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THE EFFECT OF PROBIOTIC IN MILK FERMENTATION TOWARDS DECREASING CHOLESTEROL LEVELS: IN VIVO STUDIES

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ABSTRACT

Hypercholesterolemia is a disease that is indicated by the increasing level of cholesterol in the body and also as one of the factors causing cardiovascular disease. An alternative that can be done to prevent hypercholesterolemia is by consuming fermented milk. Skim milk is fermented using probiotic of *Lactobacillus acidophillus*, *Lactobacillus plantarum* and *Streptococcus thermophilus*. The skimmed milk that has been fermented is then tested for total lactic acid bacteria, total acid, pH, and in vivo analysis for antihypercholesterolemia activity using animal model. In this study, the animal models used were male white rats which were grouped into three treatment groups, namely negative control, positive control, and fermented milk group. The result of the study shows that the total lactic acid bacteria, total acid and pH of fermented skim milk were 5.25 x10^9 CFU/mL; 2.190% and pH 4.06, respectively. From the results, fermented milk with probiotics has the ability to reduce cholesterol levels in the blood of male white rats effectively in vivo.

Keywords: Probiotic; milk fermentation; antihypercholesterolemia

INTRODUCTION

Cholesterol is a type of fat which functions as a cell protector, help produce vitamin D, and form steroid hormones (Wang *et al*., 2018). Excess cholesterol in the blood can cause hypercholesterolemia which is a condition when the level of cholesterol in the blood exceeds the normal limit of 200 mg/dl. One of the triggers for hypercholesterolemia is lifestyle changes and unhealthy eating patterns, such as high-fat food consumption. Excess cholesterol in the blood will cause cardiovascular disease and stroke (Dokic *et al*., 2015). The World Health Organization (WHO) in 2017 reported that cardiovascular disease is the world's first leading cause of death (Depkes RI, 2017).

Hypercholesterolemia can be treated in various ways. One of the safest alternatives is to change lifestyle by consuming functional foods that are able to control the amount of cholesterol in the blood, such as probiotic drinks. Probiotics are drinks containing probiotic bacteria which has health effects due to the fermentation process. Probiotics can be used as antihypercholesterolemia therapy since probiotic bacteria can increase the secretion of Bile Salt Hydrolase (BSH) enzyme which then enable the increasing excretion of bile acids (Naim, 2011; Gulzar *et al*., 2019). Apart from that, probiotic bacteria produce cholesterol dehydrogenase cofactor which functions to activate the cholesterol reductase enzyme, converting cholesterol into cosprotanol.

Cosprotanol is a sterol that cannot be absorbed by the intestine, therefore it is released through feces enabling cholesterol to go down. Several studies have shown that lactic acid bacteria can reduce cholesterol in vitro and in vivo (Naim, 2011; Octavia *et al*., 2017; Wang *et al*., 2018). Cholesterol reduction is assumed to happen due to its ability to assimilate cholesterol in the small intestine and de-conjugate bile salts. The results of a study by Oktavia *et al*., (2017) reveals that yogurt with banana flour given to wistar rats proves to decrease in triglyceride levels, total cholesterol, LDL cholesterol, and an increase in HDL cholesterol. Ishmayana *et al*. (2015) conducted a study on the effect of yoghurt consumption made with two bacterial culture and three bacterial culture on rat blood serum cholesterol levels. The study obtained a result where yoghurt made with three bacteria (*S. thermophillus*, *L. bulgaricus* and *L. acidophilus*) showed a better effect on decreasing blood serum cholesterol levels compared to two bacteria yoghurts (*S. thermophillus* and *L. bulgaricus*). Based on both studies above, therefore this study was done by using skim milk with the addition three combination of lactic acid bacteria probiotics and its activity as an antihypercholesterolemia in vivo. Skim milk contained 5% lactose which the main carbohydrate in milk that can be used by bacteria starter as energy source for growth.

MATERIAL AND METHODS

The materials used in this study include skim milk, *Lactobacillus plantarum, Lactobacillus acidophillus*, *Streptococcus thermophilus*, deMann Rogosa Sharpe Broth (MRSB) (Oxoid, Ltd, England), Pepton (Oxoid, Ltd, UK), 70% alcohol, and sterile aquades which were obtained from the Faculty of Medicine, Universitas Brawijaya. The rats *(Rattus norvegivus)* feed used was a modified AIN-93M standard feed. Male

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wistar rats *(Rattus norvegivus)* were obtained from Malang Murine Farm which weighs between 180-200 g which were examined in the in vivo testing. The equipment used in the study was an incubator, autoclave, laminar, pH meter, cholesterol strip test, digital cholesterol check tool and mouse cages.

Preparation for BAL Cultivation

Lactobacillus plantarum, *Lactobacillus acidophilus*, and *Streptococcus thermophilus* in MRS agar slant were inoculated onto MRSB media and incubated at 37ºC for 24 h. Subsequently, bacteria were centrifuged and poured into pasteurized skim milk and incubated at 37ºC for 24 h.

Preparation of Fermented Milk

Making fermented milk is done by pasteurizing skim milk at 85 ºC for 15 min, then lowering the temperature to 40ºC. Skim milk is then added to the LAB starter with a concentration of 2% (v / v). The milk is then incubated for 16 h at 37 ºC. The fermented milk is then tested for its total lactic acid bacteria, total acid, pH, and antihypercholesterolemia activity by using animal model.

Total Lactic Acid Bacteria

The total LAB measurement was based on Pelezar *et al*. (2007) method. A sample of 25 mL of fermented milk was dissolved in 225 mL of sterile pepton water and then homogenized, and 1 (P^{-1}) dilution was obtained. Furthermore, dilution 1 (P^{-1}) was taken as much as 1 mL and afterwards put in 9 mL of peptone water to obtain (P^{-2}) dilution. The dilution is carried out up to the P^{-9} dilution. From this dilution then P^{-6} to P^{-9} dilutions are taken to be inoculated on MRSA media by pour plate method and then incubated for 48 h and eventually calculated using the standard plate count method.

Total Acid

Total titrable acidity was carried out by taking a sample of 10 mL and then added with 3 drops of phenolphthalein indicator which was then titrated with 0.1 N NaOH solution until the color turned pink and when homogenized the color does not disappear (Nielsen, 2003).

pH

Measurement of the pH value was done using a pH meter (AOAC, 2005) which was previously calibrated using a buffer of pH 4 and pH 7. The measurement is done by dipping the pH meter electrode in 10 mL of fermented milk sample and waited until the number on the pH meter is stable.

Antihypercholesterolemia Condition Testing

The experimented animals used in this study were 18 male white wistar rats (*Rattus norvegivus*) weighing 180-200 g/rats. The experimented animals used were not defective and had never been used in other studies. The experimented animals were grouped into 3 treatment groups with each group consisting of 6 animals (Table 1).

Table 1. Experiment treatment groups used in the study

Groups	Treatment
Negative Control (K1)	Experimental animals were given standard AIN-93M modified feed and egg yolk induction
Positive Control (K2)	Experimental animals were given standard AIN-93M modified feed, egg yolk induction, and simvastatin
Fermented Milk (K3)	Experimental animals were given standard AIN-93M modified feed, egg yolk induction, and fermented milk

The rats were acclimatized for one week before treatment with the aim to adapt laboratory conditions. To create cholesterol conditions the rats were induced with quail yolk up to hypercholesterol conditions. During the treatment the rats were given standard modified AIN-93M feed and drinking water ad libitum. The experiment was carried out for 21 d and each rat was measured for their cholesterol levels every 7 d.

Measurement of Blood Cholesterol on Animal Tested

The sample of blood was taken through the rat's tail as much as ± 1 cc using blood lancet. The blood sample was then dropped on a cholesterol kit and waited for 150 s and the sample cholesterol level was identified.

RESULTS AND DISCUSSIONS

The objective of this study is to discover whether fermented skim milk has antihypercholesterolemia activity on male white rats (*Rattus novergicus*). Firstly, the fermented milk is characterized to determine the total number of lactic acid bacteria, the total titrable acidity, and the pH value.

Total Lactic Acid Bacteria

Based on the results of the study, the average of total lactic acid bacteria produced in the fermented skim milk was 5.25×10^9 CFU/mL. The total lactic acid bacteria were calculated using the standard plate count method. The number of these bacteria has met the standards for probiotic products with a minimum number of viable bacteria 10^6-10^7 CFU/mL (Manea *et al*., 2010).

That number is the minimum number of cells that delivers health effects for human (Syachroni, 2014). The minimum amount of probiotic strains present in food products is 10^6 CFU/g or the number of probiotic strains that must be consumed every day is approximately 10^8 CFU/g, with the aim to offset the possibility of a decrease in the number of probiotic bacteria while in the digestive system (Puryana, 2011).

The selection of probiotic bacteria must consider several aspects, especially safety and functional aspects. Safety aspects include, probiotic bacteria must maintain the digestive system healthy, non-pathogenic or safe for consumption and resistant to antibiotics. Functional aspects include the ability of living probiotic bacteria in the digestive system, resistant to acidic conditions in the stomach so that bacteria can multiply in the intestine, not cause a distorted aroma on the probiotic product made. In addition, probiotic strains must also be resistant and remain alive during product processing and storage (Puryana, 2011; Pisano *et al*., 2014).

Total Titrable Acidity

The results showed that the average total titrable acidity in fermented skimmed milk was 2.19%. Septiani *et al*. (2013) revealed that the more bacteria produce lactic acid, the higher the acid formed. The acidity of yoghurt can be measured by titration. The acidity of fermented milk due to lactose breakdown by lactic acid bacteria. The total acid titrated in food is determined by acid-base titration which will estimate the total acid concentration.

Most of these acids are organic acids that affects taste, color, microbial stability and food quality (Sadler and Murphy, 2003). Total acid will increase during storage and the peak of its increase occurs in fermented milk using mixed starter cultures. The acidic sense in yoghurt is caused by lactose metabolism by lactic acid bacteria resulting an acidic taste and casein deposition (Sawitri *et al*., 2008).

pH Value

Based on the result the average pH value of fermented milk is 4.05. The range of pH values correlates with the statement made by Farinde *et al*. (2010) which states that a good yoghurt probiotic drink has a pH value between 3.5-4.5. Sunarlim *et al*., (2007) also revealed that the lower pH value, the higher the amount of titrable acidity value. Bacterial activity can be seen through changes in pH and total titrated acid.

The longer incubation time for milk, the higher the total acid produced. This is caused by the increase of total acid alongside with the length of fermentation, allowing more time available for lactic acid bacteria to breakdown the nutrients contained in the substrate which eventually allow the accumulation of organic acids in higher amounts (Yunus and Zubaidah, 2015). Lactose in milk also has an important role in the growth of bacteria, because lactic acid bacteria survive from consuming lactose and produce lactic acid.

This acid also preserves milk and degrades lactose, enabling people who are intolerant of whole milk to consume fermented milk without getting health problems (Resnawati, 2010). The decrease in pH is a result of the fermentation process that occurs due to the accumulation of acids derived from lactic acid bacteria, meanwhile protein is used to spur the development of lactic acid bacteria, while lactose is used by starter bacteria as a carbon source and metabolism lactic acid is produced which will decrease of pH (Sintasari *et al*., 2014). The combination of starter *Streptococcus thermophilus* and *Lactobacillus acidophillus* can increase lactic acid and cause a decrease in the pH (Sunarlim *et al*., 2007). The decrease in pH is determined by the presence of H^+ ions, causing the pH to lower (Burton *et al*., 2014; Sunarlim *et al*., 2007)

Antihypercholesterolemia Activity

Based on Table 2, the average reduction percentage in cholesterol levels varies for each treatment group. It shows decrease in negative control cholesterol levels by 31.00 mg/dL positive control of 122.08 mg/dl fermented milk treatment of 169.54 mg/dl, respectively.

Table 2. Data on the average test results of the cholesterol level on the test animals

Group treatment	Average Cholesterol Level		Average decrease in Cholesterol Level	
	Start (mg/dL)	Final (mg/dL)	(mg/dL)*	(%)*
K1	248.05	217.05	31.00±7.70	12.49
K2	241.00	112.08	122.08±3.80	49.34
K3	254.21	169.54	169.54±7.11	33.31

The decrease in cholesterol levels that occurred in the positive control group was greater than the other groups. This is due to the simvastatin given which is an antihypercholesterolemia drug.

According to Harini (2009) the mechanism of simvastatin as an antihypercholesterolemia is by inhibiting 3-hydroxy-3-methyl glutaryl Coenzyme A eductase (HMG CoA Reductase), which is an enzyme that catalyzes HMG Co-A into mevalonic acid. The way it works is to inhibit the formation of cholesterol in the liver and then increases the removal of LDL from the bloodstream. The inhibition of HMG-CoA reductase causes a decrease in cholesterol synthesis and increases the number of LDL receptors found in liver cell membranes, therefore the total cholesterol and LDL cholesterol levels in the plasma falls (Setiawan *et al*., 2016). The decrease in cholesterol levels in the fermented milk group was smaller than the positive control, however supplementation of fermented milk was proven to reduce high cholesterol levels to normal. The decrease in cholesterol levels in test animals is likely due to the presence of probiotic bacteria contained in fermented milk. According to Naim (2011), the mechanism of cholesterol reduction by lactic acid bacteria is through the secretion of bile salt hydolase (BSH) enzyme.

This will result in the deconjugation of bile acids, resulting the substance becoming difficult to be reabsorbed through the enterohepatic cycle and more bile acids are excreted through faeces. This condition will result in increased cholesterol needs in the body and eventually cholesterol levels in the blood will decrease. Other studies have shown that cholesterol reduction occurs due to the ability of probiotic bacteria that can reduce cholesterol levels in the blood by absorbing some cholesterol into the cells.

Probiotic bacteria cells contain phospholipid bilayers that are able to attract cholesterol into the cells. Probiotic bacteria produce a cholesterol dehydrogenase cofactor that functions to activate the cholesterol reductase enzyme to convert cholesterol into coprostanol.

Coprostanol is a sterol that cannot be absorbed by the intestine and excreted through feces allowing cholesterol to go down (Ooi and Liong, 2010). The results of this study showed that the addition of fermented milk has antihypercholesterolemia activity which can influence in reducing cholesterol levels in male white rats induced by quail yolk.

CONCLUSION

The conclusion of this study is that the addition of probiotics from lactic acid bacteria in the fermented milk has been proved to reduce cholesterol levels in the blood of males wistar rats with an average percentage reduction in cholesterol by approximately 33.31%.

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