Therapy of Fermented Milk *Lactobacillus Casei* Strain Shirota to Level of *Malondialdehyde* (MDA) and Proteind Bands the Hearth the White Rats (*Rattus Norvegicus*) That given High Cholesterol Dietary

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Keywords: Hypercholesterolemia, Fermented Milk, MDA, Protein Profile

Abstract: Cardiovascular disease is a disease that occurs due to a disturbance in the function of the heart and blood vessels such as coronary heart disease (PJK), hypertension and stroke. One cause of PJK is a condition of hypercholesterolemia. Hypercholesterolemia is a condition which cholesterol level more than 200 mg / dL. High cholesterol dietary can cause an increase in free radicals that cause oxidative stress. Fermented milk *Lactobacillus casei* thought to contain biopeptida as antioxsidant. This study was to determine the effect of fermented milk *Lactobacillus casei* in lowering levels of MDA and repairing protein profiles of heart. Animal that used is a rat *(Rattus norvegicus,* males, aged 2-3 months, weightabout 100-250 gram. Rats divided into normal rats, hypercholesterolemia rats , hypercholesterolemia rats and therapy 1 mL and 2 mL . High cholesterol dietary used egg yolk, cholesterol pure and cholic acid that give in forcefeeding for 4 weeks.Therapy of *Lactobacillus casei* fermented milk given for 2 weeks in. Level of MDA was measured using the method of TBA and cardiac protein bands was tested using SDS-PAGE. The result of Research obtained showed *Lactobacillus casei* fermented milk therapy was significantly ($p < 0.05$) lower levels of MDA and affect cardiac protein bands of rats. Doses of 2 mL is the best dose with decreased levels of MDA by 43.11% and is able to restore protein band profiles such as *Lactobacillus casei* normal. In conclusion tha fermented milk can be used as an alternative treatment of hypercholesterolemia

1 INTRODUCTION

Cardiovascular disease is a disease that occurs due to interference withwork functions on the heart and blood vessels such as coronary heart disease (CHD), hypertension and stroke. 17.3 million people are estimated to die of cardiovascular diseasein 2008 but this number will continue to increase to 23.3 million deaths inyear 2030. In Indonesia alone the prevalence of CHD more than 800,000 people in 2013 (Riskesdas, 2013).Unhealthy lifestyles such as smoking and consuming foods high in fat can because hypercholesterolemia as one of the causes of CHD. One of research stated that in 2009-2010 sufferers of coronary heart disease (CHD) caused by hypercholesterolemia increased from 13.5% to 19.2% (Pangestika, et.al., 2014). This matter shows that in just one year there was an increase of 5.7%.

Induction of a high cholesterol diet causes an accumulation of fat in the liver an increase that also increases the amount of acetil co-A in the liver cells to producecholesterol so that an increase in cholesterol levels (Guyton, 1991). The build up of cholesterol and fat in the body is called hypercholesterolemia. The body that experiences hypercholesterolemia willbalance cholesterol levels with the synthesis of bile acids that also produced by-product in the form of free radicals which is characterized by increased levels of MDA in body (Sugiarto, et.al., 2014). Malondialdehyde (MDA) is a dialdehyde compound that hasmolecular formula C3H4O2 , results from oxidation of unsaturated fatty acids by free radicals (Winarsi, 2007). Treatment of hypercholesterolemia so far has only used anti-medication hypercholesterolemia which can reduce cholesterol levels but has side effects against other organs such as the kidneys. One study mentioned that

Putri, D., Aulanni'am, A. and Srihardyastuti, A.
Therapy of Fermented Milk Lactobacillus Casei Strain Shirota to Level of Malondialdehyde (MDA) and Proteind Bands the Hearth the White Rats (Rattus Norvegicus) That given High Cholesterol Dietary.
DOI: 10.5220/0009586901260132
In *Proceedings of the 6th International Conference on Advanced Molecular Bioscience and Biomedical Engineering (ICAMBBE 2019) - Bio-Prospecting Natural Biological Compounds for Seeds Vaccine and Drug Discovery*, pages 126-132
ISBN: 978-989-758-483-1

fermented milk including yogurt, goat milk can reduce cholesterol levels because it has antioxidants which will suppress free radicals and inhibit LDL oxidation reactions so cholesterol levels in the blood will decrease (Pangestika, et.al., 2014).

Fermented milk products are not only limited to yogurt. One of the bacteria that canused for fermenting milk is *Lactobacillus casei* Shirota strains. But the milk products that used bacterin has not been much studied. Therefore, it is necessary conducted research to determine the benefits of *Lactobacillus casei* fermented milk Shirota strains against decreased levels of Malondialdehyda (MDA) and cardiac protein profiles.

2 MATERIALS AND METHODS

2.1 Preparation of Experimental Animals

Rats were divided into 4 treatment groups and each group contained 6 rat, vizgroup 1 was rat that were not treated (normal rat), group 2 is a hypercholesterol rat (positive control / K +), group 3 is a rat hypercholesterolemia and given a fermented milk dose of 1 mL (Y1). Before receiving treatment, animal model rats (*Rattus norvegicus*) is adapted to the laboratory environment for 7-14 days.

Feeding during the adaptation period is in the form of standard feed as needed namely 20 grams of feed / head / day and drinking water. Consumption of rat feed per day rangesbetween 15-30 grams / head / day. The feed given is in the form of standard feed. Rats are placed in the cage according to treatment. This is so that high cholesterol dietsgiven through gastric sonde in each animal model can be achieved.

2.2 Preparation of Animal Model Rat (*Rattus norvegicus*) Hypercholesterolemia

Animal model of hypercholesterolemia is made by induction of hypercholesterolemia in rat (*Rattus norvegicus*) which is done by increasing blood cholesterol levels in experimental animals by giving food made from pure cholesterol as much as 2 grams, cholic acid 0.02 grams, and boiled quail egg yolk 1 gram. Which is then dissolved in distilled wateras much as 2 mL. The feeding of hypercholesterolemia in rats was carried out using sondes tomach. Induction of a high cholesterol diet is carried out for 4 weeks (Wulandari, 2013). Level measurement of rat cholesterol (*Rattus norvegicus*) is done once a week before the treatment by milk containing *Lactobacillus casei* bacterial fermentation of Shirota strains using GCU tools.

2.3 Lactobacilus Casei Fermented Milk Bacteria Shirota Strain

Lactobacillus casei fermentation of Shirota strain is administered by sonde stomach. Treat will be done if the cholesterol level in rats has reached 145mg / dL in hypercholesterolemia rat. Dosage of *Lactobacillus casei* fermented milk Shirota strain of 1 mL for group Y1 and 2 mL for group Y2.Y1 group was given *Lactobacillus casei* fermented milk therapeutic milk shirota strain as much as 1 mL per day for each mouse for 2 weeks, while the Y2 group was given*lactobacillus casei* fermented milk therapy strains of shirota as much as 2 mL per day for each rat for 2 weeks. Giving *Lactobacillus casei* fermented milk therapy Shirota strains are carried out alternately according to the treatment group, which begins the group Y1 for hypercholesterolemia rat + 1 mL therapeutic milk fermented *Lactobacillus casei* bacterial Shirota strain and continued hypercholesterolemia rat + 2 mL bacterial fermented milk therapy *Lactobacillus casei* Shirota strains in a gastric sonde.

2.4 Intake of Heart Organs

Intake of the heart organ is done after bacterial fermented milk therapy *Lactobacillus casei* Shirota strains for 2 weeks. Intake of the heart organ is carried out after surgical dislocation of the neck. The heart organ is removed for further storage in PBS (*Phosphate Buffer Saline*) for measurement of MDA levels and protein profiles.

2.5 Measurement of *Malondialdehyde* Levels (MDA) and Making the MDA Standard Curve

MDA standard curve measurements are carried out the way the MDA standard solution is taken onconcentrations of 1, 2, 3, 4, 5, 6, 7 and 8 µg / mL, each taken as much as 100 µL. The solution was put into a tube and added 550 µL of distilled water and 100 µL of distilled water TCA 10%, then homogenized. HCl 1 N was added as much as 250 µL and 100 µLNa-Thio 1% into the tube and homogenized. Then, heat for 20 minutes at 100 °C, after cold centrifugation with a speed at 5000 RPM for 10 minutes. Obtained supplies are taken and

measured at maximum wavelengths (λmax = 533 nm) using a spectrophotometer to obtain the value of the last absorbanceMDA standard curves were made. MDA standard curves are generated based on equations regression between absorbance (y) and MDA concentration (x).

2.6 Measurement of MDA Levels in Heart Organs

MDA levels were measured using the Thiobarbituric Acid method (TBA) according to the method performed (Aulanni'am, et.al., 2012). The testing begins with weighing cardiac organs weighing 0.5 grams and put into the last mortar crushed until smooth. NaCl 0.9% solution was added and homogenized. Homogenates formed were centrifuged at a speed of 8000 rpm for 20 minutes and the supernatant was taken. Supernatant taken as much as 100 µL is inserted in a microtube, added 550 µL of aquades and homogenized. Next, 100 µL TCA 10% was put into a microtube tube and homogenized. Then done addition of 100 µL HCl 1 N and 100 µL Na-Thio and homogenized with vortex.The mouth of the micro tube is covered with aluminum foil and heated at 100° C for 30 minutes in a water bath. After getting cold, centrifugation is done at 5000 RPM for 10 minutes and the supernatant is taken to be transferred to the new microtube. The absorbance samples were measured using spectrophotometer with a maximum wavelength (λmax = 533 nm). Measurement complete cardiac MDA levels can be seen in **Appendix E2.**

2.7 SDS PAGE Electrophoresis

2.7.1 Gel Preparation

Gels are made using a series of two glass plates. There are two types of gels that are made namely gel as a sample (stacking gel) and gel as a protein separation medium(separatig gel). Materials for separating gel consists of Lower Gel Buffer (LGB), T-acrylamide, distilled water, ammonium persulphate (APS), Tetramethyl Ethylene Diamine (TEMED) then dissolved into distilled water. *Separating gel* solution is poured in the place gel layer and allowed to polymerize for 10-20 minutes. Material for manufacture Stacking gel consists of the Upper Gel Buffer (UGB) , T-acryl, APS, and later TEMED dissolved in distilled water. Stacking gel that has been made is poured into a separating gel which has been polymerized then installed a comb to form a gel and wells.

2.7.2 Sample and Running Injection

The isolated and extracted heart organ is taken 15 µl, then15 µl RSB was added (1: 1 ratio) and then heated in a water bath temperature of 100° C for 5 minutes.30 µl cold extract of the heart organ was added in gel wells that already contain protein markers. The cathode is connected to the power supply whereas the anode was connected to the reservoir. The current is set at 200V for 1 hour. The process is terminated when the blue marker reached 0.5 cm below the lower limit of the gel.

2.7.3 Staining

The running gel is colored by soaking the gel in a staining solution for 30-60 minutes by matching using a magnetic stirrer. Elimination of the color is done by soaking the gel in destaining solution by shaking using a magnetic stirrer until the gel turns clear

2.7.4 Determination of Molecular Weight

Determination of molecular weight can be done by comparing the results of electrophoresis and protein markers so that the types of protein in the extract can be identified. The mathematical determination used the formula:

$$Rf = \frac{\text{Distance of protein movement from the starting place (cm)}}{\text{Color movement distance from starting place (cm)}}$$

Rf is the *Retardation factor* , then a standard curve is made with the price of Rf asthe X axis and the logarithmic price of molecular weight as the Y axis and plotted so it willthe molecular weight can be known.

2.7.5. Data Analysis

Data analysis was performed using the ANOVA test (One Way Analysis of Variance) and BNJ test (Honestly real difference) to determine whether there is a difference for the MDA level test and for the analysis of protein profiles using SDS PAGE.

3 RESULTS

3.1 Effect of Induction of High Cholesterol Diet and Therapy of Fermented Milk on Levels Rat Cholesterol (*Rattus norvegicus*)

Induction of a high cholesterol diet is done by providing feed containing quail egg yolk, pure

cholesterol and cholic acid for 4 weeks caused by an increase in cholesterol levels. Rats can be catagorized as hypercholesterol rats if the cholesterol level have exceeded 145 mg / dL. Hypercholesterolemia rats were treated with fermented milk at a dose of 1 mL and 2 mL within 2 weeks.Cholesterol levels reduction showed on Table 1.

Table 1 : Rats (Rattus norvegicus) Cholesterol Levels

Treatment	After Acclimation	After Induction	After Therapy
Normal	30,4±4,16	31,6±3,57	30±2,91
Hyper cholesterol	34±6,82	147,8±20, 04	194,4±23, 22
Hyper cholesterol + 1 mL fermented milk	31,6±2,3	191,6±17, 32	104,8±3,5 6
Hyper cholesterol + 2 mL fermented milk	31,8±4,54	182±16,76	87±3,16

Hypercholesterolemia rat experienced a significant increase in cholesterol levels after induction by high cholesterol diet. Induction of a high cholesterol diet contains saturated fats resulting in increased levels of triglycerides in the blood whereas triglycerides themselvesis a cholesterol precursor. Saturated fats will increase LDL levels and decrease levels of HDL. Rat with hypercholesterolemia were given fermented milk therapy with a dose of 1 mL and 2 mL. Fermented milk therapy could reduce the cholesterol levels of rats.

3.2 Effect of Induction of High Cholesterol Diet on MDA (*Malondialdehyde*) Levels of Rats (*Rattus norvegicus*) Hypercholesterolemia

MDA levels were obtained after measurement using the TBA methodhypercholesterolemia MDA levels in rat hearts will experience whereas in rat treated MDA levels of rats heart will decrease.

Table 2: MDA levels of the rat heart induced by a high cholesterol diet

Treatment	Average of MDA Levels (µg/mL)	MDA Levels	
		% Increase	% Decrease
Normal	1.47±0,06[a]	-	-
Hyper cholesterol	7,81±0,23[b]	81,17	-
Hyper cholesterol + 1 mL fermented milk	5,97±0,78[c]	-	23,56
Hyper cholesterol + 2 mL fermented milk	4,44±1,42[d]	-	43,11

From table 2 showed that the induction of a high cholesterol diet increased MDA levels of rat heart (7.93 ± 0.259) µg / mL or experiencing 85.88% of MDA levels of normal rat heart organ that is equal to (1.12 ± 0.213) µg / mL. MDA levels of rats hypercholesterolemia which has been treated with 1 mL of fermented milk is (5.11 ± 0.701) mg / dL or decreased when compared with hypercholesterol rat by 35.51%whereas in hypercholesterol rat treated with mL fermented milk, MDA levels were obtained by (3.88 ± 0.721) mg / dL which decreased by 50.99%.

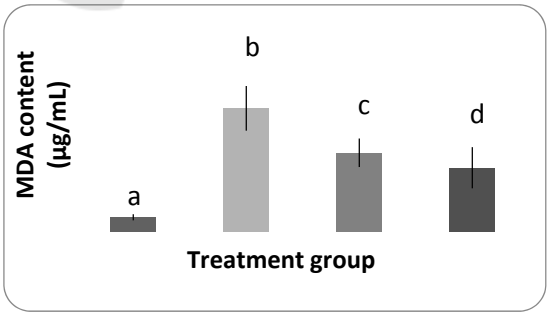


Figure 1 : MDA content of rat heart

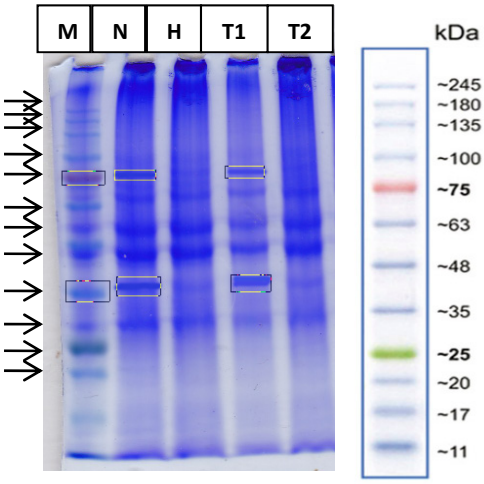
Statistical analysis using One-Way ANOVA showed that the treatment of fermented milk significantly ($p < 0.05$) can reduce MDA levels rat (*Rattus norvegicus*) hypercholesterol induced high cholesterol diet as shown in table 2. Test results using Tukey or

Honestly Significant Difference (BNJ) showed that normal rat and hypercholesterol rat were significantly different so the notation were different.In hypercholesterol rat and rat that have been given therapy show a difference

significant so that the notation is different whereas in rat given ratshypercholesterolemia + 1 mL therapy and rat hypercholesterolemia + 2 mL therapy showed significant difference so the notation was different. Feeding hypercholesterolemia in *Rattus norvegicus* rat will increase the amount of free radicals in the body is indicated by an increase in MDA levels (Wulandari et.al., 2013). MDA levels are an indicator of the presence of free radicals caused by lipid peroxidation process. Normally, free radicals are produced by the body in small amounts as a result of various metabolic processes in the body. Free radicals produced by some of the constituent components of cells, such as mitochondria, plasma membranes, lysosomes,endoplasmic reticulum and nucleus. Free radicals produced are the resultthe side effects of oxidation or cell metabolism that take place during respiration cells and digestion. Fermented milk therapy reduced MDA levels allegedly because of the presence of active biopeptides. Active biopeptides in fermented milk have the potential to play a role antioxidants by capturing reactive oxygen species (ROS) compounds so that the effect of free radicals will decrease.

3.3 Effect of Induction of a High Cholesterol Diet on the Protein Profile of Rats (*Rattus norvegicus*) Hypercholesterolemia

White rat heart protein profiles were measured using the SDS-PAGE method. Band results protein can be seen in figure 2.



M : Marker
N : Normal
H : Hypercholesterol
T1: Hypercholesterol + 1 mL fermented milk
T2: Hypercholesterol + 2 mL fermented milk

Figure 2: SDS-PAGE results

The results in figure 2 and table 3 showed normal rat, and hypercholesterol rat + 2 mL fermented milk therapy there is no severe protein molecules 70 kDa and 23 kDa whereas in hypercholesterol rat and hypercholesterol rat+ 1 mL therapy, there are proteins with molecular weights of 23 kDa and 70 kDa

Table 3 : Molecular Weight (MW) Loss

MW	N	H	H+1 mL Fermented Milk	H+2 mL Fermented Milk
100 kDa	√	√	√	√
85 kDa	√	√	√	√
70 kDa	-	√	√	-
66 kDa	√	√	√	√
48 kDa	√	√	√	√
35 kDa	√	√	√	√
25 kDa	-	√	√	-
17 kDa	√	√	√	√

(MW: Molecular Weight; N: Normal; H: Hypercholesterol)

A protein with a molecular weight of 23 kDa can be said to be CRP (C- *Reactive Protein*) which is a class of pentraxin protein that binds calcium with defense

properties immunological.The formation of CRP due to an inflammatory response in the heart whichproves that induction of a high cholesterol diet increased ROS production. Protein with a molecular weight of 70 kDa is the HSP protein (Heat Shock Protein). The HSP is a protein which results from genetic based responses to induce genes encoding molecular chaperons, proteases and other proteins that are important in recovery and defense and cell response from various physiological disorders and environment. The appearance of protein with BM 70 kDa in hypercholesterolemia rat were an inflammatory response from free radicals due to the induction of a high cholesterol diet.In therapy 1 protein bands with molecular weights of 23 kDa and 70 kDa are still formed, however,the band formed is thinner than the protein band formed in rat hypercholesterolemia. This showed that hypercholesterolemia rat + 1 mL fermented milk therapy. Fermentation has the effect of reducing cholesterol levels so that protein bands were formed not as thick as a hypercholesterol rat. Therapy 2 did not show the formation of protein bands at a molecular weight of 23 kDa and 70 kDa. This happened due to the active biopeptides that played a role as an antioxidant suppresses inflammation that occured so that the rat proteins band profiles hypercholesterolemia treated with 2 mL of fermented milk approached normal rat protein band profiles. This showed that 2 mL of fermented milk therapy has a decreased effect which is more effective so as not to form protein bands with molecular weights of 23 kDa and 70 kDa.

4 CONCLUSIONS

Based on the results of this research, it can be concluded that the higher the *Lactobacillus casei* fermented milk therapy, the greater MDA levels decrease. The best dose of therapy is 2 mL of fermented milk. *Lactobacillus casei* fermented milk therapy can suppress the expression of CRP and HSP so that the therapeutic protein profile resembles the normal rat protein profile.

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