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Potential of Lactic Acid Bacteria from Wong Tea can Reduce Rats' Blood Serum Cholesterol Levels

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Abstract

Lactic Acid Bacteria (LAB) isolated from wong tea which have the potential to be probiotic candidates generally have the ability to reduce cholesterol levels. This research uses BAL isolate from wong tea as a probiotic drink that can lower cholesterol. The LAB isolate used is resistant to low pH, resistant to microbial activity (S. aureus and E. coli), and resistant to bile salts. The aim of this research was to determine the effect of giving LAB from wong tea to reduce blood serum cholesterol levels in mice in vitro. The research design used was a Randomized Block Design (RAK) with 5 treatments giving BAL and each treatment was repeated 4 times. Giving BAL for 14 (fourteen) days is carried out in batches. Total LAB was carried out using the surface method on MRS agar, Gram staining using the staining method, and cholesterol levels were measured using a cholesterol kit using the CHOD-PAP enzymatic method. The total research results of Wong tea's LAB ranged from 1.23 x 109 to 2.20 x 109 cfu/ml. The isolation process obtained by Gram staining showed positive Gram staining results in the form of rods. Based on analysis of variance, data on blood serum cholesterol levels of mice in each treatment showed that cholesterol levels before the intervention were not significantly different ($p>0.05$) and cholesterol levels after the intervention showed a significant difference ($p<0.05$).

Contents

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1 Introduction

Wong tea is a drink resulting from fermented sugar water (sweet tea water) which is fermented using a starter from palm wine (wong) mushrooms. If Jake palm wine is left for 6 months, clots (nata) will form so that it can be used as a starter in making wong tea. Wong (mushroom) tea is a fermented drink originating from Bali, especially Gianyar, which tastes slightly sour. The development of functional food regarding probiotics is currently continuing to be developed through research. Local foods as functional foods include wong tea which has been developed as a probiotic candidate drink based on research (Antarini et al., 2022).

The condition of hypercholesterolemia can increase the risk of coronary heart disease (cardiovascular disease). WHO (2011) estimates that by 2030, cardiovascular disease will affect around 23.6 million people worldwide. This occurs as a result of the lifestyle patterns and lifestyles of modern society through changes in daily food consumption without regulating dietary patterns and controlling nutritional intake in a balanced manner according to needs. The results of Basic Health Research (Riskesdas) in 2013 showed that the highest prevalence of cardiovascular disease in Indonesia was coronary heart disease at 2,650,340 people (0.5%). The 2018 Riskesdas results show that the prevalence of cardiovascular disease in Indonesia has increased to 1.5%. Several studies show that reducing 1% of cholesterol in the blood can reduce the risk of coronary heart disease by 2-3% (Maryati et al., 2016). Probiotic bacteria are non-pathogenic, consumable microbes that provide positive benefits for the health of their hosts. Probiotics are one of the functional foods that can be developed which have a positive influence in lowering cholesterol.

Previous research results show that several mechanisms of probiotics in providing hypocholesterolemic effects include enzymatic deconjugation of bile acids by probiotic bile salt hydrolase activity, assimilation of probiotic cholesterol, co-precipitation of cholesterol with bile deconjugation, binding of cholesterol to probiotic cell walls, incorporation of (incorporation) of cholesterol into the probiotic cell membrane during growth, conversion of cholesterol to coprostanol and production of short-chain fatty acids (SCFA) from fermentation by probiotics in the presence of prebiotics (Ooi & Liong, 2010). The ability of probiotic bacteria to deconjugate bile salts is an important aspect for these bacteria to be able to survive against bile salts in the digestive tract. The type of bacteria commonly used as probiotics is lactic acid bacteria (Sujaya et al., 2008). The research aims to determine the effect of Lactic Acid Bacteria (LAB) originating from wong tea as a probiotic drink in reducing blood serum cholesterol levels in mice in vitro.

2 Materials and Methods

Isolate used. The isolates used were LAB isolates TW17, TW18, TW25, and TW27 which were isolated from wong/mushroom tea drinks.

Procedure

Refreshment of living matter

The stock of identified LAB isolates stored in 30% glycerol at -20oC, was taken in one loop and inoculated in a test tube containing 5 ml of MRS broth media. The reaction tube was incubated in an aerobic atmosphere for 24 hours at 37oC. Positive results are indicated by the appearance of turbidity in the test tube. Next, a confirmation test is carried out to ensure that the isolate has not changed. These tests include gram staining

and morphology. If no change occurs, then this positive result (culture) will be used for the next testing stage.

Calculate total LAB using the agar cup method

Total bacteria were determined using the surface method. A total of 100 µl of the sample was put into the Eppendorf which contained 900 µl of physiological salt solution (NaCl 0.85%), so that a 10-1 dilution was obtained, then shaken until homogeneous, then pipetted 100 µl and put into the Eppendorf which contained 900 µl physiological salt solution, so that a dilution of 10-2 is obtained, and so on to obtain a greater dilution. From the desired dilution, 100 µl was pipetted into a petri dish containing bacteria and then grown in MRS media (Klein et al., 1998; Wang et al., 2014; Tamai et al., 1996).

Into the previously prepared MRS agar medium, 60 ppm Bromcresol Purple (BCP) was added as a pH indicator. then spread over the entire surface of the media (surface spread method) with a bent glass rod. The petri dishes that have been planted are then put into the incubator upside down and incubated at 37oC for 24 hours. Lactic Acid Bacteria (LAB) colonies will appear as colonies surrounded by a yellow zone, then isolated and streaked on MRS agar media. The pure isolate is then stored in a glycerol solution with a final concentration of 15% and stored as a culture stock at a temperature of – 20oC for working culture which is made in the form of a stab culture (Sujaya et al., 2008; Nuryady et al., 2013; Nur et al., 2015). Observe and count the population (total bacteria). Total Number of Bacteria = Number of colonies per plate x 1/dilution factor

Identification of microbes using the gram stain test

One cell of pure isolate was grown in LB media (Lactose broth) and then incubated at 37°C for 24 hours. Then a Gram stain test is carried out, and after drying it is viewed under a microscope.

Treatment of White Rats (R. norvegicus)

a) Acclimatization stage of white rats (R. norvegicus)
   In this study, 42 male White Rats (R. norvegicus) aged 5 weeks with a weight of 110 grams were used which were obtained from a breeding place on Jalan Pulau Moyo XV, Gang Tegal Carik no 2, South Denpasar (Mr. Gede Wiranatha). Before being given treatment, the experimental animals were acclimatized for 35 days, which included; cage, age, diet, and body weight. At this acclimatization stage, mice were given standard food in the form of a mixture of ground corn, green bean sprouts, lard oil, and egg yolk (50:30:10:10). Rats were given food every day weighing 40 grams. Mice were placed in cages made from plastic tubs measuring 50 cm x 30 cm x 10 cm.
b) Preparation of bacterial suspension (identified LAB)
   The culture (LAB isolate) that had grown in MRS broth media was vortexed (to obtain a homogeneous culture), then 1 ml was taken with a micropipette, placed in an Eppendorf and centrifuged at 5000 rpm for 7 minutes to separate the cell mass from the supernatant. The supernatant was discarded and the cell mass obtained was washed 2 times with saline solution (NaCl 0.85%) to remove media residues. Washing was carried out by adding 1 ml of saline to the cell mass, vortexing until homogeneous, and centrifuging at 5000 rpm for 7 minutes. In the final stage, the cell mass was dissolved with 1 ml of saline, so that a suspension concentration of approximately 108 cfu/ml was obtained (Nursini, 2010).

In vitro cholesterol assimilation test

The ability to reduce cholesterol levels of the five isolates used was determined using the Cholesterol Oxidase Phenol Aminoantipyrin (CHOD-PAP) enzymatic method.

a) Collecting Rat Blood
   Blood sampling was carried out in the orbital sinus of the eye using microhematocrit. The blood collection procedure according to (Nugroho, 2021), is:

1) Prepare a microhematocrit tube.
2) Scratch the microhematocrit into the orbital sinus or medial canthus of the eye under the eyeball towards the optic foramen, while the other end of the hematocrit tube acts as a blood reservoir.
3) Rotate the microhematocrit until it injures the plexus. If the microhematocrit is rotated 4 times then it must be returned 4 times.
4) The blood that comes out can be immediately collected in a tube and ready to be used for research purposes.
5) Fill in the identification on each container tube.

b) Serum collection
1) Prepare tools and materials.
2) Blood that has been collected in a tube without anticoagulant (red tube) is allowed to sit for 15 minutes.
3) Then centrifuge at 3000 rpm for 5 minutes.
4) The clear, light yellow layer at the top is serum, immediately collect it using a micropipette.
5) Then put it in another clean and dry tube.
6) Put a label on each tube.

c) Checking Cholesterol Levels
Method: CHOD-PAP; with Procedure:
1) Prepare the tools and materials used and condition them to an experimental temperature of 37°C.
2) Prepare a spectrophotometer with an absorbance of 0 using distilled water.
3) Prepare 3 test tubes that have been labeled blank, standard, sample.
4) Pipette each into a tube:

	Blank	Standard	Sample
Reagent	1000 µL	1000 µL	1000 µL
Standard	-	10 µL	-
Sample	-	-	10 µL

5) The mixture is homogenized and incubated for 5 minutes at room temperature.
6) The absorbance of the standard solution is read with a spectrophotometer at a wavelength of 505 nm.
7) The standard absorbance is recorded, then the cholesterol content in each sample is calculated.

Data analysis

The results of checking blood cholesterol levels were analyzed statistically using analysis of variance (ANOVA) and to find out whether there were real differences between the treatments, they continued with the Least Significant Difference (BNT).

3 Results and Discussions

Total LAB

Isolation was carried out after the plates were spread out and incubated for 24 hours at 37oC. Colonies that are suspected to be LAB are then streaked onto new MRS Agar media to obtain single colonies. Total LAB was taken from LAB isolates from The Wong with sample codes TW17, TW18, TW25, and TW27. The total number of LAB identified can be seen in Table 1.

Table 1
Total LAB in LAB isolates used for intervention decreased rat cholesterol levels

Isolate LAB	Total LAB (cfu/ml)
TW17	1.23×10^9
TW18	2.20×10^9
TW25	2.08×10^9
TW27	1.48×10^9

Table 1 shows that the total LAB of Teh Wong ranges from 1.23 x 109 to 2.20 x 109 cfu/ml. In this study, the total BAL in LAB isolates from Teh Wong ranged from 1.23 x 109 to 2.20 x 109 cfu/ml. The total requirement for lactic acid bacteria of 106-108 cfu/ml is appropriate. The total amount of LAB is still included in the probiotic content limits recommended in probiotic product standards, namely 105 - 109 colonies/ml, so Wong tea in this study as a probiotic drink meets the requirements. In order to create a functional food product that is good for health, the product must contain a live starter of 107-109 cfu/ml (Kim et al. 2019).

Gram staining

The LAB isolate used showed positive Gram staining results and was rod-shaped. For more details, LAB morphology can be seen in Table 2.

Table 2
Hasil gram staining BAL dari isolate Teh Wong

No	LAB Isolate	Bacterial Morphology	Gram Staining
1.	TW17	Bacillus Long Gram +	Gram +
2.	TW18	Bacillus Long Gram +	Gram +
3.	TW25	Bacillus Long Gram +	Gram +
4.	TW27	Bacillus Long Gram +	Gram +

Table 2 shows that the LAB isolates from Teh Wong are rod-shaped (Bacillus). The results of observations of microscopic characterization using Gram staining on MRSA media, the results of identification using Gram staining on LAB isolates from Teh Wong showed that all LAB isolates were purple in color and classified as Gram-positive, namely bacillus (stem) type bacteria. This is in accordance with the opinion of Waluyo, in (Putri et al., 2020), who states that Gram-positive bacteria have cell walls in the form of thick peptidoglycan. When decaying with alcohol, the pores of the cell wall narrow due to decolorization so that the cell wall still holds the violet crystals. The results of microscopic characterization observations using Gram staining showed that the 4 LAB isolates were purple in color and bacillary in shape.

Cholesterol levels

As a candidate probiotic drink, lactic acid bacteria isolate has the ability to lower cholesterol. In this research, the treatment was given to LAB isolates originating from fermented wong tea. Cholesterol levels before (pre) intervention giving BAL isolate and after (post) intervention giving LAB isolate as well as the reduction in blood serum cholesterol levels in mice can be seen in Table 3

Table 3
Cholesterol levels before and after the intervention of giving LAB isolates as well as decreased blood cholesterol levels in mice

No	Treatment	Cholesterol levels (mg/dL)		Reduced cholesterol (mg/dL)
		Pre-intervention (Pre)	Post-intervention (Post)	
1	P1	29.90a	21.30a	8.60a
2	P2	37.13a	29.15b	7.98a
3	P3	36.93a	31.75c	5.18a
4	P4	35.55a	32.50c	3.05a
5	P5	37.50a	33.83c	3.67a

Note: different letters behind the average value indicate a significant difference at the 5% test level.

Based on Table 3, it shows that the cholesterol level before the intervention by administering LAB isolate was 29.9 mg/dL – 37.50 mg/dL, and the blood serum cholesterol level in mice after the intervention was 21.30 – 33.83 mg/dL, so there was a decrease in cholesterol levels by 3.05 – 8.60 mg/dL. Based on the results of variations in cholesterol levels before intervention, it was not significantly different ($p > 0.05$). Based on the results of variance data on cholesterol levels from each treatment with $p < 0.05$ which is presented in Table 3, the cholesterol levels after the intervention of giving LAB isolates showed that the cholesterol levels were significantly different (significant), while the decrease in cholesterol levels showed that they were not significantly different ($p > 0.05$) between treatments (Antarini et al., 2018; Diza et al., 2016; Korhenen, 2010).

Cholesterol levels were measured after being given food high in fat and cholesterol for 14 days, then pre- and post-intervention cholesterol levels were analyzed. For more details, see Figure 1.

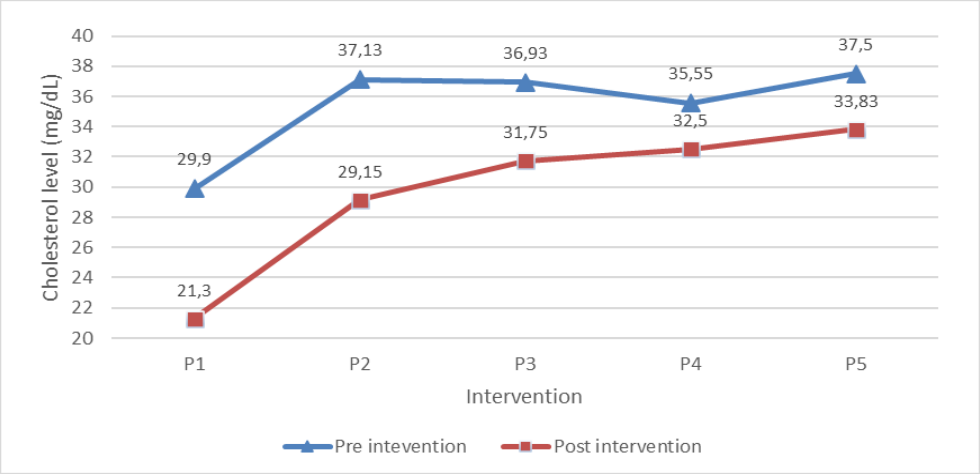

Figure 1. Cholesterol levels before and after intervention by administering LAB isolate to mice

In this study, administering BAL isolate to mice resulted in a significant reduction in rat blood serum cholesterol levels after administering LAB isolate. The results of the study showed that cholesterol levels before intervention were 29.9 – 37.5 mg/dL, and cholesterol levels after intervention were 21.3 – 33.83 mg/dL. Based on the results of variance analysis, it shows that before the intervention there was no significant difference ($p > 0.05$).

The results of the variation in cholesterol levels after the intervention showed that there was a significant difference in cholesterol levels between treatments ($p < 0.05$), while the reduction in cholesterol levels based on the difference between the amount of cholesterol before the intervention and the amount of cholesterol after the intervention was 3.05 – 8.60 mg/dL. This research is in line with research (Fadhilah et al., 2015), that

LAB can reduce cholesterol levels significantly, but the effect of type/composition does not significantly influence it.

One of the lactic acid bacteria that has the potential to be a probiotic candidate is the bacterium Lactobacillus acidophilus. This bacteria can attach to the epithelial cells of the digestive tract, can also be found in the human intestine, and can be isolated from the feces of healthy babies aged 1-2 months, besides that it can also be found in breast milk. L. acidophilus is classified as a lactic acid bacteria which is homofermentative because this bacteria ferments sugars or carbohydrates which only become lactic acid through the glycolysis pathway. Research shows that this bacteria is able to bind cholesterol (Tortuero et al., 1997; Ding et al., 2017; Servili et al., 2011).

Giving Lactobacillus to reduce cholesterol levels can be done through several mechanisms. According to (Widajati, 2020), there are several mechanisms for reducing cholesterol by LAB activity. The first mechanism is that fermented products by LAB inhibit cholesterol synthesis thereby reducing cholesterol production. The second mechanism is through the elimination of bile salts through feces, where deconjugated bile salts are not absorbed by the intestines and are more easily excreted from the digestive tract compared to conjugated bile salts. This results in more cholesterol being needed to synthesize bile salts again, thereby lowering cholesterol levels. The third mechanism is the ability of LAB to bind cholesterol, thereby preventing the absorption of cholesterol back to the liver. Some types of LAB have cell walls that are able to bind cholesterol in the small intestine before the cholesterol is absorbed by the body (Albano et al., 2018; Budiani et al., 2017; Iranmanesh et al., 2014). Results Probiotics are one of the functional foods that can be developed which have a positive influence in lowering cholesterol. The factor causing the decrease in cholesterol levels in the blood is the deconjugation of bile salts due to the activity of bile salt hydrolase (BSH) which is possessed by the bacteria Lactobacillus sp. Probiotic cells have the ability to deconjugate bile salts which are associated with cholesterol in the blood and digestive tract. Bile salts will be deconjugated into bile acids which cannot be absorbed and are secreted with feces if probiotic cells have BSH activity. The higher the activity of BSH in deconjugating bile acids, the more bile acids will be released. The body will take cholesterol in the blood to be used as a precursor for the synthesis of new bile salts so that cholesterol levels in the blood will decrease (Astuti, 2019 in Andriani et al., 2020).

Previous research results show that several mechanisms of probiotics in providing hypocholesterolemic effects include enzymatic deconjugation of bile acids by probiotic bile salt hydrolase activity, assimilation of probiotic cholesterol, co-precipitation of cholesterol with bile deconjugation, binding of cholesterol to probiotic cell walls, incorporation of (incorporation) of cholesterol into the probiotic cell membrane during growth, conversion of cholesterol to coprostanol and production of short-chain fatty acids (SCFA) from fermentation by probiotics in the presence of prebiotics (Ooi & Liong, 2010).

The ability of probiotic bacteria to deconjugate bile salts is an important aspect for these bacteria to be able to survive against bile salts in the digestive tract. The type of bacteria commonly used as probiotics is lactic acid bacteria. Several mechanisms of BAL in lowering cholesterol include the ability of LAB to assimilate cholesterol and deconjugate bile salts. The ability to deconjugate bile salts is related to the activity of bile salt hydrolase (BSH) which is produced by lactic acid bacteria (Jensen et al., 2012; Argyri et al., 2013; Antarini et al., 2022).

Several studies have shown that some LAB can reduce cholesterol in vitro and in vivo. In hyperlipidemic subjects, the general effect of consuming probiotics is a decrease in cholesterol levels, whereas in normal subjects, the effect that generally occurs is a decrease in triglyceride levels (Agestiawan et al., 2014; Febrianti et al., 2016). The effect of lactobacilli on reducing cholesterol is thought to be due to their ability to assimilate cholesterol in the small intestine and deconjugate bile salts. Short-chain fatty acids produced by lactobacilli can inhibit hepatic cholesterol synthesis and cholesterol distribution in the plasma and liver. Deconjugation of bile acids has been suggested as one of the main activities of gut microbes that can be considered as probiotics.

Bile acids are synthesized in the liver from cholesterol and secreted as conjugates of glycine and taurine into the duodenum and will play a role in facilitating fat absorption and following enterohepatic circulation. During circulation in the digestive tract, bile salts can undergo modification by intestinal microbes, namely deconjugation of bile salts by bile salt hydrolysis enzymes (Bile Salt Hydrolase-BSH) by releasing amino acid residues and forming deconjugated bile acids (especially cholate and quenodeoxycholate) (Bawole et al., 2018).

Cholesterol in the intestine will be converted into coprostanol so that it cannot be absorbed by the intestine and will come out with the feces. The use of the cholesterol reductase enzyme produced from LAB isolate cultures to reduce the amount of cholesterol absorbed in the animal's intestines will not reduce the quality of the product produced, and will not cause serious side effects because the enzyme is a derivative of protein which at high temperatures will denature. The cholesterol reductase enzyme mixes with the cytosol of LAB, is easy to extract because it is soluble in water (Desmazeaud, 1996; Ramdani, 2015).

4 Conclusion

1) Total Lactic Acid Bacteria (LAB) for Wong tea ranges from 1.23 x 109 to 2.20 x 109 cfu/ml. Gram staining on BAL isolates showed positive Gram staining and rod-shaped (bacillus) results.
2) The results of the study showed that cholesterol levels before intervention were 29.9 – 37.5 mg/dL, and cholesterol levels after intervention were 21.3 – 33.83 mg/dL. The results of variance analysis showed that after intervention with the administration of BAL isolate, there was a significant difference ($p < 0.05$) in the blood serum cholesterol levels of mice.

Suggestions

To obtain more significant results in reducing cholesterol levels, treatment with LAB isolate should be extended for more than 14 days. It is also necessary to carry out an initial examination of the blood serum cholesterol levels of mice before being given high cholesterol feed.

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