-cholesterol with soy yogurt, or high-cholesterol with synbiotic soy yogurt diet for 5 wk. Blood samples were collected to measure lipids concentration and oxidative and antioxidative status.

Results: Proximate composition of SCLBP-formulated soy yogurt exhibits a marked difference from control soy yogurt in terms of total solids, moisture, protein, fat, ash, carbohydrate, and energy content. Results indicated that the 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity (75.28%) in synbiotic yogurt containing 2% SCLBP was significantly greater ($P \leq 0.05$) compared with control soy yogurt (52.98%). In mice with hypercholesterolemia that were fed synbiotic soy yogurts, the yogurts had a favorable effect in reducing blood cholesterol, triglycerides, and low-density lipoprotein cholesterol and lipid peroxidation in liver. These led to a significant decrease of the atherogenic index compared with soy yogurt (control) only. Treatment with synbiotic soy yogurt cultures ameliorates lipid peroxidation in liver.

Conclusions: These results indicated that the synbiotic soy yogurts have beneficial effects against hypercholesteroemia and can be used as a therapeutic agent in hypercholesteremic patients.

Introduction

Probiotics are defined as living microbial supplements that beneficially affect the host animals by improving its intestinal microbial balance [1]. Probiotics could improve gut health by inhibiting the growth and attachment of pathogenic bacteria. In addition, probiotics have promoting effects against many kinds of diseases such as hypertension, diabetes, cancer, allergic reaction, and gout [2]. Probiotics have been found to improve hypercholesterolemia and decrease cholesterol level in animal models [3]. Human studies, on the other hand, have exhibited contradictory results. Probiotic food products provide a novel non-pharmacologic alternative to reduce hypercholesterolemic risk factors [4,5]. Prebiotics are indigestible fermented food substances that selectively stimulate the growth, composition, and activity of microflora in the gastrointestinal tract and thus improve hosts' health and well-being [1]. Combinations of both probiotics and prebiotics are known as synbiotics. Consumption of synbiotics has synergistic effects through improving the survival of existing probiotic in the colon and activating the growth of other new probiotics strains [6]. In addition synbiotics have antimicrobial, anticancer, antiallergic, and immune-stimulating properties [7]. They improve absorption of minerals, prevent diarrhea, and optimize assimilation of nutrients [8].

In developed and developing countries cardiovascular disease (CVD) is a severe problem. Atherosclerosis is the underlying cause of

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CVD. Hypercholesterolemia is one of the major risk factors of atherosclerosis, and low-density lipoprotein cholesterol (LDL-C) is the major cause of onset of the atherogenic process. Results of the studies conducted by El-Sayyad et al. [9] revealed that maternal diabetes and hypercholesterolemia developed hepatic disease during fetal life. High cholesterol can also be a predictor of diabetes; elevated cholesterol levels are often seen in people with type 2 diabetes with insulin resistance. People with type 2 diabetes tend to have increased triglycerides, decreased high-density lipoprotein cholesterol (HDL-C), and sometimes increased LDL-C. High insulin levels act to increase LDL-C, which tends to form plaques in arteries and decrease the number of HDL-C particles that help to clear out fibrous plaques before they break off to cause atherosclerosis [9]. Dietary modification lowers hypercholesterolemia, and in particular soy foods are emphasized as part of a cardioprotective diet because increased consumption of soy foods is associated with improved plasma lipid profile via the lowering of LDL-C resulting in reduction of CVD [10]. The beneficial bioactive compounds of soy have attracted attention from researchers for their efficacy in disease prevention [11–15]; therefore soy consumption has been recommended as a favorable dietary technique to reduce cholesterol, and more studies are needed in this area.

Lactobacilli and bifidobacteria are found in the gut and are considered as potential probiotic strains because of their beneficial effect on blood cholesterol reduction [16]. Many studies have been performed on the total plasma cholesterol (TC) and LDL-C lowering effect of soy milk containing lactobacilli and bifidobacteria [13,17]; however, relatively little is yet known regarding the cholesterol-lowering effect of soy yogurt containing bifidobacteria [18]. According to El-Sayyad [2], significant reduction in TC (7.2%) and LDL-C (10.3%) was identified in hypercholesterolemic rats fed with yogurt supplemented with *Bifidobacterium pseudocatenulatum G4* or *B. longum* for 8 wk. Excessive generation of free radicals reduces the body's antioxidant system, leading to increased oxidative stress as seen in situations like hypercholesterolemia, which leads to remarkable alterations in livers. It is well established that hypercholesterolemia can induce severe fatty degeneration in the hepatocytes, known as non-alcoholic fatty liver disease [19]. Although a few studies have reported the cholesterol-lowering effects of synbiotic diets [1,20] and synbiotic yogurts [3] in animals and humans, there is no information on cholesterol-lowering effects of synbiotic soy yogurts containing probiotic microorganisms and prebiotic ingredients. Synbiotic capsule containing *LactoBacil Plus* (SCLBP) is a synbiotic supplement containing probiotic lactobacilli, bifidobacteria, and *Saccharomyces* and prebiotic fructo-oligosaccharide. The aim of this study was to develop a synbiotic soy yogurt containing synbiotic capsule *LactoBacil Plus* to investigate the effects of newly developed bioyogurt on the plasma lipid profiles of hypercholesterolemic mice.

Materials and methods

Chemicals and reagents

Soybean seeds were purchased from MP Biomed Products. Commercially available milk curd cultures were purchased from Microbial Type Culture Collection (MTCC) and Gene Bank (Chandigarh, India) (*Lactobacillus delbrueckii* ssp. *bulgaricus* [MTCC 911] and *Streptococcus thermophilus* MTCC 1938]). SCLBP manufactured by Swiss Garnier Life Sciences Limited (marketed by Organon [India], a subsidiary of Schering–Plough Corporation) was purchased from a local medical shop in Kolkata, West Bengal, India. All solvents, reagents, and distilled water used throughout the experiments were obtained from Merck. Media required for microbiological studies were procured from HiMedia Laboratories Pvt., Ltd.

Bacteria and growth conditions

Lyophilized starter cultures were purchased from MTCC, Chandigarh, India, were activated by triplicate sub culturing in de Mann Rogosa Sharpe broth using 1% inoculum and incubated at 37°C for 18 to 24 h.

Preparation of SCLBP-incorporated soy yogurts

Soy milk from whole soy seeds was prepared according to the procedure of Sengupta et al. [12]. The soy milk was homogenized (Remi Motors–RQ–122) and pasteurized at 80°C for 15 min. It was then cooled to 40°C and was aseptically inoculated with 2 g of hard gelatin capsules of SCLBP (synbiotic capsule *LactoBacil Plus* containing *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* 1.5 billion, *B. longum* and *Bifidobacterium bifidum* 1.5 billion, *Saccharomyces boulardii* 0.10 billion, and fructo-oligosaccharide 100 mg) per 100 mL of soy milk. The inoculated soy milk was then poured into 100 mL sterile transparent food-grade plastic cups with lids and incubated at 37°C for 6 h. The obtained SCLBP-formulated synbiotic soy yogurt was refrigerated at 4°C for 28 d. Control soy yogurt was prepared from whole soy seeds with 2% starter culture. Two experimental soy yogurts were manufactured and designated as control soy yogurt without incorporation of SCLBP (SYC) and soy yogurt containing 2% SCLBP (SSY).

Proximate compositions of control and synbiotic soy yogurt

The proximate compositions (protein, moisture, total solids, and ash content) of two types of experimental soy yogurts were carried out in triplicate using the standard methods of the Association of Official Analytical Chemists [21]. Fat was determined according to Bligh and Dyer's method [22]. Carbohydrate content was calculated according to the equation 100 – (moisture + crude protein + lipid + ash). Energy values were obtained using the Atwater formula [23].

Antioxidant properties of control and synbiotic soy yogurt

Extracts of control and synbiotic soy yogurts were prepared and their antioxidant activities were analyzed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. This method was based on the capture of the DPPH radical by antioxidants, producing a decrease in absorbance at 515 nm [24]. Estimation of polyphenol content in control and synbiotic soy yogurts was carried out using Folin-Ciocalteu reagent [24]. The Fe^{3+} reducing power of control and synbiotic soy yogurts were determined using ferric-reducing antioxidant power (FRAP) assay [25]. The reducing power was determined by measuring the absorbance at 593 nm with a spectrophotometer (V-630 UV-VIS Spectrophotometer, JASCO). A standard curve of ascorbic acid was generated with concentrations ranging from 0 to 200 mg/mL. The reducing power was expressed as milligrams per gram of control and synbiotic soy yogurts.

Microbiological properties of control and synbiotic soy yogurt

Microbiological analyses of soy yogurts samples were evaluated during 28 d of storage at 4°C. Serial decimal dilutions in sterile peptone water (0.1%) were prepared from each soy yogurt sample (1 g). Enumeration was done over selected culture media by taking 1 mL aliquots. Plate count agar was used for total viable count as described by the American Public Health Association [26]. Enumeration of the foodborne pathogen *Escherichia coli* was conducted in violet red bile agar using the spread plate method after aerobic incubation at 35°C for 5 d. *Salmonella* spp. was also enumerated by using respective selective media recommended by American Public Health Association [26]. The count of lactobacilli and bifidobacteria in synbiotic soy yogurts (SYC and SSY) that did not contain yogurt culture was enumerated by the pour plate method on lactobacilli–Mann Rogosa Sharpe agar medium under anaerobic conditions according to the method described by Samona and Robinson [27]. The samples were incubated at 37°C for 72 h. Yogurt cultures *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* were enumerated according to Elli [28]. Viable bacteria counts were expressed as log cfu/g of soy yogurt sample.

Diets

The freshly prepared control and synbiotic bio-soy yogurts were used for the animal experiments. The diets were prepared according to Reeves [29] of the American Institute of Nutrition (AIN); however, some modifications related to source and concentration of lipids and carbohydrates were performed following the standard AIN-93G. The diets consisted of different ingredients, which, along with the energy of different experimental diets containing experimental soy yogurts, are shown in Table 1.

Experimental work

Six-wk-old healthy Balb/c male mice (Mus musculus) with 20 to 25 g body weight were used in the feeding experiment for evaluating the nutritional quality of the control and fortified soy yogurts. Mice were indivdually housed in cages at 21 ± 2°C and humidity of 65% to 75% with a 12:12 h light-dark cycle. All the mice were acclimatized on AIN-93G diet for 1 wk to stabilize metabolic conditions before the feeding experiments. The mice were divided into four groups (six in each group) that did not exhibit any significant differences in the initial body

Table 1
Composition of four tested experimental diets received by Balb/c mice groups in this study, according to the standard AIN-93G diet

Ingredients (g/100 g)	Group I	Group II	Group III	Group IV
Casein	20.0	20.0	13.0	13.0
Cornstarch	39.8	38.8	37.3	37.3
Dextrinized cornstarch	13.2	12.9	12.9	12.9
L-cystine	0.3	0.3	0.3	0.3
Sucrose	9.5	9.5	9.5	9.5
Soybean oil	7.0	7.0	7.0	7.0
Cellulose	5.0	5.0	5.0	5.0
Vitamin mix	1.0	1.0	1.0	1.0
Minerals mix	3.5	3.5	3.5	3.5
Cholesterol	0.00	1.25	1.25	1.25
Choline bitartarate	0.25	0.25	0.25	0.25
Cholic acid	0.00	0.5	0.5	0.5
Soy yogurt	0.00	0.00	8.5 (SYC)	8.5 (SP I)
Total	100	100	100	100
Energy (kcal/100 g)	372.2	368.5	371.15	371.63

AIN, American Institute of Nutrition; SCLBP, synbiotic capsule *LactoBacil Plus*; SSY, synbiotic soy yogurt; SYC, soy yogurt control.
Group I, AIN-93G diet (placebo group); group II, AIN-93G diet + 1.25% cholesterol + 0.5% cholic acid (negative control); group III, AIN-93G diet + 1.25% cholesterol + 0.5% cholic acid and SYC (positive control); group IV, AIN-93G diet + 1.25% cholesterol + 0.5% cholic acid and SSY (2% SCLBP per 100 mL of soy milk).
These diets supplied equal amounts of carbohydrate, protein, fat, and dietary fiber. Thus the diets were formulated to be isocaloric.

weight and serum total cholesterol level from each other. The placebo group (group I) were fed the AIN-93G diet only throughout the entire feeding period. The hypercholesterolemic group (AIN-93G) was fed a diet containing 1.25% cholesterol and 0.5% cholic acid for 4 wk. After 4 wk, all hypercholesterolemic mice (with 200 mg/dL of blood cholesterol) were randomly assigned into three dietary treatments for 5 wk. Mice in the negative control group (group II) were fed an AIN-93G diet containing 1.25% cholesterol and 0.5% cholic acid, whereas mice in positive control group (group III) were fed on control soy yogurt (SYC) in addition to AIN-93G diet and 1.25% cholesterol and 0.5% cholic acid. Mice in group IV were fed symbiotic soy yogurt containing SCLBP capsule (2%) along with AIN-93G diet and 1.25% cholesterol and 0.5% cholic acid. A high-cholesterol diet was supplemented with 8.5% soy yogurt (SYC and SSY). Composition of experimental diets and the detail of the groups are given in Table 1. All the mice except group I were fed cholesterol throughout the experiment.

Experimental feeding was continued for 5 wk with ad libitum access to the diet and water. The diet consumed by each mouse was determined by deducting the leftover and spilled food from the total amount supplied per day. Feed intake of individual mice was measured daily by excluding leftover and collecting spilled food during the entire period to determine the impact of individual experimental diets. Deionized water was provided with the help of graduated drinking bottles. All the experiments were carried out as approved by the Institutional Animal Ethics Committee (IAEC) of the ICMR-NICED (PRO/143/November 2017–December 2019).

Body weight and food intake of mice fed different experimental diets

After feeding treatment, all mice appeared healthy and there was no evidence of toxicity. Feeding and growth performance were monitored by recording initial body weight, final body weight, feed intake, and body weight gain according to Chapman [30].

Sampling procedure

At the end of the 5-wk experimental period, blood samples were collected from tail veins of overnight fasted mice under anesthesia. The serum was separated by centrifugation at 3500 × g for 15 min after allowing the blood to stand for at least 30 min at room temperature and stored at −80°C until analysis.

Biochemical analyses of serum of mice fed different experimental diets

Serum fasting glucose was determined according to the method of Trinder [31] using reagents kits purchased from Mediclone Biotech Pvt. Ltd, India. Serum TC was determined according to the method of Allian [32], and serum triglyceride (TG) levels were determined according to the method of Fossati and Prencipe [33]. Serum HDL-C was determined according to the method of Burstein [34]. Serum LDL-C was calculated by the Friedewald equation [35] and serum very low density lipoprotein (VLDL) was determined according to Norbert's formula [36]. Atherogenic index was calculated by using equation 1.

$$\text{Atherogenic index} = \log\left(\frac{TG}{HDL-C}\right) \quad (1)$$

Assay of blood serum liver enzymes of mice fed different experimental diets

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed according to the method of Reitman and Frankel [37]. Estimation of alkaline phosphatase was done by Bowers and McComb [38]. Total bilirubin was assayed according to the method of Pearlmen and Lee [39]. γ-Glutamyl transferase (GGT) was assayed according to the method of Persijn and Van der Silk [40] using reagents kits purchased from Mediclone Biotech Pvt. Ltd, India.

Evaluation of oxidative stress of mice fed different experimental diets

Livers were immediately perfused with ice cold 0.1-M phosphate buffered saline to prepare a 10% suspension. This suspension was then centrifuged at 16 000 × g for 30 min in a cooling chamber at 0°C. The supernatant was employed to assess the parameters of oxidative stress after estimating the protein content. Lipid peroxidation (LPO), reduced glutathione (GSH), super oxide dismutase (SOD), and catalase (CAT) activity of liver tissue homogenate were measured by Ohkawa [41], Marklund and Marklund [42], and Aebi [43], respectively.

Statistical analysis

Data are presented as means ± SD. Statistical analysis was performed by using multivariant analysis of variance with the Origin Pro 8 software package for Windows. The means were compared between groups by Tukey's post hoc test. All analyses were carried out in triplicate. Values of $P < 0.05$ were considered significant.

Results

Proximate compositions of control and synbiotic soy yogurts

Proximate composition of both control soy yogurt (SYC) and synbiotic soy yogurt (SSY) prepared from soy milk obtained from whole soy seeds is shown in Table 2.

As expected, the total solid contents recorded for SYC were significantly lower than that of SSY throughout the storage period because no prebiotics were added in SYC. Table 2 shows that total solid contents of SYC and SSY decreased during the total storage period of 28 d. Fat content in SYC and SSY decreased gradually during storage. In the present study SSY had significantly lower fat content than SYC during storage. Our results indicated that protein content was lower in SYC. Incorporation of prebiotic significantly increased the protein content of synbiotic yogurt. We found that protein content of SYC decreased gradually during the storage period of 28 d, and in the case of SSY, after 7 d of storage, protein content gradually decreased during the overall storage period. Maximum protein content was found in SSY at 7 d of storage and minimum protein content was identified in SYC at 28 d of storage, respectively. Table 2 indicated that SCLBP-formulated SSY had higher energy content than SYC during the storage period.

Antioxidant activities of control and synbiotic soy yogurts

Antioxidant properties of control and soy yogurt samples during 28 d of storage are presented in Figure 1 indicating that DPPH radical scavenging activity of SYC decreased while that of SSY increased gradually during the storage period. Figure 1 also reveals that SSY had higher free radical scavenging activity compared with that of SYC. SSY had the highest value of DPPH radical scavenging activity on the seventh day of storage, followed by SYC. The capacity of soy yogurts to act as reducing components was evaluated by the FRAP assay. Antioxidant property measured by FRAP assay is also presented in Figure 1, which indicates that the FRAP value of synbiotic soy yogurt was significantly higher than the control

Table 2
Proximate compositions of SYC and SSY stored at 4°C for 28 d

Proximate compositions (g/100 g)	Day	SYC	SSY
Total solids	0	12.27 ± 0.25*	14.20 ± 0.11†
	7	11.57 ± 0.05*	13.13 ± 0.03†
	14	10.88 ± 0.05*	13.02 ± 0.05†
	21	8.79 ± 0.06*	12.18 ± 0.05†
	28	8.08 ± 0.07*	11.17 ± 0.02†
Moisture	0	87.7 ± 0.01†	85.80 ± 0.02†
	7	88.43 ± 0.05†	86.87 ± 0.03*
	14	89.12 ± 0.02†	86.98 ± 0.05*
	21	91.21 ± 0.05†	87.82 ± 0.02*
	28	91.92 ± 0.01†	88.83 ± 0.01*
Protein	0	4.73 ± 0.14*	5.72 ± 0.07†
	7	4.52 ± 0.02*	5.75 ± 0.05†
	14	4.23 ± 0.01*	5.53 ± 0.06†
	21	3.59 ± 0.01*	5.02 ± 0.02†
	28	3.12 ± 0.14*	4.91 ± 0.02†
Fat	0	3.40 ± 0.03*	2.61 ± 0.01†
	7	3.21 ± 0.01*	1.93 ± 0.03*
	14	2.96 ± 0.05†	1.78 ± 0.02†
	21	1.63 ± 0.01†	1.72 ± 0.06†
	28	1.54 ± 0.02†	1.65 ± 0.25
Total ash	0	0.60 ± 0.03†	0.92 ± 0.08†
	7	0.60 ± 0.05*	0.91 ± 0.07†
	14	0.58 ± 0.05†	0.89 ± 0.10†
	21	0.55 ± 0.03†	0.88 ± 0.03†
	28	0.53 ± 0.09†	0.86 ± 0.02
Carbohydrate	0	3.49 ± 0.04†	4.95 ± 0.07†
	7	3.24 ± 0.02†	4.81 ± 0.05*
	14	3.11 ± 0.07*	4.80 ± 0.04†
	21	3.02 ± 0.09*	4.56 ± 0.05†
	28	2.89 ± 0.01*	3.75 ± 0.06†
Energy (kcal/100 g)	0	63.05 ± 0.10†	64.93 ± 0.50†
	7	59.12 ± 0.20†	58.40 ± 0.30†
	14	55.22 ± 0.20†	56.14 ± 0.41†
	21	40.35 ± 0.10*	52.66 ± 0.30†
	28	37.17 ± 0.40*	48.55 ± 0.40†

SCLBP, synbiotic capsule *LactoBacil Plus*; SSY, synbiotic soy yogurt (2% SCLBP per 100 mL of soy milk); SYC, soy yogurt control.
Results are expressed as mean ± SD ($n = 3$).
*,†Mean values having different superscript symbols in rows are significantly different ($P < 0.05$).

yogurt. It was also found that FRAP of SSY decreased gradually during the storage period, whereas FRAP of SYC was increased.

Microbiological properties of control and synbiotic soy yogurts

Table 3 lists the viable count of probiotic bacteria and yogurt culture in the experimental products. The results in Table 3 indicate that count of lactic acid bacteria decreased in soy yogurt without supplementation during refrigerated storage up to 28 d. No coliform bacteria or *Salmonella* were detected in any of the soy yogurt samples in the present study.

Body weight, food intake, water intake, and urine volume of mice fed different experimental diets

Body weight and food intake in each group of all mice fed different experimental diets were shown in Figure 2. The results revealed that non-significant changes were found in initial body weight of all the mice, whereas final weight, gain in weight, and food intake per day were significantly lower in hypercholesterolemic mice (group II) compared with mice fed a normal diet (group I). At the end of the feeding period the final body weights of hypercholesterolemic mice that fed on soy yogurt (groups III and IV) were significantly higher than the mice continued on only a hypercholesterolemic diet (group II). Group IV had a greater increase in body weight (9.64 ± 0.95; $P < 0.05$) compared with group II (7.26 ± 2.56) than group III (9.64 ± 0.95; $P < 0.05$). Mice in group II had hypophagia (Fig. 2B) and hypodipsia (Fig. 2C) compared with group I. Interestingly, groups II, III, and IV had hypouria (Fig. 2D). As indicated in Figure 2, food and water intake was significantly greater for hypercholesterolemic mice that fed on soy yogurt (groups III and IV) compared with the mice continued fed only the hypercholesterolemic diet (group II). Blood serum lipid profiles of mice fed different experimental diets are shown in Table 4, which indicates that intake of 1.25% of cholesterol in the diet caused a significant increase in TC in the mice of groups II, III, and IV compared with mice fed the AIN-93G diet (group I), indicating that hypercholesterolemia was successfully induced in groups II, III, and IV. But a reduction in the total cholesterol was identified in hypercholesterolemic mice that fed on soy yogurts (groups III and IV), and the reduction was greatest with the SCLBP-fortified synbiotic soy yogurts (group IV) compared with the control soy yogurt (group III). Mice in group IV had lowered their elevated TC to 25.89%, whereas it was only 19.01% for group III compared with group II. The animals in group I, which did not receive cholesterol in the diet, did not have a significant difference in the values of total cholesterol during the entire feeding period. These results are interesting because they indicate that a decrease in the total cholesterol level occurred in groups III and IV compared with group II because of the use of soy yogurt that was taken from outside. It seems that soy yogurts, which are the fermented product, do not interfere with the synthesis of endogenous cholesterol.

Blood serum lipid profiles of mice fed different experimental diets

Table 4 also shows that the hypercholesterolemic mice (groups II, III, and IV) exhibited an elevated TG, VLDL-C, and LDL-C but a decreased HDL-C compared with group I. Consumption of soy yogurts significantly reduced TG, VLDL-C, and LDL-C while increasing HDL-C in groups III and IV compared with group II. This reduction in TG, VLDL-C, and LDL-C and elevation in HDL-C was greatest with hypercholesterolemic mice that fed on SSY.

Blood serum AST, ALT, alkaline phosphatase, GGT, and total bilirubin levels of mice different fed experimental diets

Table 5 shows significant differences in liver enzyme levels and reveals that AST, ALT, alkaline phosphatase (ALP), and GGT activities and total bilirubin level in all the hypercholesterolemic mice (groups II, III, and IV) increased compared with those of the mice fed control diet (group I). Consumption of soy yogurts (groups III and IV) significantly reduced the activities of the elevated liver enzymes, and maximum reduction was identified in hypercholesterolemic mice fed 2% SCLBP-fortified symbiotic soy yogurt.

Liver oxidative stress biomarkers and antioxidant parameters (LPO, GSH, SOD, and CAT) of mice fed different experimental diets

As shown in Table 6, hypercholesterolemic mice (group II) had large decreases in SOD and CAT levels compared with group I. In our study a significant increase in thiobarbituric acid reactive substances such as malondialdehyde level was identified in hypercholesterolemic mice (group II), indicating higher lipid oxidation in hypercholesterolemic mice. In the present study, a significant increase in SOD (40.82%) and CAT (102.1%) concentrations was identified after administration of the SCLBP-fortified synbiotic soy yogurt compared with a hypercholesterolemic diet. In addition, with regard to malondialdehyde (MDA) concentration, mice consuming soy yogurt and synbiotic soy yogurt had lower MDA level

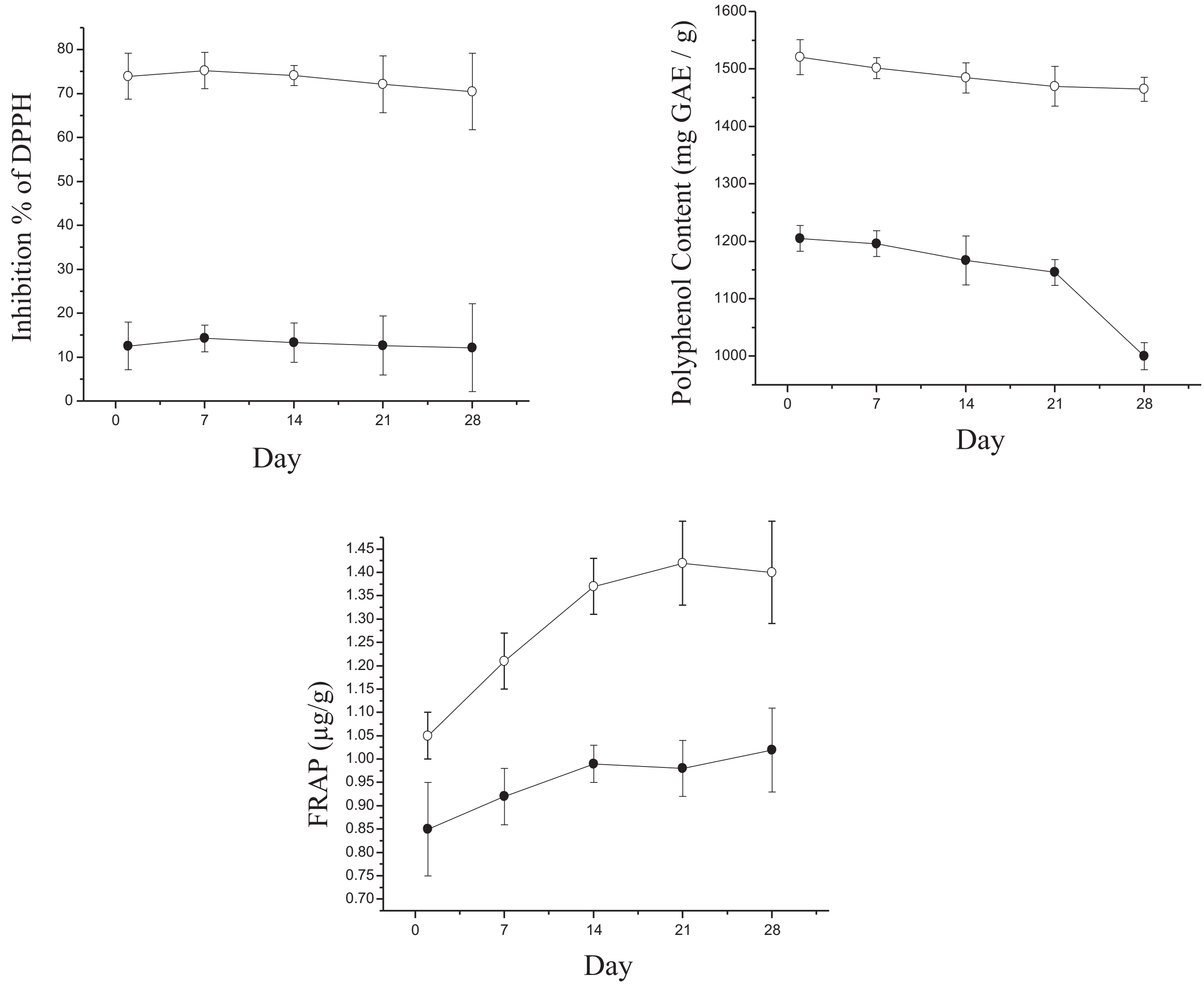

Fig. 1. Antioxidant activities of SYC and SSY stored at 4°C for 28 d. DPPH, 1,1-diphenyl, 2-piryl hydrazyl; FRAP; ferric-reducing antioxidant power; GAE, gallic acid equivalent; SCLBP, synbiotic capsule *LactoBacil Plus*; SSY (open circles), synbiotic soy yogurt (2% SCLBP per 100 mL of soy milk); SYC (filled circles), soy yogurt control.

than did those on normal and hypercholesterolemic diets. This decrease was greater in group IV than group III. GSH in hypercholesterolemic mice decreased significantly compared with that of normal mice (group I). Our results indicated that compared with hypercholesterolemic mice (group II), hypercholesterolemic mice fed control soy yogurt (group III) and synbiotic soy yogurt (group IV) exhibited higher GSH levels, and this improvement was greater in group IV than group III.

Discussion

High blood cholesterol is generally considered a risk factor for cardiovascular disease. Therefore development of fermented symbiotic yogurt with the suitable prebiotics and probiotics to improve serum cholesterol levels has attracted much interest in recent years.

In the present study a significant decrease in total solid content was identified in SYC compared with SSY throughout the storage

Table 3
Microbiological properties of SYC and SSY stored at 4°C for 28 d

Microorganisms/Day	SYC					SSY				
	0	7	14	21	28	0	7	14	21	28
TPC (log cfu/mL)	1.8×10^6	2.4×10^6	4.4×10^6	4.6×10^6	4.2×10^6	2.6×10^6	3.3×10^6	4.6×10^6	5.2×10^6	4.8×10^6
Lactic acid bacteria (log cfu/mL)	16.29×10^6	15.01×10^6	12.14×10^6	8.25×10^6	7.26×10^6	18.09×10^6	19.78×10^6	20.15×10^6	19.68×10^6	18.06×10^6

SCLBP, synbiotic capsule *LactoBacil Plus*; SSY, synbiotic soy yogurt; SYC, soy yogurt control; TPC: total plate count.
Results are expressed as mean ± SD ($n = 3$).
Salmonella spp. (log cfu/mL), *Escherichia coli* (log cfu/mL), and coliform (log cfu/mL) are the missing determination in both yogurts.

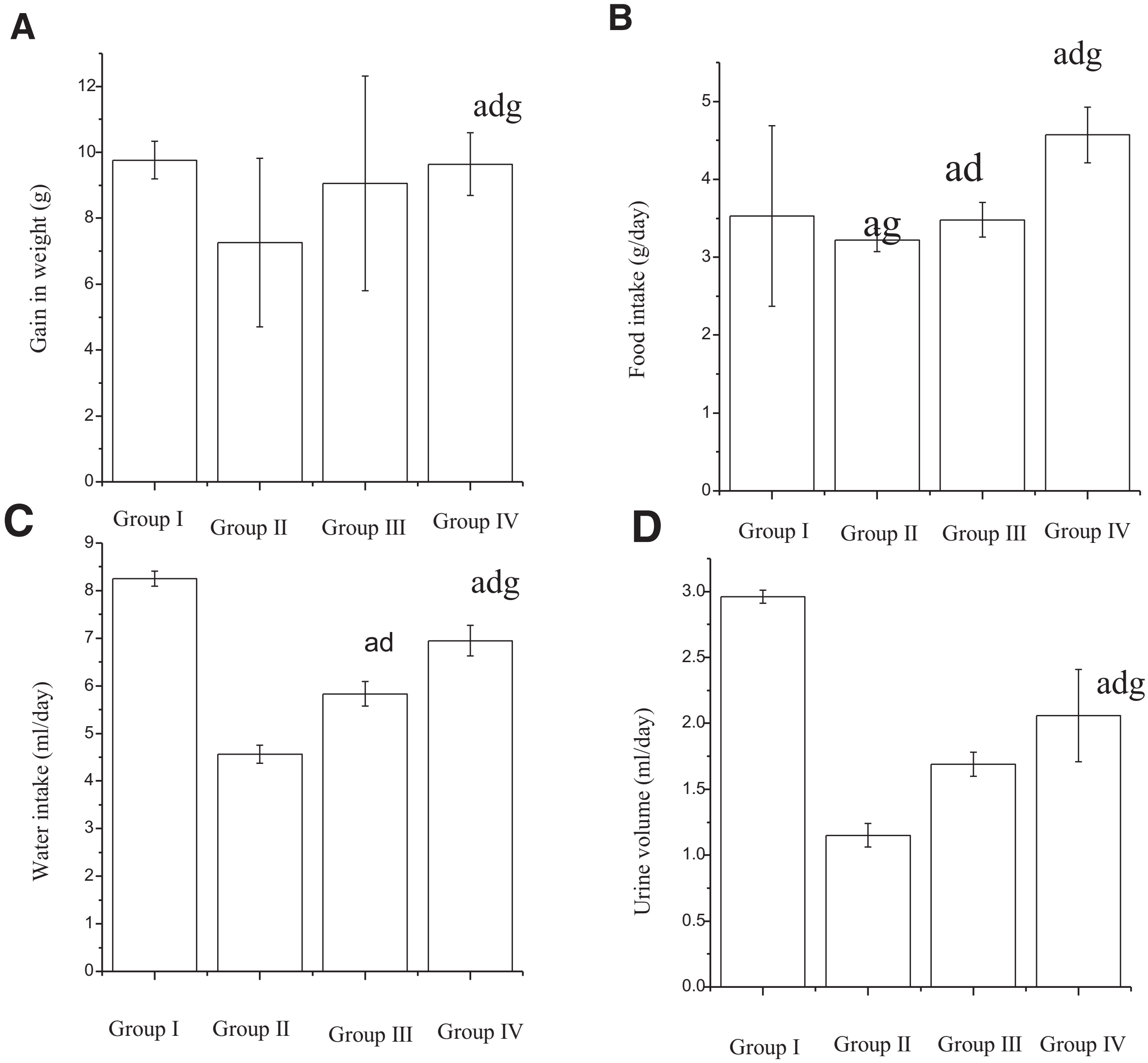

Fig. 2. Body weights gain (A), food intake (B), water intake (C), and urine volume (D) of mice fed different experimental diets. Group I, AIN-93G diet (placebo group); group II, AIN-93G diet + 1.25% cholesterol + 0.5% cholic acid (negative control); group III, AIN-93G diet + 1.25% cholesterol + 0.5% cholic acid and SYC (positive control); group IV, AIN-93G diet + 1.25% cholesterol + 0.5% cholic acid and SSY (2% SCLBP per 100 mL of soy milk). The data are significantly different at ap < 0.05, bp < 0.01 and cp < 0.001 vs Group I, at dp < 0.05, ep < 0.01 and fp < 0.001 vs Group II, at gp < 0.05, hp < 0.01 and ip < 0.001 vs Group III; AIN, American Institute of Nutrition; SCLBP, synbiotic capsule *LactoBacil Plus*; SSY, synbiotic soy yogurt; SYC, soy yogurt control.

Table 4
Blood serum lipid profiles of mice fed different experimental diets

Parameters	Group I	Group II	Group III	Group IV
FBS (mg/dL)	106.33 ± 7.23	109.45 ± 4.56	106.66 ± 2.25	105.29 ± 6.29
TC (mg/dL)	$139.66 \pm 10.57^{\S,\#}$	$236.57 \pm 8.47^{*,\#}$	$191.58 \pm 3.58^{\dagger,\S}$	$168.47 \pm 10.29^{\dagger,\P,\dagger\dagger}$
TG (mg/dL)	$112.46 \pm 2.75^{\S,\#}$	$148.12 \pm 9.57^{*,\#}$	$126.54 \pm 4.65^{*\S}$	$126.47 \pm 4.29^{\dagger,\S,\dagger\dagger}$
HDL-C (mg/dL)	$84.61 \pm 0.72^{\S,\#}$	$70.19 \pm 0.95^{*,\#}$	$72.54 \pm 0.26^{*}$	$87.19 \pm 3.29^{\S}$
VLDL-C (mg/dL)	$22.49 \pm 0.55^{\S,\#}$	$28.41 \pm 0.46^{*,\#}$	$25.30 \pm 0.26^{*,\S}$	24.65 ± 0.49
LDL-C (mg/dL)	$32.56 \pm 0.96^{\S,\#}$	$137.97 \pm 2.26^{*,\#}$	$93.74 \pm 0.85^{\ddagger,\|}$	$88.26 \pm 0.83^{*,**}$
A I	$0.12 \pm 0.06^{\S,\#}$	$0.32 \pm 0.05^{*,\#}$	$0.24 \pm 0.05^{*}$	$0.16 \pm 0.04^{*,\S}$

AI, atherogenic index; AIN, American Institute of Nutrition; FBS, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SCLBP, synbiotic capsule *LactoBacil Plus*; SSY, synbiotic soy yogurt; SYC, soy yogurt control; TC, total cholesterol; TG, triglyceride; VLDL-C, very low density lipoprotein cholesterol.

Group I: AIN-93G diet (placebo group); group II: AIN-93G diet + 1.25% cholesterol + 0.5% cholic acid (negative control); group III: AIN-93G diet + 1.25% cholesterol + 0.5% cholic acid and SYC (positive control); group IV: AIN-93G diet + 1.25% cholesterol + 0.5% cholic acid and SSY (2% SCLBP per 100 mL of soy milk).

The data are mean ± SD and significantly different at $^{*}P<0.05$, $^{\dagger}P<0.01$, and $^{\ddagger}P<0.001$ versus group I; at $^{\S}P<0.05$, $^{\P}P<0.01$, and $^{\|}P<0.001$ versus group II, at $^{\#}P<0.05$, $^{**}P<0.01$, and $^{\dagger\dagger}P<0.001$ versus group III.

Table 5
Blood serum AST, ALT, ALP, GGT, and total bilirubin levels of mice fed different experimental diets

Parameters	Group I	Group II	Group III	Group IV
AST (IU/L)	121.14 ± 3.07 $^{§,\#}$	230.15 ± 2.93 †,**	196.56 ± 5.56*,§	164.98 ± 7.50†,¶
ALT (IU/L)	62.10 ± 1.43 $^{§,\#}$	132.20 ± 2.10 ‡,††	123.11 ± 6.29*,§	90.59 ± 2.41*,§
ALP (IU/L)	0.01 ± 0.012 $^{§,\#}$	0.065 ± 0.019 $^{†,\#}$	0.039 ± 0.002 *,§	0.054 ± 0.012
GGT(IU/L)	56.03 ± 3.41$^{§,\#}$	142.45 ± 3.23†	127.19 ± 6.31*,§	85.29 ± 7.69†,¶
Total bilirubin (IU/L)	1.50 ± 0.17$^{§\|\|}$	2.30 ± 0.05†	1.91 ± 0.05*,§	1.72 ± 0.01*,§

AIN, American Institute of Nutrition; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; SCLBP, synbiotic capsule *LactoBacil Plus*; SSY, synbiotic soy yogurt; SYC, soy yogurt control.
Group I: AIN-93G diet (placebo group); group II: AIN-93G diet + 1.25% cholesterol + 0.5% cholic acid (negative control); group III: AIN-93G diet + 1.25% cholesterol + 0.5% cholic acid and SYC (positive control); group IV: AIN-93G diet + 1.25% cholesterol + 0.5% cholic acid and SSY (2% SCLBP per 100 mL of soy milk).
The data are mean ± SD and significantly different at $^{*}P<0.05$, $^{†}P<0.01$, and $^{‡}P<0.001$ versus group I; at $^{§}P<0.05$, $^{¶}P<0.01$, and $^{\|}P<0.001$ versus group II; and at $^{\#}P<0.05$, $^{**}P<0.01$, and $^{††}P<0.001$ versus group III.

period. This result is similar to results reported by Kumari et al. [44], who developed plain yogurt using cow's milk and a synbiotic yogurt with probiotic *Bifidobacterium animalis* ssp. *lactis* BB-12 using cow's milk and boiled rice. They found that total solid content was lowest in plain yogurt. According to the result of present study, synbiotic soy yogurt exhibited significantly lower fat content than control soy during storage. This might be due to the use of fat by the probiotic bacteria present in SCLBP-fortified soy yogurt [45]. In contrast, Kumari [44] reported that the average fat content in plain yogurt was lower than rice-incorporated synbiotic yogurt. Results of this study revealed that addition of prebiotics and probiotics to yogurt caused an increase in protein content compared with control yogurt without the addition of SCLBP. Similarly Kumari [44] reported that average protein content in plain yogurt was lower than rice-incorporated synbiotic yogurt.

The DPPH method and FRAP assay were employed to evaluate the antioxidant potential of different experimental soy yogurts. The reducing capacity of a yogurt may serve as a significant indicator of its potential antioxidant activity. In DPPH radical scavenging activity assay method, the antioxidative capacity was expressed in terms of percentage reduction of free radicals. DPPH radical scavenging activity of an antioxidant is responsible for their hydrogen or electron donating ability [46]. In the presence of an antioxidant this radical scavenging activity is visually noticeable as a change in the color of DPPH (from purple to yellow). It has been commonly used to test the ability of compounds as free radical scavengers or hydrogen donors and to evaluate the antioxidative activity of dairy and non-dairy foods [47,48]. Whole soy seeds had antioxidants presumably as a result of the presence of isoflavones (genistein and daidzein) in soy. SSY formulated with SCLBP contributed to enhancing the DPPH radical scavenging activity compared with SYC, which clearly indicated that probiotic strains could possibly provide additional dietary sources of antioxidants. These results were similar to those noted by Mahmoudi et al. [49], who reported that a mixture of probiotic *Lactobacillus* strains exhibited antioxidant activities by DPPH methods in the presence of prebiotics (fructo-oligosaccharides and lactulose). In the FRAP assay, antioxidants present in the experimental soy yogurts formed a blue Fe2 +-TPTZ complex because of reduction of the Fe^{3+}-TPTZ complex, resulting an increase in the absorbance. SCLBP-fortified soy yogurt presented higher ferric-reducing antioxidant power than that of control soy yogurt. The strong reducing power of SSY may be due to increased availability of hydrogen ions as a result of metabolic reactions of probiotic microorganisms present in SSY. These findings indicated that probiotic microorganisms have strong potential antioxidant properties and possibly provide additional dietary sources of antioxidants.

The synbiotic yogurts (SSY) developed in this study contained probiotic cultures and control yogurt (SYC) contained ordinary yogurt starters. It was already documented that yogurts containing live probiotics provide therapeutic properties to the host when they are consumed in appropriate quantity [50]. Thus it is necessary for probiotic cultures to survive in yogurts during their shelf life before being consumed. The minimum acceptable level of viable probiotic cultures in 100 g of probiotic product should be 10^6 to 10^9 cfu/g at the time of consumption. During the storage period, good viability of the probiotic culture was identified in SSY. In the present study the synbiotic yogurts contained the minimum level of probiotic cultures suggested previously. Because the probiotic culture we used in this study caused a significant ($P < 0.05$) reduction in serum total cholesterol, triglycerides, and LDL-C levels in the mice fed a cholesterol-enriched diet compared with control soy yogurt, we predict that higher levels of these bacteria probably would have more potential to reduce total cholesterol and HDL-C in hypercholesteremic patients. The results are similar to the findings of Shaghaghi et al. [7] on the effects of inulin and lactulose containing synbiotic yogurt on survival of probiotic growth. Mahrous et al. [51] found that the addition of oat to yogurt as a prebiotic caused an increase in the number of probiotics cultured compared with control yogurt. Results of the study by Mahrous et al. [51] concerning the presence of coliform bacteria and *Salmonella* were in agreement with the study conducted by Abhari et al. [1] with rice-incorporated synbiotic yogurt. Undesirable levels of yeast and mold and detection of no coliforms in any of the yogurt samples probably were due to use of a good hygienic environment during the development of yogurt in this study.

Table 6
Liver oxidative stress biomarkers and antioxidant parameters (LPO, GSH, SOD, and CAT) of mice fed different experimental diets

Parameters	Group I	Group II	Group III	Group IV
LPO (MDA nmoles/g)	38.85 ± 0.19$^{§,\#}$	76.75 ± 0.75*	68.26 ± 0.31$^{†,§,\#}$	73.56 ± 0.48$^{*,§,\#}$
GSH (nmoles/g)	54.20 ± 0.37 $^{§,\#}$	35.54 ± 0.59$^{*,\#}$	40.26 ± 1.29$^{*,\#,\|}$	50.05 ± 0.48*,§,
SOD (units/mg/protein)	56.01 ± 0.79 $^{§,\#}$	37.85 ± 1.84$^{*,\#}$	38.01 ± 0.35$^{*,§,\|}$	55.14 ± 0.16$^{*,§,\|}$
CAT (H_2O_2 consumed/mg protein/min)	1.96 ± 0.0 1 $^{§,\#}$	0.95 ± 0.005$^{*,\#}$	1.22 ± 0.09*,§	1.92 ± 0.006$^{*,\|,**}$

CAT, catalase; GSH, reduced glutathione; LPO, lipid peroxidation; MDA, malondialdehyde; SCLBP, synbiotic capsule *LactoBacil Plus*; SOD, superoxide dismutase; SSY, synbiotic soy yogurt; SYC, soy yogurt control The data are mean ± SD and significantly different at $^{*}P<0.05$, $^{†}P<0.01$, and $^{‡}P<0.001$ versus group I; at $^{§}P<0.05$, $^{¶}P<0.01$, and $^{\|}P<0.001$ versus group II; and at $^{\#}P<0.05$, $^{**}P<0.01$, and $^{††}P<0.001$ versus group III.

The present investigations concerning the hypocholesterolemic effect of prebiotic in combination with probiotic are in the same line as several in vitro trials. Because of the presence of soy protein, isoflavones, and saponin, the effect of soy yogurt as a functional food for the improvement of hypolipidemia is well documented [12,13]. Kim et al. [16] investigated the beneficial effects of a probiotic mixture containing *Lactobacillus* (*L. ruteri* and *L. plantarum*) on cholesterol-lowering efficacy in hypercholesterolemic rats. They found that after supplementation of the probiotic mixture in hypercholesterolemic rats, significant reductions of TC, TG, VLDL-C and LDL-C levels and increased HDL-C were identified. Mahrous et al. [51] found that cholesterol and bile acid levels in the serum of mice fed with synbiotic yogurt fermented by probiotic Lactacidophillus P106 with 7% oat decreased significantly. Abhari et al. [1] reported that administration of symbiotic diets containing probiotic *Bacillus coagulans* and prebiotic inulin significantly reduced serum cholesterol and LDL-C and increased HDL-C of hypercholesterolemic rats, but they found no influence of probiotic *Bacillus coagulans* on lipid profiles. Mahmoudi et al. [52] found that 20 selected probiotic *Lactobacillus* strains exhibited cholesterol-removing properties via cholesterol assimilation in the presence of the prebiotics fructo-oligosaccharides and lactulose. Ooi and Liong [53] found that the synbiotic diet that contained *Lactobacillus casei*, fructo-oligosaccharide, and maltodextrin beneficially altered cholesterol levels and produced a healthier bowel microbial population. Schaafsmaet al. [54] reported that daily consumption of synbiotic milk containing *L. acidophilus* and fructo-oligosaccharide resulted in a significant decline in TC, LDL-C, and LDL-to-HDL ratio. *Lactobacillus* spp. and *Bifidobacterium* spp. comprise the main probiotic organisms, and the SCLBP-formulated soy yogurt manufactured in this study is a source of these probiotics. El-Sayyad [2] reported that administration of yogurt supplemented with *Bifidobacterium pseudocatenulatum* G4 or *B. longum* to hypercholesterolemic rats for 8 wk led to significant reductions in TC and LDL-C by 7.2% and 10.3%, respectively.

A number of cholesterol removal mechanisms by probiotic have been proposed, including assimilation of cholesterol by growing cells, binding of cholesterol to cellular surface, incorporation of cholesterol into the cellular membrane, deconjugation of bile via bile salt hydrolase, and coprecipitation of cholesterol with deconjugated bile [55]. But the exact mechanisms remain unclear and controversial.

Serum TC concentrations had a direct effect on development of experimental atherosclerosis. Clinical and epidemiologic studies found that elevated LDL-C concentrations were a primary risk factor for development of atherosclerosis [53]. Decreased plasma LDL-C concentrations were a potential mechanism that contributed to reducing atherosclerosis in mice administered with synbiotic soy yogurt compared with regular soy yogurts.

The activities of plasma AST, ALT, alkaline phosphatase, and GGT reflect the state of liver function. Elevations of these enzymes are usually signs of liver disease. These results were in agreement with the result obtained by Sartang et al. [56], who reported that twice daily administration (2×250 g) of fortified synbiotic yogurt significantly reduced serum levels of hepatic enzymes. Ngongang et al. [57] found that hyperlipidemic Wister rats that did not receive probiotic strains *Lactobacillus plantarum* and *Lactobacillus pentosus* registered higher amounts of two transaminase enzyme (ALT and AST) activities. It was expected that the addition of SSY to a hypercholesterolemic diet would be effective for the recovery of hepatic function by improving lipid metabolism or delaying the hepatic disorder.

Cellular antioxidative enzymes LPO, GSH, SOD, and CAT are used as liver oxidative stress biomarkers. In hypercholesterolemic mice (group II), reduction of these enzymes activity was associated with the accumulation of highly reactive free radicals, which reduced functional activity of cell membrane [58]. Elevated levels of lipid peroxides and the reduction of antioxidant enzymes have been reported to occur in hypercholesterolemic mice [59]. An increase of MDA levels in animals fed with a high cholesterol diet has been previously reported [60]. Elevation of lipid peroxidation was reduced at a maximum rate with synbiotic soy yogurt administration because of its probiotic bacterial activity, antioxidant activity, and presence of prebiotics such as fructo-oligosaccharides [61]. Apart from enzymatic antioxidants, non-enzymatic antioxidants such as GSH played a vital role in protecting cells from oxidative damage. Lowering of GSH in hypercholesterolemic mice was due to the increased utilization of these antioxidants for quenching enormous free radicals produced in hypercholesterolemia. This result suggested that synbiotic soy yogurt exhibited greater antioxidant and antiradical effects relative to soy yogurt. These findings further support the research of Tulk et al. [62], who reported an increase in antioxidant status in aging mice on supplementation with synbiotic yogurt.

Conclusions

The present work was conducted to develop synbiotic soy yogurt combining the health benefits of synbiotic capsule *LactoBacil Plus* with the fructo-oligosaccharides prebiotics. In conclusion, our findings suggested that synbiotic soy yogurt has the potential to reduce serum total cholesterol, triglycerides, and LDL-C levels in the mice fed a cholesterol-enriched diet compared with control soy yogurt. In addition, the synbiotic yogurt exhibited better antioxidant activities. SCLBP-formulated soy yogurts had higher total solids, protein content, and energy content and higher viability of lactic acid bacteria than control soy yogurts during 28 d of storage at 4°C. Results of the present study therefore indicated that the synbiotic soy yogurt could be consumed as functional food for hypercholesterolemic patients. Administration of synbiotic soy yogurt shows promise as an alternative method for prevention and treatment of cardiovascular diseases.

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