Research Article

Protease and Lipase Enzyme Activity of Probiotic Yogurt and its Effect on Protein, Lipid, and Cholesterol Level of Chicken Egg Yolk

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Abstract | As a probiotic, yogurt has protease and lipase enzyme activity, which can increase digestive tract enzymes in laying hens. In this study, it is hoped that probiotics can increase protein levels and reduce lipid and cholesterol levels in chicken egg yolks. This study aims to determine the activity of the protease and lipase enzymes from yogurt and the effect and level of use of probiotic powder, which can increase protein and reduce lipid and cholesterol levels in chicken egg yolks. The study was conducted using a completely randomized design (CRD) with five treatments and eight replications. The treatment consisted of T0: basal diet, T1: basal diet + 2% probiotic powder B1 (*Bifidobacterium* spp. and *L. acidophilus*), T2: basal diet + 3% probiotic powder B1, T3: basal diet + 2% probiotic powder B2 (*L. bulgaricus, S. termophilus, L. acidophilus and Bifidobacterium bifidum*), and T4: basal ration + 3% probiotic powder B2. Data were analyzed using Analysis of Variance (ANOVA) and continued with Duncan's multiple range test. The results showed that probiotic powder had a significant effect (P≤0.05) on increasing the protein and cholesterol, and decreasing the lipid content of egg yolks.

Keywords | Yogurt, Protease, Lipase, Probiotic, Protein, Lipid, Egg yolk

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INTRODUCTION

Eggs are a type of food that has essential value because it is a source of protein and fat. Eggs are consist ed of 3 main parts: Shell, albumen, and yolk. The protein content in eggs is relatively high, but the lipid and cholesterol content, especially in the egg yolks, is also high. One effort to increase protein and reduce lipid and cholesterol levels in egg yolks is by mixing probiotics with feed for laying hens. Probiotics is a term that refers to microorganisms that provide benefits to humans and animals. These microorganisms play a role in the intestinal microbial balance and are essential in maintaining health. Probiotics also produce antibacterial compounds which can control intestinal pathogens as competition for nutrition and adhesion sites (Adriani *et al.*, 2023). Probiotic can widen the surface area of the intestinal villi and also produce protease and lipase enzymes, improving and increasing the absorption of proteins and lipids in the digestive tract (Widjastuti *et al.*, 2021). Protease enzymes can hydrolyze peptide bonds in proteins into oligopeptides and amino acids. Meanwhile, the lipase enzyme can hydrolyze ester bonds from lipids and fats into fatty acids, glycerol and other alcohols (Liu *et al.*, 2004).

The small intestine absorbs more accumulated amino acids when the protease enzyme's activity increases. Amino acids absorbed by the small intestine and synthesized by the liver will be transported to the blood ovary and later help compose the yolk. Lipase can reduce triglyceride levels in blood and eggs. By decreasing triglycerides, the total lipids in eggs will decrease. In addition, lactic acid bacteria (LAB) may efficiently inhibit the activity of acetyl CoA carboxylase, an enzyme involved in the rate of fatty acid production. There is a positive link that less acetyl CoA carboxylase release results in less fatty acid formation. Reduced fatty acid is associated with lower triglyceride levels (Cavallini *et al*., 2009; Adriani *et al*., 2018). Probiotics can also produce the Bile Salt Hydrolase enzyme, which contribute to cholesterol reduction by hydrolyzing or breaking the C-24 N-Acyl amide bond formed between bile acids and amino acids in conjugated bile salts.

Yogurt is an example of probiotics. Yogurt is a product obtained from milk fermentation using a consortium of lactic acid bacteria, such as *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *Bifidobacterium bifidum*, which can increase blood protein on broiler chicken (Adriani *et al*., 2021). Yogurt can also be made from a consortium of *Bifidobacterium* spp. and *Lactobacillus acidophilus*, which can enhance gut enzymatic activity and increase protein levels in blood (Lesmana and Adriani, 2020). These bacteria can increase enzymes in the digestive tract so that protein digestion and lipid digestion will improve. The use of probiotics in liquid dosage form is less effective because they are not entirely consumed and will remain in drinking containers, whereas probiotics in dry form are a new and effective alternative and can be mixed into rations. Dry probiotics can use yogurt media, which is done by drying it into powder form. Drying aims to extend storage and make distribution easier. A simple drying method that can be used is the spray drying method, which uses maltodextrin coating and skim milk so that microbes with higher viability and survival percentage can be produced during the storage period.

Previous studies have shown that probiotics could increase laying hen blood protein and decrease blood total lipid (Mateova *et al*., 2009). However, other studies mentioned that implementing probiotics in feed did not significantly affect total lipid content (Hadaddin *et al*., 1996; Kalavathy *et al*., 2008). Therefore, research into the enzyme activities of various combinations of probiotic strains should be evaluated and their effect on egg yolk quality.

In the current study, we aim to evaluate the enzyme activities of two type of consortium; the first consortium consists of *L. Bulgaricus*, *S. Termophilus*, *L. Acidophilus* and *Bifidobacterium bifidum* and the second consortium consist of *L. Acidophilus* and *Bifidobacterium* spp. (contains three strains) and also their effect on the protein and lipid levels of chicken egg yolk. We hypothesized that the usage of probiotics in feed can increase egg yolk protein levels and decrease egg yolk lipid levels.

MATERIALS AND METHODS

Probiotic yogurt preparation

There are two consortiums used in this study, namely B1 and B2. The probiotics that will be used for the B1 consortium are *Bifidobacterium* spp. and *Lactobacillus acidophilus*, as well as the B2 consortium, namely *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacterium bifidum* as much as 7,5% (v/v) were inoculated into 250 ml of De Man Rogosa and Sharpe (MRS) media and then incubated at 37°C for 24 hours. Fresh milk is heated (pasteurized) by heating fresh milk at 70-80 °C for 30 minutes. Milk that has been heated is then cooled to a temperature of 37-40°C, then 5% consortium bacteria is added, then homogenized. The fermentation or incubation process is carried out for 24 hours at room temperature.

Analyzing activity of protease enzyme

Protease activity was tested by adding a total of 0.1 mL of supernatant with 0.5 mL of casein and 0.9 mL of 0.1 M phosphate buffer pH 7. The reaction mixture was incubated at 40°C for 30 minutes. Incubation was stopped by adding 0.5 mL of 0.1 M trichloroacetic acid (TCA). The supernatant was separated from the precipitate using a centrifuge at 12,000 rpm for 30 minutes. The supernatant absorbance was measured using a spectrophotometer at a wavelength of 280 nm. As a blank, the enzyme samples were replaced with distilled water. Protease activity units were measured using the following formula:

$$\text{Protease Activity Units} = \frac{\text{Assay absorbance - Blank absorbance}}{\text{0,001 x hydrolysis time (minutes) x enzyme volume(ml)}}$$

Analyzing activity of lipase enzyme

Lipase activity was tested by mixing 2 mL of olive oil, 4 mL of phosphate buffer pH 7, 1 mL of crude enzyme solution (supernatant). The mixture was incubated at 4°C for 30 minutes. After incubation, the enzyme-substrate mixture was inactivated by adding 10 ml of acetone: ethanol (1:1) solution, then titrated with 0.05 M NaOH by adding 2-3 drops of 1% phenolphthalein as an indicator. The titration was stopped when a pink colour was formed, and lipase activity units were measured using the following formula:

$$\text{Lipase Activity Units} = \frac{\text{(Assay Titration - Blank Titration) x M NaOH x 1000}}{\text{enzyme volume (ml) x hydrolysis time (minutes)}}$$

Making probiotic powder

The process of making probiotic powder, probiotic yogurt is added with encapsulant ingredients (skim milk and maltodextrin), sterile distilled water (1/2 of the total volume of solution), then stirred and homogenized. Once homogeneous, the mixture is dried using a spray dryer with an inlet temperature of 160°C and an outlet of 65-70°C to produce probiotic yogurt in powder form.

Experimental design

This study uses Completely Randomized Design (CRD). The experiment used laying hens aged 31 weeks, which were reared for four weeks. Laying hens were given five treatments and repeated eight times so that there were 40 experimental units with each cage containing 1 laying hen. Each cage was labeled with a treatment and repetition number to facilitate observation and data collection. Probiotic mixed feed was given 2 times in the morning and evening as much as 120 grams/head/day and drinking was given ad libitum. Feed and drinking water container are always observed and cleaned to prevent disease. The list of treatments can be seen in Table 1.

Table 1: List of treatments.

Category	Treatment
Control	Basal feed only (no probiotics)
T1	Basal feed + 2% B1 probiotic powder
T2	Basal feed + 3% B1 probiotic powder
T3	Basal feed + 2% B2 probiotic powder
T4	Basal feed + 3% B2 probiotic powder

Analyzing protein and lipid level of chicken egg yolk

Chicken eggs are collected at the end of second week and fourth week. The protein level of chicken egg yolk analysis used the Kjeldahl method, while the lipid level analysis used the Sokhlet method. The cholesterol level analysis used Biolabo reagent CHOD PAP 80106.

Statistical analysis

Statistical data analysis involved calculating the arithmetic mean, standard error, and Student's t-test using and post-hoc Duncan test SPSS software. Differences were significant at $p<0.05$.

RESULTS AND DISCUSSION

Protease and lipase enzyme activity of probiotic yogurt

The result of protease and lipase enzyme activity anaylisis of probiotic yogurt can be seen in Table 2.

The results of Yogurt B1 and B2 protease enzyme activity were 367.6 units/ml and 256.5 units/ml, respectively. B1 yogurt has higher protease enzyme activity compared to B2 yogurt. In this study, another parameter is pH or acidity level, where B1 yogurt has a lower pH or higher acidity level compared to B2 yogurt, in accordance with Amani *et al.* (2013), which states that yogurt with bacteria that have high proteolytic activity will produce a low pH or high acidity level. This difference in proteolytic activity is caused by differences in the types of lactic acid bacteria used as starters, in accordance with the opinion of Shihata and Shah (2000) that certain combinations or starter cultures, as well as the strains of lactic acid bacteria used, have different protease enzyme activity values. Even though B1 yogurt has less microbiota, the bacteria Bifidobacterium spp. has a more complete strain so the protease enzyme is higher than B2 yogurt. Processing methods, additional ingredients, and storage conditions can also influence the proteolytic activity of yogurt.

Table 2: Protease and lipase enzyme activity of probiotic yogurt.

Variable	Probiotic yogurt	
	B1	B2
Protease enzyme activity (U/ml)	367,6	256,5
Lipase enzyme activity (U/ml)	3,57	4,2

However, B1 yogurt has lower lipase enzyme activity than B2 yogurt. Tattu *et al.* (2021) stated that the more acidic the environmental atmosphere, the lower the lipase enzyme activity. This is in line with research where the pH of yogurt B1 is lower than yogurt B2, so the lipase enzyme activity of yogurt B1 is lower than yogurt B2. This difference in lipolytic activity is caused by differences in the type of lactic acid bacteria used as a starter, in accordance with the opinion of Serra *et al.* (2008) that the level of lipolysis and free fatty acid content produced in yogurt can vary based on factors such as processing methods, bacterial strains, and storage conditions.

Protein, lipid and cholesterol level of chicken egg yolk

The result of protein, lipid, and cholesterol level analysis of chicken egg yolk can be seen in Table 3.

Egg yolks from laying hens in this study contain higher protein levels compared to egg yolk protein levels in general. According to Tien *et al.* (2015), egg yolk protein content is 16%. There was an increase in protein levels in T1, T2, T3 and T4. These increases in protein levels is caused by increased activity of protease enzymes in the digestive tract. Protease enzymes break down peptide chains in feed protein to release amino acids needed by the body. When the activity of the protease enzyme increases, the levels of amino acids absorbed by the small intestine also increase. Increased absorption can lead to increased levels of amino acids available for protein synthesis, potentially increasing egg yolk protein content (Amani *et al.*, 2013). After being absorbed by the small intestine, amino acids are transported to the liver to synthesize new proteins and vitellogenin, a precursor of egg yolk protein (Craig and Yilmaz, 2018).

Table 3: Protein, lipid and cholesterol level of chicken egg yolk.

Experimental period (Weeks)	Groups				
	T0	T1	T2	T3	T4
Protein (%)					
2	15.81	16.40	17.08	17.03	17.10
4	16.11	16.83	17.57	17.41	17.57
2-4	15.96[a]	16.61[b]	17.32[c]	17.22[c]	17.34[c]
Lipid (%)					
2	37.32	34.23	34.92	31.84	29.62
4	32.01	29.60	31.67	27.38	27.78
2-4	34.69[c]	31.92[bc]	33.30[c]	29.61[ab]	28.71[a]
Cholesterol (mg/dL)					
2	294,26	291,78	263,31	188,75	215,59
4	155,48	153,34	310,16	314,95	373,93
2-4	224,87[a]	222,56[a]	286,74[bc]	251,85[ab]	294,76[c]

Different superscripts indicate there is a real difference.

Increased egg yolk protein levels can also result from improved digestive tract health. Probiotics can modulate the microbiota in the gut by encouraging the growth of good bacteria. This modulation of the gut microbiota helps restore the balance of microorganisms and increases healthy microbial communities (Ma *et al*., 2023). Probiotics can also inhibit the growth and activity of pathogenic bacteria in the intestine. Production can stimulate the production of antimicrobial substances, compete for binding sites with pathogens, and limit pathogen access to nutritional sources. This process helps prevent colonization and overgrowth of harmful bacteria (Wang *et al*., 2021). In addition, probiotics can modulate the immune response in the intestine so that it can have a positive effect on the immune system of laying hens. Probiotics can increase immune tolerance, reduce inflammation, and promote a balanced immune system (Mirnawati *et al*., 2023). This ability of probiotics will ultimately influence protein synthesis and quality positively so that protein levels in eggs will increase.

Egg yolks from laying hens in this study contain lower lipid levels compared to egg yolk lipid levels in general. According to Tien *et al*. (2015), egg yolk lipid content is 30%. The decrease in lipid levels in egg yolk can be caused by the activity of lactic acid bacteria. Lactic acid bacteria can synthesize esterase enzymes and lipase enzymes, which can break down ester bonds or bonds connecting fatty acids and glycerol into esterified forms so that triglycerides in the blood are reduced, which causes a decrease in total lipid levels in egg yolk. Another mechanism for reducing egg yolk lipids is because lactic acid bacteria produce inhibitors of Acetyl CoA Carboxylase, which is an enzyme that plays a role in the rate of fatty acid synthesis. Inhibition of Acetyl CoA Carboxylase will result in a decrease in fatty acid formation. A decrease in fatty acids will result in a decrease in triglyceride levels in egg yolk (Cavallini *et al*., 2009; Adriani *et al*., 2010). T4 treatment produces the lowest egg yolk lipid content compared to other treatments, this is proven by the lipase enzyme activity of B2 yogurt, which is used in T4, which is higher than B1 yogurt.

The reduction in fat content in egg yolk can also be caused by the ability of probiotics to regulate the expression of genes involved in lipid metabolism. Probiotics that can produce Short Chain Fatty Acid (SCFA) can increase the entry of SCFA in the liver. Increased SCFAs in the liver can lead to downregulation of angiopoietin-like protein 4 (ANGPTL4), which inhibits circulating lipoprotein lipase (LPL) (He and Shi, 2017). Lipoprotein lipase is an enzyme that plays a role in hydrolyzing triglycerides in circulating lipoproteins, such as very low-density lipoprotein (VLDL) and chylomicrons, releasing nonesterified fatty acids. Lipoprotein lipase is found in adipose tissue, muscle and heart tissue but not in the liver (Pirahanchi *et al*., 2023). Decreased ANGPTL4 will ultimately increase circulating lipoprotein lipase. Increasing lipoprotein lipase can result in a more efficient breakdown of triglycerides so that the levels of triglycerides circulating in the blood will decrease. This will decrease triglyceride levels in the egg yolk, so the total lipids in the egg also decrease.

Egg yolks from laying hens in this study produced lower cholesterol levels than egg yolk cholesterol in general. Egg yolk from laying hens contain cholesterol of 17.08 mg/100mg egg yolk or 308.29 mg/dL (Han and Lee, 1992). This can be caused by the ability of probiotics to produce the Bile Salt Hydrolase (BSH) enzyme, which works by deconjugating bile salts so that bile acids are not properly absorbed in the intestine (Ljungh *et al*., 2005). Bile acids deconjugated will be excreted in the feces so that the amount of bile acids returning to the liver will decrease. Then, the liver will take cholesterol from the body to synthesize bile salts. This process will cause a decrease in cholesterol in the blood and ultimately reduce cholesterol levels in egg yolks.

The lowest cholesterol levels are found in T1, while the highest cholesterol levels are found in P4. The increase in cholesterol in T2, T3, and T4 is thought to be due to increased hormone production. Probiotics can increase the production and regulation of steroid hormones, including estrogen, which is involved in vitellogenin synthesis (Mazanko *et al*., 2019). Estrogen receptors in the liver help regulate lipid metabolism, including the synthesis and breakdown of cholesterol. The process of vitellogenesis is influenced by sexual maturity. When chickens reach sexual maturity, there is an increase in gene expression related to the synthesis of egg yolk precursors and reproductive hormone receptors (Cui *et al*., 2020). During egg production, the chicken liver synthesizes apolipoprotein and vitellogenin, the main components of egg yolk. Large amounts of egg yolk precursors, such as cholesterol, are synthesized in the liver, mainly under the influence of estrogen. This causes estrogen to directly increase cholesterol, as a precursor to egg yolk, if the amount of estrogen increases. Yolk precursors are then secreted into the bloodstream and taken up by the growing oocyte via receptor-mediated endocytosis.

CONCLUSIONS AND RECOMMENDATIONS

B1 yogurt, with the most complete strains, has a higher proteolytic activity but also has lower lipolytic activity than B2 yogurt. Adding probiotic powder can increase the protein level of chicken egg yolk while also decreasing the lipid level. The increase in protein level can be caused by the ability of lactic acid bacteria to produce protease enzymes and increase proteolytic activity in the small intestine. The decrease in lipid levels can be caused by the increase of lipolytic activity in the small intestine and the increase of SCFA in the liver.

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NOVELTY STATEMENT

Probiotics in the laying hen diet have been thoroughly researched and shown to provide significant benefits. On the other hand, using probiotics in early-phase laying hens contributing to the increasement of the cholesterol of chicken egg yolk is a novelty.

According to research, there are benefits to administering probiotics at the proper dosage and at the right time to increase the protein level of chicken egg yolk and also decrease the lipid level of chicken egg yolk. The data will aid researchers in conducting their research and determining the results.

AUTHOR'S CONTRIBUTION

All authors contributed equally to this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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