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Therapy of *Lactobacillus casei* Shirota strain Fermented Milk to Rats with High-Cholesterol Diet toward Cholesterol Levels and SOD Activity

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ABSTRACT

This study aimed to determine the fermented milk of Lactobacillus casei Shirota strain potential on cholesterol levels, SOD activity, LPL enzyme activity, and cardiac histopathology. This study used 25 rats (Rattus novergicus) Wistar strain aged 2-3 months, weighing 150-200 grams. These rats were divided into 5 groups: negative control group, positive control group with high-cholesterol diet for 21 days, and therapy group with 1.0 ; 1.5 ; 2.0 mL/rat/day of fermented milk for 14 days. The analysis methods used in this study are One Way ANOVA and Tukey. The results showed that the fermented milk of Lactobacillus casei Shirota strain had a strong effect on blood cholesterol levels and increasing the SOD activity. This study also showed that the fermented milk of Lactobacillus casei with 2.0 mL/rat/day is the most effective dose to decrease the total cholesterol and increase the SOD activity.

Keywords: cholesterol, fermented milk of Lactobacillus casei Shirota strain, total cholesterol, SOD

INTRODUCTION

Cholesterol is a steroid alcohol on the class of lipids (fats) which play a part as a source of calories. In addition, cholesterol is a component of fat found in the blood of animals and humans that produced by the liver and used as an energy producer. It is also the wall-forming cells in the body and as a basic ingredient of the steroid hormones formation in the body. Cholesterol is the precursor of all steroids, such as corticosteroids, sex hormones, bile acids, and vitamin D.

Consuming lots of high-cholesterol diet can lead to the increasing of cholesterol levels. It causes hypercholesterolemia, atherosclerosis in the arteries, and blood vessels constriction in the heart, brain, kidneys, and eyes when it reaches beyond the normal limits. Hypercholesterolemia is a condition when the blood cholesterol level exceeds the normal value of 200 mg/dL. In the body, cholesterol found in lipoproteins, such as chylomicrons, which contains triglycerides, VLDL (very low density lipoprotein) to transport triglycerides. The triglycerides formed by the heart, LDL (low density lipoprotein) or beta-lipoprotein consisting of 25% protein and 40% cholesterol and HDL (high density lipoprotein) or alpha lipoprotein contain 50% protein and 20% cholesterol [1].

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Fermented milk is a biotechnology product that uses bacteria for fermentation. Consumption of fermented milk can reduce the lipid concentration in the blood [2]. Fermented milk used lactic acid bacteria *Lactobacillus casei* (*L. casei*) Shirota strain has a positive effect as an antioxidant. Several studies of fermented milk, such as yoghurt (using *Lactobacillus bulgaricus* and *Streptococcus thermophillus*) that lowered the cholesterol levels in the blood have been widely studied, but studies on fermented milk of *L. casei* Shirota bacteria have not been investigated yet.

High cholesterol in body can increase the production of ROS (Reactive Oxygen Species). ROS is a major contributor to the formation of oxidative stress. Oxidative stress is a metabolism disorder caused by an imbalance between antioxidants with free radicals that cause tissues damages. SOD (Superoxide Dismutase) has a significant role in protecting cells from free radicals and regulating the balance of toxic oxygen [3]. This study was investigated the effects of fermented milk of *Lactobacillus casei* Shirota strain potential on cholesterol levels and SOD activity to rats.

EXPERIMENT

Chemicals and instrumentation

This study used male rats of *Rattus norvegicus* Wistar strain (2-3 months old) with weight ranges of 150-200 g, commercial fermented milk of *Lactobacillus casei* Shirota strain. The equipment used in this study include laminar air flow, test tubes, incubators, micropipette, gavage, Eppendorf tubes, vortex, scissors, pipette, object glasses, spatula, surgical equipment, freezer, refrigerator, ELISA plate and *Biosystems* total cholesterol kit to check the levels of blood cholesterol in experimental animals. The materials used in this study include the cholesterol standard solution, distilled water, NaCl 0.9%, PFA (paraformaldehyde) 10%, aluminum foil, phosphate buffer (pH 7.4), standard feed of SP, HE dye (hematoxylin-eosin) , alcohol 80%, 90% and 95%, phosphate buffer (pH 6.8), and 0.1 N NaOH.

Acclimation of Experimental Animals

Experimental animals used were male rats (*Rattus novergicus*) Wistar strain aged 2-3 months with a body weight of 150-200 grams which have been approved by the ethical acceptance of UB's Research Commission No. KEP-600-UB. Animals were divided into 5 groups: negative control group (healthy rats), the positive group (sick rats), therapy of *Lactobacillus casei* Shirota strain fermented milk group of 1 mL/rat/day, 1.5 mL/rat/day, and 2 mL/rat/day.

Induction of High Cholesterol Diet

High-cholesterol diet consisting of 1 gram of pure cholesterol, 1 gram of quail egg yolk, 0.02 grams of colic acid, 0.3 mL of cooking oil, and 2 mL of water. The induction of high-cholesterol diet was conducted in 21 days consecutively intraperitonieally.

Measurement of Total Cholesterol Levels

Measurement of total cholesterol was performed using the BioSiystem SA Costa Brava Kit, 30. The reagent composition of the kit is 0.5 mmol/L sodium cholate, 28 mmol/L of phenol, cholesterol esterase > 0.2 U/mL, cholesterol oxidase > 0.1 U/mL, peroxidase > 0.8

U/mL, 4-aminoantipyrine 0.5 mmol/L employed with standard pH and cholesterol, which is 200 mg/dL (5.18 mmol / L). The reagent set up as follows:

	Blank	Standard	Sample
Standard Cholesterol	-	10 µL	-
Sampel	-	-	10 µL
Reagen	1.0 mL	1.0 mL	1.0 mL

Furthermore, the reagents were being homogenated and incubated at room temperature for 10 minutes. Its absorbance was measured with a wavelength of 500 nm.

Measurement of SOD activity

Determination of SOD activity samples was performed using Biovision Superoxide Dismutase Assay Kit. 20 mL of rat's serum were inserted into the sample wells. Then, 20 mL of distilled water put in blank wells (blank 1 and blank 3) and then added by 200 mL of working solution in each well. On the blank 2 and 3 added 20 mL of dilution buffer. Next, it added by 20 mL of enzyme working solution in each wells and blank 1 then incubated for 20 minutes at a temperature of 37^{o}C. The result was read using a micro-plate reader.

DATA ANALYSIS

Data obtained from measurements of serum total cholesterol and serum SOD activity was analyzed using SPSS version 16.00 to test the analysis of variance (ANOVA) and further analysis by Tukey's test (α = 5%).

RESULT AND DISCUSSION

Decreasing Total Cholesterol Levels via Therapy of Fermented Milk containing *Lactobacillus casei* strain Shirota in Rats Serum with High Cholesterol Diet

The calculation results of the total cholesterol levels in Table 1 shows that the lowest cholesterol levels found in mice with 2mL/rat/day with a decline of 36.3%, while the treatment group of 1.0 mL/rat/day and 1.5 mL /rat/day showed a decrease by 28.9% and 34.6% respectively. According to Table 1, a negative control group (healthy rats, given standard feed only) had total blood cholesterol levels of 48.8 ± 0.84 mg/dL, which means total blood cholesterol levels is relatively normal. Group B as a positive control (sick rats) that was fed with high-cholesterol diet had higher levels of total blood cholesterol of ± 83.8 mg/dL. Normal cholesterol level of rats is 10-54mg/dL, and if the cholesterol levels of rats exceed these ranges, rats were suffered from hypercholesterolemia. Group C, D and E are rats group that were fed with a high-cholesterol diet and treated with fermented milk containing *Lactobacillus casei* strain Shirota with the dose of 1.0, 1.5, 2.0 mL/rat/day per day for 14 consecutive days and found a decreasing total cholesterol levels as shown in Table 1. Group C as a group 1 rat therapy (rats given treatment by 1 mL of fermented milk) resulted in decreasing of total cholesterol level significantly compared with group B (positive control) that is 59.6 ± 1.14 mg / dL. Group D as a group 2 rat therapy is was rats treated with 1.5 mL of fermented milk resulted in decreasing of total cholesterol level compared with group B (positive control) that is 54.8 ± 1.14 mg / mL. Last, group E as group 3 rat therapy was rats treated with 2 mL of fermented milk and resulted in a decreasing of total cholesterol levels compared with group B (positive control) that is 53.4 ± 1.14 mg / dl.

Table 1. Total cholesterol level of rat serum with high-cholesterol diet that were treated by fermented milk containing *Lactobacillus casei* Shirota strain

Group	The Average of Total Cholesterol Level in Blood (mg/dL)	Increasing of Total Cholesterol Levels in Blood towards Negative Control (%)	Decreasing of Total Cholesterol Levels in Blood towards Positive Control (%)
Healthy (Negative control)	48.8±0.84	-	-
Sick (Positive control)	83.8±0.84	71.72	-
1.0 mL/rat/day dose therapy	59.6±1.14	-	28.9
1.5 mL/rat/day dose therapy	54.8±1.30	-	34.6
2.0 mL/rat/day dose therapy	53.4±1.14	-	36.3

Cholesterol was converted into IDL (Intermediate Density Lipoprotein) through enzyme lipoprotein lipase activity then it will be turned into LDL within 2-6 hours [4]. Moreover, some of the cholesterol was excreted in the form of bile acids into the gallbladder. Feeding a high-cholesterol diet for a period of time could trigger the formation of plaque as the beginning phase of cardiovascular disease. An enzyme that plays a major rule in cholesterol biosynthesis is a HMG-CoA (Hydroxymethylglutarate-CoA) reductase. *Lactobacillus casei* Shirota strain fermented milk containing lactic acid bacteria that produced statins compound that could inhibit the HMG-CoA reductase enzyme so the formation of cholesterol was restrained [5].

Statins are bioactive compounds synthesized by some micro-organisms which are known to inhibit cholesterol biosynthesis by inhibiting the HMGR (HMG CoA Reductase) enzyme [6]. Previous research stated that the discovery of *Lactobacillus* (lactic acid bacteria) as a statin-producing bacterium was a novelty because previous statin-producing microorganisms are known as fungi such as *Aspergillus terreus*, *Penicillium brevicompactum*, *Streptomyces roseochromogenus*, *Streptomyces carbophilus*, *Streptomyces halstedii*, *Actinomadura sp.*, *Streptomyces sp.*, and *Bacillus megaterium* [7].

Cholesterol synthesis inhibition by fermented milk can occur because of BSH (bile salt hydrolase) enzyme production that can deconjugate bile acids, so it can be eliminated through the feces with cholesterol. Deconjugation of bile acids were employed by BSH enzyme produced free or deconjugate bile salt in the form of free colic acid which is poorly absorbed by small intestine and had a low solubility, so it engendered a decreasing of bile salts that returned into liver and causing a decreasing in total cholesterol. The cholesterol-lowering mechanism was through the C-24 of N-acyl amides termination contained in conjugate bile salts. For lactic acid bacteria, BSH enzymes were useful as a defense in bile acids. Cholesterol is useful as compilers of bacterial membrane so that it will adsorb or assimilate the cholesterol that leads to cholesterol levels decreasing.

Cholesterol levels in the blood that decreased were due to the fermented milk contained lactic acid bacteria, wherein the lactic acid bacteria contained in fermented milk can degrade cholesterol to coprostanol by lactic acid bacteria. Coprostanol is a substance that cannot be absorbed by the intestine, so the coprostanol and the rest of cholesterol can be excreted along with the feces, so it will lead to the declining of total cholesterol levels.

Increased activity of SOD by fermented milk of *Lactobacillus casei* Shirota strain therapy in rat blood serum with high-cholesterol diet

The calculation result of the total cholesterol levels in Table 2 showed that the treatment of 2 mL/rat/day fermented milk therapy lead to the highest increasing of SOD activity in rats, which is 60.65%., while the treatment group of 1.0 mL/rat/day and 1.5 mL/rat/day showed increasing of SOD activity to 21.52% and 38.65% respectively.

Table 2. Superoxide Dismutase (SOD) activity of white male rats (*Rattus norvegicus*) blood serum in all groups that treated with fermented milk containing *Lactobacillus casei* Shirota strain

Group	Average of SOD Activity (U/mL) ($\bar{X}$ ±SD)	Decreasing of SOD activity (%)	Increasing of SOD activity (%)
Negative (healthy)	96.18 ± 1.28	-	-
Positive (sick)	56.4 ± 0.94	41.31	-
1.0mL/rat/day therapy	68.54 ± 1.12	-	21.52
1.5mL/rat/day therapy	78.2 ± 1.28	-	38.65
1.0mL/rat/day therapy	90.56 ± 1.28		60.65

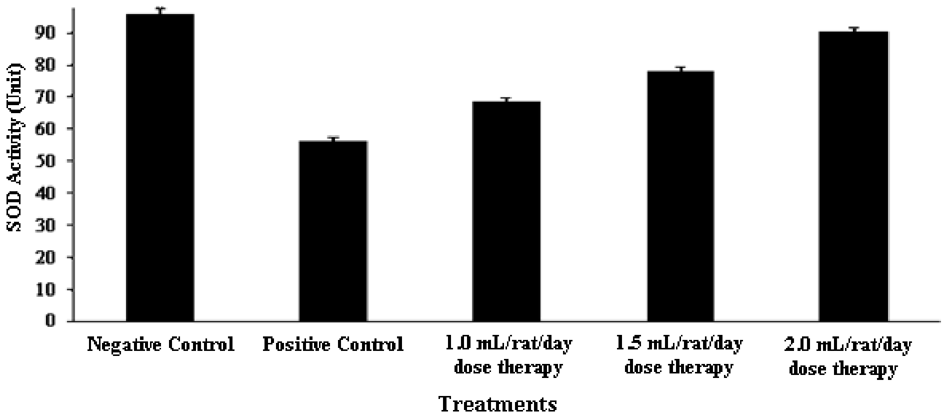

Figure 1. Superoxide Dismutase (SOD) activity of white male rats (*Rattus norvegicus*) blood serum in all groups that treated with fermented milk of *Lactobacillus casei* Shirota strain

The results of this study indicated that the treatment of *Lactobacillus casei* Shirota strain fermented milk can increase the levels of SOD rats fed with high-cholesterol diet. SOD activity of the positive control group of rats a diet high in cholesterol) is 56.4 ± 0.94 units less than the negative control group of rats that is 96.18 ± 1.28 units. Meanwhile, group of rats that had been treated with 1.0 ; 1.5 ; 2.0 mL/rat/day fermented milk had SOD activity of 68.54 ± 1.12 units, 78.2 ± 1.28 units, 90.56 ± 1.28 units respectively. These results indicated that treatment can increase the SOD enzyme activity levels, and ROS production can be suppressed by *Lactobacillus casei* Shirota strain fermented milk that containing lactic acid bacteria which can produce BSH enzymes and bioactive peptides [8].

Statistical data analysis result showed significant effects. It can be seen from the results of total cholesterol level statistical analysis of rats groups treated with variation dose of fermented milk in the rat blood serum with a level ($p < 0.05$) decrease 36.3% and SOD activity increase 60.65%. Increased activity of rats serum SOD along with the given of therapeutic *Lactobacillus casei* Shirota strain in fermented milk with higher dose.

CONCLUSION

Therapy of *Lactobacillus casei* Shirota strain in the fermented milk can decrease the blood cholesterol levels and increase the SOD activity of rats fed with a high-cholesterol diet. This treatment can enhance the SOD enzyme activity levels, and ROS production can be suppressed by *Lactobacillus casei* Shirota strain which contained in fermented milk that comprises lactic acid bacteria which can produce BSH enzymes and bioactive peptides.

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