Effect of synbiotic biscuit on decreasing cholesterol levels in Wistar rats

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Abstract. Synbiotic biscuits made of a composite flour (banana corm, sweet potatoes, and black soybean) contain dietary fiber, inulin, and fructo-oligosaccharide as prebiotic and *Lactobacillus acidophilus* as a probiotic. Prebiotic and probiotic have the ability on decreasing cholesterol level, thus further research is needed to prove the effect on cholesterol level. The aim of this research was to evaluate the difference effect of synbiotic biscuit's amount of decreasing cholesterol levels in Wistar rats. This research used a true experimental method with descriptive explanatory research, then continued by paired t-test analysis and regression-correlation analysis. The experiment consisted of 4 treatment: A (fed standard food), B (fed simvastatin 0.018 mg/200g body weight), C (fed 5 gram of synbiotic biscuit in 10 mL aquadest), and D (fed 10 gram of synbiotic biscuit in 10 mL aquadest). The result showed that feeding 5 g of synbiotic biscuit in 10 mL aquadest gives an effect on *L. acidophilus* growth by 97.1%. Feeding 10 gram of synbiotic biscuit in 10 mL aquadest give an effect on *L. acidophilus* growth by 95.7%, decreasing total cholesterol by 45.12% and LDL by 31.11%. Feeding 0.018 mg/200g BW of simvastatin give an effect on decreasing bodyweight by 20.24% and increasing HDL by 51.53%.

1. Introduction

Synbiotic products are products that contain prebiotic and probiotic. Synbiotic biscuits are one example of synbiotic products. Synbiotic biscuits are biscuits that contain probiotic cultures and prebiotic compounds which can increase the functional value of the product [1]. Food products can be considered as synbiotic products if they contain probiotic at least 7 Log CFU g⁻¹ and can produce 6-8 Log CFU g⁻¹ in feces [2].

Synbiotic biscuits made of a composite flour (*banana corm, sweet potatoes, and black soybean*) contain *Lactobacillus acidophilus* microcapsules as probiotic and dietary fiber, inulin, and fructo-oligosaccharide (FOS) as prebiotic about 0.630 grams, 0.509 gram and 0.298 grams in 10-gram synbiotic biscuit [3]. Prebiotics are known to be fermented by probiotic when they enter the digestive system and give health effects on the body. Based on that theory, synbiotic biscuits can be categorized as functional food because of their compounds that can provide health effects on the body.

Functional foods are processed food that contains one or more functional components that have certain physiological functions, proven to be harmless and beneficial to health based on scientific studies [4]. This healthy functional effect must be proven in a series of chemical and biochemical tests with in vitro and in vivo systems both in animal and clinical trials in humans [5]. Now a day, synbiotic biscuits have not been studied and scientifically proven regarding their effect on health, so they cannot be claimed as functional food products yet.
Prebiotic and probiotics that contained in synbiotic biscuits are known to have the potential on decreasing cholesterol. Probiotic bacteria can decrease cholesterol by assimilating cholesterol [6] and deconjugation of bile salts. The mechanism of lactic acid bacteria on decreasing cholesterol is increasing the secretion of the Bile Salt Hydrolase (BSH) enzyme. The BSH enzyme separates glycine or taurine from steroids and produces deconjugated bile acids such as cholic acid which is less absorbed by the small intestine, so it can decrease cholesterol level. Prebiotics in the intestine will be fermented by probiotics to produce SCFA which can decrease cholesterol [7].

Based on the description above, in vivo research is needed to determine the effect of biscuit synbiotic's amount (banana corm, sweet potatoes, and black soybean) on blood cholesterol levels in Wistar rats.

2. Materials and methods

2.1. Material

In this study, animals used were male Wistar rats (Rattus norvegicus) with a bodyweight of 150-200 g, and aged 2-3 months obtained from the medical faculty of Padjadjaran University. Banana corm was from Cikuda Tanjungsari, sweet potato are varieties and black soybean AKIBE 1 was from the agriculture faculty of Padjadjaran University, culture of L. acidophilus was from SITH ITB, L. acidophilus microcapsules, standard rat food (511), simvastatin, PTU, and high cholesterol diets that made from a mixture of 1.5% egg yolk, 10% goat fat, 1% oil, and standard feed (511) up to 100%. The chemicals reagent used were reagent kits for cholesterol analysis using the CHOD-PAP method (Good's buffer pH 6.70, 4-Aminoantipirin, Phenol, Cholesterol oxidase, and Peroxidase) obtained from the medical faculty of Padjadjaran University, LDL-Cholesterol reagent (R1: detergent 1, cholesterol oxidase, cholesterol esterase, peroxidase, 4-aminoantipyrine, ascorbic acid oxidase; R2: MES buffer, detergent 2, and HSDA or DSBmT), and HDL-Cholesterol reagents (R1: Cholesterol oxidase, Peroxidase, HSDA or DSBmT, and ascorbate oxidase R2: Cholesterol esterase, 4-aminoantipyrine, and detergent) obtained from Biotest. Physiological NaCl 0.85%, MRS agar (Merck), MRS broth (Oxoid), glacial acetic acid, CaCO3 and aquadest.

2.2. Animal study

The experimental animals (rats) were divided into four groups, each group consisted of three rats. The rats were adapted for one week and fed ad libitum with standard food 511 (20g/day) and aquadest. After 7 days, the body weight and the blood of the rats were taken to determine cholesterol levels. The rats fasted for 12 hours before blood taking. Furthermore, rats were induced with a high cholesterol diet (1.5% yolk, 10% goat fat, 1% oil, and standard food (511) up to 100%) and propylthiouracil (PTU) 0.1% for 14 days.

After 14 days, feeding cholesterol-high diets were stopped and continues by feeding standard food 511 and 4 different treatments until day 28th. Group A was given aquadest and standard food 511 (negative control), group B was given simvastatin 0.018 mg/200g BB/day (positive control), group C was given 5-gram synbiotic biscuits in 10 mL of aquadest, and group D was given 10 grams of synbiotic biscuits in 10 mL of aquadest. After that, the blood of the rats taken from capillary blood vessels by cutting the end of the rat's tail to determine cholesterol levels on day 29th. The rats fasted for 12 hours before blood taking.

2.3. Total cholesterol levels

The determination of total cholesterol levels was conducted using chod-pap method [8]. A 1000 µL of cholesterol reagent kit was pipetted into cuvettes, then 10 µL samples were added, 10 µL standard and 10 µL distilled water into each cuvette. Incubate the sample for 20 minutes at 20 - 25°C or 10 minutes at 37°C, then read the absorbance value with a spectrophotometer with a wavelength of 546 nm. Enter the absorbance value into the following formula:

\[
\text{Cholesterol concentration (mg/dL)} = \frac{\text{(A sample)}}{\text{(A standard)}} \times \text{standard concentration (mg/dL)}
\]
2.4. Determination of HDL and LDL levels
HDL and LDL levels are determined by Colorimetric Enzymatic Direct Assay Method [9] using Automatic Architect c4000. Blood samples were centrifuged at 3500 rpm for 10-15 minutes. After that, the samples were inserted into the rack and then into the Automatic Architect c4000. The samples code and type of test are entered through the keyboard and the machine will automatically take the shelf and do the test according to the program.

2.5. Number of L. acidophilus bacteria with tpc method
One gram of feces was inserted into 3 mL of man rogosa sharpe broth (MRSB) medium which had been set to pH 4 with glacial acetic acid, then incubated at 37 °C for 1-2 hours. After that, dilution was carried out by moving 0.1 mL of the sample into 0.9 mL Physiological NaCl 0.85% to 10⁻⁸. The number of L. acidophilus was calculated using the spread plate method on MRS agar which had been mixed with 1% CaCO₃ and then incubated at 37°C for 48 hours anaerobically. The number of colonies that were grown was observed by performing the Total Plate Count (TPC) calculation technique.

2.6. Statistical analysis
This research used a true experimental method with a descriptive explanatory research, then continued by paired T-Test samples for observations on body weight and blood and regression analysis and correlation for observation in feces of rats.

3. Results and discussion

3.1. Body weight of rats
The results of the statistical analysis showed that all of the treatment had an effect on the weight of the rats. Table 1 shows that giving simvastatin by 0.018 mg/200 g BB (group B) and feeding 10 g of synbiotic biscuits in 10 mL aquadest (group D) was significantly different on body weight loss among the other group based on statistical tests, while feeding standard food (group A) and 5 g of synbiotic biscuits in 10 mL aquadest (group C) was not significantly different on body weight loss. The biggest body weight loss was experienced by the rats that giving simvastatin about 0.018 mg/200 g BB (group B), which was decrease by 20.24%. Then followed by feeding standard food (group A), feeding 10 g of synbiotic biscuits in 10 mL aquadest (group D), and the last wass feeding 5 g of synbiotic biscuits in 10 mL aquadest (group C) with the value of weight loss by 13.64 %, 11.93% and 9.14%. Group C had lower different on body weight loss compared to group D. This could be caused by the amount of prebiotic that contained in 5 g of synbiotic biscuits (C) was lower than the prebiotic in 10 g of synbiotic biscuits (D), thus affecting on the percentage of rats weight loss. Prebiotic compounds are known to decrease body weight by increasing free fatty acids and cholesterol to deconjugated bile acids form. Dietary fiber decreases body weight by increasing free fatty acids and cholesterol to bile acids form in the digestive system, then released them through feces [10]. So, consuming dietary fiber can reduce body weight [11]. Bacteria in our gut also display a range of enzymatic activities capable of acting on bile acids and, to a lesser extent, cholesterol [12].

| Treatment | Body Weight of Wistar Rat (g) | Average of Decrease (%) |
|-----------|------------------------------|-------------------------|
|           | Before Treatment | After Treatment |               |
| A         | 245.000 ± 43.55ᵃ     | 214.000 ± 58.92ᵃ     | 13.641       |
| B         | 222.670 ± 19.89ᵇ     | 177.330 ± 20.03ᵇ     | 20.241       |
| C         | 225.000 ± 8.19ᵃ      | 204.330 ± 12.70ᵃ     | 9.136        |
| D         | 232.667 ± 34.96ᵃ     | 205.333 ± 34.99ᵇ     | 11.939       |

Note:ᵃᵇ indicates the average treatment marked with lowercase letters with the same horizontal direction is not significantly different according to the T level test of 5%.
Feeding 5 g (group C) and 10 g (group D) of synbiotic biscuits in 10 mL aquadest had a lower percentage of weight loss compared to group A that giving standard food. This can be caused by the presence of \( L.\ acidophilus \) in the synbiotic biscuit group, while group A does not have it. Probiotic can suppress changes in body weight by fermenting prebiotics that contained in synbiotic biscuits as a source of oligosaccharides to SCFA form, so the body weight loss that happen will be lower than the group without probiotics. SCFA can activate free fatty acid receptor-2 (FFAR2). Activation of FFAR2 by SCFA also suppresses insulin signals in adipose tissue by inhibiting Akt phosphorylation which resulted in inhibiting lipid storage in adipose tissue and triggering lipid and glucose metabolism in other tissues so the changes in body weight can be suppressed [13].

### 3.2. Total cholesterol levels of rats

The results of the statistical analysis showed that all of the treatment had an effect on the total cholesterol levels of rats.

| Treatment | Total Cholesterol (mg/dl) | Average of Decrease (%) |
|-----------|---------------------------|-------------------------|
|           | Before Treatment          | After Treatment         |
| A         | 99.467 ± 4.47 \(^a\)     | 74.600 ± 5.40 \(^b\)    | 25.028 |
| B         | 86.267 ± 0.95 \(^a\)     | 70.634 ± 10.65 \(^a\)   | 18.199 |
| C         | 104.467 ± 2.10 \(^a\)    | 68.467 ± 9.81 \(^b\)    | 34.414 |
| D         | 119.933 ± 10.71 \(^a\)   | 65.567 ± 7.65 \(^b\)    | 45.118 |

Note: \(^a\) \(^b\) indicates the average treatment marked with lowercase letters with the same horizontal direction is not significantly different according to the T level test of 5%.

Table 2 shows that feeding 5 g of synbiotic biscuits in 10 mL aquadest (group C), 10 g of synbiotic biscuits in 10 mL aquadest (group D), and standard food (group A) in rats for 7 days were able to decrease total blood cholesterol levels significantly while giving simvastatin 0.018 mg/200 g BB (group B) was not significantly different based on statistical tests.

Feeding 10 g of synbiotic biscuits in 10 mL aquadest was the highest decrease of total blood cholesterol by 45.12%, then followed by feeding 5 g of synbiotic biscuits (group C) in 10 mL aquadest, feeding standard food (group A), and the last was giving simvastatin about 0.018 mg/200 g BB with the percentage of decrease by 34.41%, 25.03%, and 18.20%. The decreasing of cholesterol levels in rats that feeding by 5 g and 10 g of synbiotic biscuits in 10 mL aquadest were caused by the presence of prebiotics (dietary fiber, inulin, and FOS) and probiotics (\( L.\ acidophilus \)) in biscuits, where Lactobacillus were known can decrease cholesterol because its cholesterol assimilation ability in the small intestine and deconjugation of bile salts.

In the mechanism of cholesterol assimilation, lactic acid bacteria will take or absorb cholesterol and make it unite with the bacterial cell membranes, so the bacteria will resistant to lysis. Probiotic bacterial cells contain phospholipid bilayer which is able to attract cholesterol into their cells. In addition, probiotic bacteria also produce cholesterol dehydrogenase cofactor which has functions to activate the cholesterol reductase enzyme to convert cholesterol to coprostanol. Kosprotanol is a sterol that cannot be absorbed by the intestine and will release through feces, so the cholesterol level in the body will decrease [13].

The mechanism of decreasing cholesterol by lactic acid bacteria occurs by increasing the secretion of the Bile Salt Hydrolase (BSH) enzyme which can increase the excretion of bile acids. The BSH enzyme separates glycine or taurine (forming taurocholate acid) from steroids and produces deconjugated bile acids such as cholic acid which is less absorbed by the small intestine than conjugated bile acids. Furthermore, bile acids will be excreted through the feces and it will make more cholesterol needed to synthesize bile salts, so the cholesterol level in the blood will decrease [7].

Besides the probiotic effects, the decrease of cholesterol is also caused by prebiotics in synbiotic biscuits which have an ability to decrease cholesterol by decreasing cholesterol absorption in the intestine.
Prebiotics decrease absorption by stimulating excretion through feces and producing short-chain fatty acids (SCFAs), especially propionic acid which can inhibit the fatty acid synthesis and cholesterol in the liver, thereby decreasing blood cholesterol levels [14]. Cholesterol reduction by simvastatin occurs because of the statin compound which can inhibit the formation of HMG Co A into mevalonate, so cholesterol synthesis will be inhibited and it causes a decrease of cholesterol concentration in liver cells and increases LDL receptors (E, Apo-B-100) [15].

3.3. HDL levels of experimental mice

The results of statistical analysis showed that all of the treatments had an effect on HDL levels in rats.

Table 3. Changes in rats HDL levels during the treatment period.

| Treatment | HDL (mg/dl) | Average of Decrease (%) |
|-----------|-------------|------------------------|
|           | Before Treatment | After Treatment |                 |
| A         | 21.667 ± 2.31 | 23.667 ± 0.58 | 10.221          |
| B         | 16.000 ± 4.00 | 24.000 ± 4.58 | 51.528          |
| C         | 20.667 ± 3.06 | 24.333 ± 2.52 | 18.611          |
| D         | 19.667 ± 1.53 | 25.667 ± 1.16 | 30.820          |

Note: a, b indicates the average treatment marked with lowercase letters with the same horizontal direction is not significantly different according to the T level test of 5%.

Table 3 shows that giving simvastatin 0.018 mg/200 g BB (group B) and feeding 10 g of synbiotic biscuits in 10 mL aquadest (group D) were the group that had a significantly different effect on increasing HDL levels while feeding 5 grams of synbiotic biscuits in 10 mL aquadest (group C) and standard food (group A) were not significantly different on increasing HDL levels based on statistical tests.

Giving simvastatin about 0.018 mg/200 g BB was the highest increase of HDL levels by 51.53%, then followed by feeding 10 g of synbiotic biscuits in 10 mL aquadest (group D), 5 g of synbiotic biscuits in 10 mL aquadest (group), and the lowest was feeding standard food with increases of HDL by 30.82, 18.61 and 10.22%. The increasing of HDL levels in rats that feeding by 5 g and 10 g of synbiotic biscuits in 10 mL aquadest were caused by prebiotic compounds and L. acidophilus that contain in the synbiotic biscuits. L. acidophilus has an ability to increase HDL levels, starting with fermenting the carbohydrates into lactic acid. As lactic acid increases, the pH of the environment becomes low and it causes the other microbes not to grow up well. In the same condition, the increase of H⁺ ions in the intestine causes an increase in water bonds with lipids through lipoprotein (HDL) [13].

Increased HDL levels also occur because of the automatic response of HDL to function in reverse cholesterol transport (RCT), where HDL can increase excess cholesterol reflux from peripheral tissue and return to the liver to be excreted through bile [16].

3.4. LDL level of experimental mice

The results of the statistical analysis showed that all of the treatments had an effect on LDL levels in rats.

Table 4. Changes in rats LDL levels during the treatment period.

| Treatment | LDL (mg/dl) | Average of Decrease (%) |
|-----------|-------------|------------------------|
|           | Before Treatment | After Treatment |                 |
| A         | 13.000 ± 4.58 | 11.000 ± 3.61 | 13.445          |
| B         | 16.000 ± 4.36 | 12.000 ± 1.73 | 23.382          |
| C         | 12.667 ± 0.58 | 10.667 ± 0.53 | 16.026          |
| D         | 14.000 ± 1.73 | 9.667 ± 2.08 | 31.111          |

Note: a, b indicates the average treatment marked with lowercase letters with the same horizontal direction is not significantly different according to the T level test of 5%. 

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Based on table 4, feeding standard food (group A), giving simvastatin 0.018 mg/200 g BB (group B), and feeding 5 g of synbiotic biscuits in 10 mL aquadest (group C) was not significantly different on decreasing LDL levels, while feeding 10 g of synbiotic biscuits in 10 mL aquadest (group D) was significantly different on decreasing LDL levels based on statistical tests. Feeding 10 g of synbiotic biscuits in 10 mL aquadest (group D) was the highest decrease of LDL levels by 31.11%, then followed by giving simvastatin about 0.018 mg/200 g BB (group B), feeding 5 g of sybiotic biscuits in 10 mL aquadest (group C), and the lowest was feeding standard food with decreases of HDL by 23.38, 16.03 and 13.45%. Decreasing of LDL blood levels in rats that feeding by 5 g and 10 g of synbiotic biscuits in 10 mL aquades were caused by the prebiotic compound in synbiotic biscuits such as inulin about 5.09 g/100 g. Inulin can decrease LDL blood levels by inhibiting the activity of lipogenic enzymes in the liver, which causes reduced of triglyceride synthesis. Decreased production of triglycerides causes a decrease in VLDL secretion. Decreasing synthesis and secretion of VLDL in the liver will decreasing LDL levels of serum cholesterol, because VLDL will be converted into IDL (Intermediate Density Lipoprotein) / residual of VLDL after the breakdown of triglycerides. IDL can be absorbed directly by the liver via LDL receptors or can be converted to LDL [17].

Probiotics that contained in synbiotic biscuits also influence the reduction of LDL blood levels. The mechanism of probiotics in decreasing LDL occurs by fermentation of inulin by probiotics and produce short-chain fatty acids such as propionic acid. Propionic acid decreases cholesterol synthesis in the liver by inhibiting the activity of HMG-CoA reductase enzyme that plays a role in cholesterol synthesis in the liver. Decreasing cholesterol production will inhibit the synthesis and secretion of VLDL. Decreasing the synthesis and secretion of VLDL can reduce LDL levels because LDL is a metabolic product of VLDL [14]. Mechanisms enzymatic deconjugation of bile acids by bile-salt hydrolase of probiotics. Bile is a water-soluble end product of cholesterol in the liver, stored and concentrated in the gallbladder, and released into the duodenum upon ingestion of food. Cholesterol is used to synthesize new bile acids in a homeostatic response, resulting in the lowering of serum cholesterol [14].

3.5. Number of probiotic bacteria in feces

The results showed that there was a relationship between the duration of synbiotic biscuits feeding and the amount of L. acidophilus from the synbiotic biscuit in Rats. This can be seen from the positive slope value of the rats feces that fed by 5 g and 10 g of synbiotic biscuits in 10 mL aquadest, which is 0.1493 and 0.1296, meaning that feeding 5 g and 10 g synbiotic biscuit in 10 mL aquadest could increase the number of L. acidophilus bacteria in rats feces by 0.1493 Log CFU g⁻¹ and 0.1296 Log CFU g⁻¹. The duration of synbiotic biscuits feeding affected the growth of L. acidophilus bacteria by 97.1% for group C and 95.7% for group D.

The number of L. acidophilus in the rats feces that fed by 5 g of synbiotic biscuits in 10 mL aquadest (group C) was 5.6 Log CFU g⁻¹ at the beginning of the treatment period (hypercholesterolemia condition) and 6.5 Log CFU g⁻¹ at the end of the treatment period or increase by 0.9 Log CFU g⁻¹, while rats that fed by 10 g of synbiotic biscuits in 10 mL aquadest (group D) was 5.8 Log CFU g⁻¹ at the beginning of the treatment period (hypercholesterolemia condition) and 6.6 Log CFU g⁻¹ at the end of the treatment period or increase by 0.8 Log CFU g⁻¹. These results are in accordance with the study showing the population of BAL in the cel mucosa of rats given probiotics reached 6.31–6.52 log CFU/cm², while in negative and positive control’s rats only reached 5.59–5.72 log CFU/cm² [18].
Figure 1 shows that an increase in the number of *L. acidophilus* in both treatment groups along with the duration of treatment until day 7th. This increase was influenced by prebiotic compounds such as dietary fibers, FOS, and inulin that contain in synbiotic biscuits. Undigested prebiotics in the upper intestine, when entering the large intestine will be hydrolyzed and fermented by probiotics produce short-chain fatty acids (SCFA) in the form of acetic acid, propionate, and butyrate [19]. SCFA can increase the growth of probiotic bacteria in the intestine. Increasing the number of beneficial bacteria in the colon requires a source of carbohydrates that cannot be digested and those carbohydrates will be used as a substrate for the growth of probiotic bacteria in the colon [20].

Beside prebiotics, the number of probiotic bacteria in feces is also influenced by the ability of these bacteria to attach to the digestive tract. Lactobacillus spp. produce adhesin compounds that make them attach to the intestinal mucosa [21]. The ability of bacteria to attach to the digestive tract for each species, strain, and even for cell types in the same organism is different, its depend on the chemical composition of microbial cell walls varies greatly. The decrease of *L. acidophilus* in the beginning of the treatment by feeding 5 g and 10 g synbiotic biscuits in 10 mL of aquadest allegedly caused by the bacteria that contained in synbiotic biscuits were still adapting to the environment and competing in obtaining nutrients between the bacteria itself and other probiotic bacteria that naturally exist in the intestine, so the bacterial growth at the beginning will be on the slow growth curve. The increase of *L. acidophilus* bacteria in the next day can be caused by that bacteria entering the fast growth curve phase, so they have inhabited the intestine and is able to balance the composition of the intestinal flora [22].

4. Conclusion
The duration of feeding 5 g of synbiotic biscuits in 10 mL aquadest affected the growth of *L. acidophilus* bacteria by 97.1% with the number of bacteria at the end of the treatment period by 6.5 Log CFU g⁻¹. While the duration of feeding 10 g of synbiotic biscuits in 10 mL aquadest affected the growth of *L. acidophilus* by 95.7% with the number of bacteria at the end of the treatment period by...
6.6 Log CFU g⁻¹, decreasing total cholesterol by 45.12% and LDL by 31.11%. Giving simvastatin about 0.018 mg/200 g BB affected the body weight loss by 20.24% and increasing HDL by 51.53%.

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