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The Potential of Probiotic (*Bacillus subtilis* and *Bacillus licheniformis*) and Black Soldier Fly Larvae Combination as a Supplement for Late-Phase Layer Hens: Antimicrobial and Enzyme Activities

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ABSTRACT

This study aimed to characterize the combination of probiotics (*Bacillus subtilis* and *Bacillus licheniformis)* and Black Soldier Fly Larvae (BSFL) as a potential supplement for late-phase laying hens through antimicrobial, protease, and lipase activity tests. The research used a completely randomized design with three treatments (T1=1 Pro: 1 BSFL, T2=1 Pro: 2 BSFL, and T3=2 Pro: 1 BSFL) and six replications. The ratio of T3 (2 Pro: 1 BSFL) showed significant sensitivity against *S. aureus* ($P<0.05$) but showed no significant difference against *E. coli* and *S. typhimurium*. Additionally, the highest lipase activity was significantly ($P<0.05$) found in T3. In contrast, the T2 ratio (1 Pro: 2 BSFL) resulted in significantly ($P<0.05$) highest protease activity compared to the other combinations. This finding suggest that the T2 (1 Pro: 2 BSFL) can improve productivity in late-phase laying hens through enzyme stability, especially protease.

Keywords: BSF larvae, Probiotic, Protease Activity, Lipase Activity, Antimicrobial Activity.

INTRODUCTION

In accordance with the egg-laying cycle, egg production begins to decline slowly after reaching the peak phase. The production of late-phase laying hens has decreased due to the degradation of physiological system functions, especially in the reproductive and digestive tracts. Maintaining production in late-phase laying hens is important to reduce replacement costs, especially in small-scale farmers. Egg productivity depends on nutrient absorption in the small intestine, which is influenced by the gut microbiota. The gut microbiota not only aids in nutrient absorption but also enhances immunity and prevents colonization by harmful pathogens, making it a key factor in maintaining productivity (Ricke et al. 2022). In addition, reproductive health in late-phase laying hens declines due to the oviduct and reproductive tract infections, which not only reduce egg production but also affect egg quality (Yan et al. 2019). One of the efforts to maintain the integrity and function of cells is by providing high protein as a feed supplement. Protein plays a role in cell regeneration, body tissue formation, egg formation, and vital metabolic processes such as enzymes, hormones and antibodies (Beski et al. 2015). This high-protein supplementation can be BSFL.

BSFL or *Hermetia illucens* have attracted attention as a source of protein for livestock feed, including laying hens. BSFL has a 40-50% protein content, making BSFL a good choice in fulfilling protein needs. Research has indicated that incorporating various forms of BSFL, such as defatted larvae (Mwaniki et al. 2018), full-fatted larvae (Chu et al. 2020), dried larvae (Liu et al. 2020), and live larvae (Tahamtani et al. 2020) into feed has the potential to enhance both the productivity and egg quality in laying hens. In addition, BSFL is rich in fat, vitamins and minerals essential for livestock productivity (Spranghers et al. 2017; Nekrasov et al. 2019; Shumo et al. 2019). BSFL does not contain pathogenic factors. BSFL produces active

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peptides that are safe to use as supplements for laying hens. These peptides are classified as Antimicrobial Peptides (AMPs) and possess antimicrobial characteristics. In addition to AMPs, the antimicrobial properties of BSFL also come from its saturated fatty acid content, especially lauric acid (Harlystiarini et al. 2019). BSFL also has a significant potential for lipase and protease activity that affects livestock digestion. Feed substrates and environmental conditions significantly influence enzyme activity. BSFL is advantageous for late-phase laying hens experiencing a decline in productivity. It can provide nutritional support to maintain egg quality, body health and production.

The use of BSFL as a feed supplement, especially for late-phase laying hens is still limited. Supplementation of BSFL individually to laying hens has disadvantages because it can reduce digestibility and palatability due to the high-fat content in BSFL (Kroeckel et al. 2012). This will affect fat levels in eggs and blood. Adding probiotics like *B. subtilis* and *B. licheniformis* can minimize these deficiencies by helping to increase digestibility and nutrient absorption. Both bacteria secrete lipase enzymes that can degrade fat so that fat levels in the blood and eggs will decrease.

Probiotics have long been recognized as essential for preserving intestinal health and enhancing feed digestion in chickens. Probiotics are living microorganisms that confer health benefits upon consumption in adequate amounts (Hill et al. 2014; Phupaboon et al. 2024). Probiotics like *B. subtilis* and *B. licheniformis* produce lipase, protease enzymes, and antimicrobial compounds. Supplementing laying hens with a combination of *B. subtilis* and *B. licheniformis* aims to improve the balance of intestinal microflora, which can improve digestive health, egg quality, and the efficiency of nutrients (Kumalasari et al. 2023). Probiotics can inhibit pathogenic bacteria and support the growth of beneficial bacteria in the chicken intestine, thereby expanding the surface of the intestinal villi and increasing the number of goblet cells, ultimately improving nutrient absorption (Adriani et al. 2019; Feng and Liu 2022). In addition, probiotics help regulate the immune system, produce antimicrobial compounds, prevent pathogens from adhering to the intestinal wall, and compete with pathogenic bacteria (Ahasan et al. 2015; Adriani et al. 2023; Usman et al. 2024).

BSFL and probiotics have similar properties, increasing productivity and suppressing the activity of pathogenic bacteria. Supplementation of high protein (16-17%) and probiotics combination can strengthen the humoral response, contributing to increased resistance to infection and production (Anwar et al. 2012; Anwar et al. 2015). The combination of probiotics and BSFL has potential due to their different but complementary mechanisms of action, making them more effective than using individually. This study aimed to characterize the combination of BSFL and probiotics to supplement late-phase laying hens through antimicrobial, protease and lipase activity assays. This study is the initial step before applying a combination of probiotics and BSFL in feeding trials of late-phase laying hens.

MATERIALS AND METHODS

Ethical approval

This study did not require ethical approval since it did not involve animals.

Probiotic preparation

This study used *B. subtilis* ATCC 19659 and *B. licheniformis* ATCC 12759, obtained from IPB Culture Collection, Institut Pertanian Bogor University, Indonesia. Each bacterium was incubated in Nutrient Broth at 37°C for 24h. The two inoculants were mixed in a 1:1 ratio and re-incubated in fresh Nutrient Broth at 37°C for 24h.

BSFL preparation

BSFL was grown using fermented coconut dregs (F-CD) and fish waste (FW) substrates for 14 days. The nutritional content of BSFL in this study was dry matter (33.31%), crude protein (44.54%), ether extract (14.27%), and crude fiber (5.32%). BSFL were extracted by soaking the larvae in the sterile phosphate-buffered saline (PBS) pH 7.3 for 1h at room temperature (25ºC) in the dark (Anjarwati et al. 2019). The mixture was centrifuged at 25°C, 10.000rpm for 15min. The supernatant was collected and sterilized using the syringe filters PTFE 0.22µm.

Experimental design

The research was conducted from May to August 2024 at the Biotechnology Research and Testing Laboratory, Faculty of Animal Husbandry, Universitas Padjadjaran, Indonesia. The combination of probiotics (Pro) and BSFL was carried out by incubating according to the treatment. The experiment focuses on determining the BSFL and probiotic ratio combination through antimicrobial, lipase, and protease activities. This study used an experimental design with a completely randomized design with three treatments and six replications. The treatments include (T1)=1 Pro: 1 BSFL, (T2)=1 Pro: 2 BSFL, and (T3)=2 Pro: 1 BSFL.

Table 1: Enzyme and antimicrobial activities on BSFL and Probiotic

Items	BSFL	Probiotics
Enzyme activities		
Protease activity (U/mL)	1.48	1.21
Lipase activity (U/mL)	0.750	1.33
Antimicrobials Activities		
E. coli (mm^2)	241.22	6.97
S. typhimurium (mm^2)	188.10	26.41
S. aureus (mm^2)	144.10	87.81

BSFL with crude protein 44.54% and extract ether 14.27%; Probiotics are combination of 1 *B. subtilis* : 1 *B. licheniformis.*

Antimicrobial activity test

The antimicrobial activity test was performed using the agar well diffusion method (Atipairin et al. 2022). The test involved the Pro-BSFL mixture and pathogenic bacteria, including Gram- negative strains (*Escherichia coli* ATCC 25922 and *Salmonella typhimurium* ATCC 14028) and a Gram-positive strain (*Staphylococcus aureus* ATCC 29213), chlorampenicol 500ppm was used as the positive control. Samples were added to test tubes containing physiological saline solution and thoroughly mixed using a vortex. The saline solution or bacterial culture was adjusted to achieve the same turbidity as the 0.5 McFarland standard. Once the bacterial suspension matched the turbidity of the McFarland 0.5 standard, 40µL of the sample was introduced into wells on Nutrient Agar plates inoculated with the pathogen bacteria. The plates

were incubated at 37°C for 8h. The inhibition zones around the wells indicated bacterial growth inhibition, measured using callipers (Wulandari et al. 2024).

Determination of protease activity

Protease activity was assessed using an enzymatic method, following Bergmeyer et al. (1983). For each treatment, 42µL of supernatant was combined with 42µL of distilled water and 42µL of Tris-HCl buffer in a microtube. The mixture was incubated at 37°C for 30min. Subsequently, 84µL of TCA (Sigma-Aldrich, USA), 1mL of a solution containing a 50:1 ratio of $NaCO_3$ to $CuSO_4 \cdot 5H_2O$, and 270µL of Folin-Ciocalteu reagent (Merck) were added. The resulting solution was centrifuged (Sigma 1-16K, Sigma-Aldrich, Osterode am Harz, Germany) at 13,000rpm and 4°C for 10min. Absorbance was recorded at 540nm using a spectrophotometer (Agilent Cary 60 UV-Vis Spectrophotometer, USA). A blank was prepared in the same manner as the sample, except the 42µL of sample was replaced with 42µL of distilled water. Tyrosine solution (5mM; Sigma-Aldrich, USA) was the standard, with a 500–6000µmol calibration range. One unit of protease activity was defined as the enzyme amount required to produce one µmol of tyrosine per minute under the assay conditions.

Determination of lipase activity

Lipase activity was assessed using a titrimetric method. The supernatant from each treatment was obtained by centrifugation at 6000rpm for 3min. One mL of supernatant from each treatment was mixed with 2g of palm cooking oil and 4mL of 0.05M phosphate buffer solution in an Erlenmeyer flask. This mixture was then homogenized with a magnetic stirrer for 60min. Next, 10mL of an acetone solution (1:1) was added and stirred until homogeneous. A 1% phenolphthalein indicator, 2-3 drops, was added. Titration was performed using a 0.05 N KOH solution dissolved in alcohol. The titration was stopped when the solution turned pink, and the color persisted for 1min, indicating the endpoint. The volume of KOH used was recorded. The blank solution was prepared similarly to the sample, except a mixture of acetone and alcohol (1:1) was added at the start before homogenizing with a magnetic stirrer for 60min.

Statistical Analysis

Data were statistically analyzed using Analysis of Variance (ANOVA) and mean comparisons were performed using Duncan Multiple Range Test with $P<0.05$ significance level. SPSS software (IBM SPSS Statistic, USA) version 25 was used to analyze the data.

RESULTS AND DISCUSSION

Antimicrobial activity on combination Pro-BSFL

The data in Table 2 shows that the highest inhibition zone area of the treatment combination against *E. coli* (155.67mm^2) and *S. aureus* (192.26mm^2) was found in T3, and against *S. typhimurium* (120.38mm^2) was found in T2. While the lowest inhibition zone area against *E. coli* (99.74mm^2) and *S. typhimurium* (95.31mm^2) was found in T1, and against *S. aureus* (122.72mm^2) was found in T2. The data show that the combination treatment had a non-significant inhibition zone against *E. coli* and *S. typhimurium* ($P>0.05$) but was significant against *S. aureus* ($P<0.05$). Although the inhibition zones against *E. coli* among treatments were not significantly different, the T3 treatment showed a superior effect. Likewise, T3 showed a significantly larger inhibition zone against *S. aureus* ($P<0.05$) compared to other treatments. On the other hand, T2 showed superiority in inhibiting *S. typhimurium*, because it contains more BSFL than probiotics. BSFL has antimicrobial compounds, namely lauric acid and AMPs.

The combined potential of probiotics and BSFL could significantly improve gut health in late-phase laying hens by reducing pathogen load, which increases the risk of age-related diseases. According to Wang et al. (2020), late-phase laying hens are more susceptible to bacterial pathogens due to age-related decline in immune function and physiological stress from prolonged egg production. The combined use of probiotics and BSFL may provide broader antimicrobial effects due to their different mechanisms of action.

Table 2: Inhibition zone of combination Pro-BSFL against pathogens

Treatments	*E. coli* (mm^2)	*S. typhimurium* (mm^2)	*S. aureus* (mm^2)
Control (+)	528.62	422.52	433.52
T1	99.74±11.46a	95.31±13.15a	143.62±2.15a
T2	121.99±20.58a	120.38±26.34a	122.72±12.98a
T3	155.67±23.84a	109.53±9.29a	192.26±0.58b
P-value	0.20	0.63	0.002

Control (+)=chloramphenicol 500 ppm, T1=1 Pro: 1 BSFL, T2=1 Pro: 2 BSFL, T3=2 Pro: 1 BSFL. Values (mean+SE) with different alphabets within the same column differ significantly.

BSFL primarily reduces pathogens through digestion and the production of bioactive compounds. Antimicrobial peptides in BSFL interact with bacterial cell membranes, binding to lipids and integrating into the cytoplasmic membrane, disrupting acid and protein synthesis (Park et al. 2014; Jozefiak et al. 2016). Additionally, lauric acid in BSFL exhibits strong antibacterial properties by destabilizing bacterial membranes and accelerating hydrogen ion influx, compromising cell integrity. Probiotics, such as *B. subtilis* and *B. licheniformis*, complement these effects by enhancing gut health through direct interactions with the immune system and gut microbiota. These probiotics possess anti-inflammatory and antioxidant properties, effectively preventing pathogen adhesion to intestinal epithelial cells (Pezsa et al. 2022; Oleinikova et al. 2024). Their antimicrobial mechanisms include disrupting cell wall synthesis, inhibiting protein synthesis, and interfering with nucleic acid metabolism in pathogens (Zhen et al. 2019; Yesilyurt et al. 2024). Probiotics also inhibit pathogenic bacteria by producing antimicrobial substances, competing for binding sites, and limiting access to nutrients, thereby preventing colonization and overgrowth (Wang et al. 2021). Furthermore, probiotics promote long-term benefits by supporting gut microbiota balance, contributing to systemic health.

These findings suggest that combining different antimicrobial agents, such as probiotics and BSFL, can potentially enhance their effectiveness against pathogenic bacteria. This combination could be an innovative

approach to improving poultry gut health by utilizing synergistic effects that boost beneficial microbiota and extend the duration of antimicrobial activity.

Protease activity on combination Pro-BSFL

Table 3 shows that protease activity varied among three treatments, ranging from 1.39 to 1.51U/mL. Treatment T2 had the highest protease activity (1.51U/mL) and the lowest T1 (1.39U/mL). The data shows that T2 was significantly higher (P<0.05) than T1 and T3 (Fig. 1).

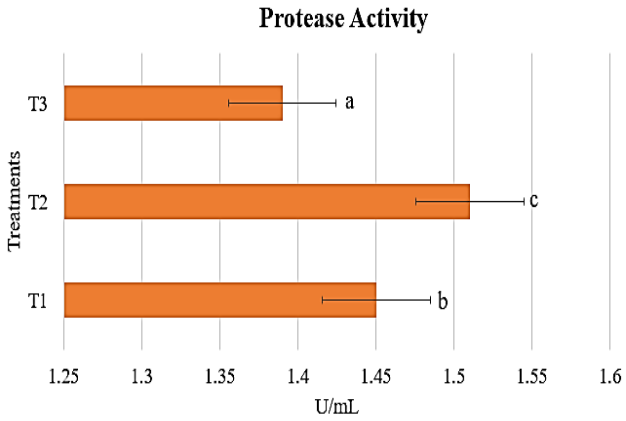

Fig. 1: Protease Activity of Pro-BSFL Combination. Treatments are (T1) 1 Pro: 1 BSFL, (T2) 1 Pro: 2 BSFL, and (T3) 2 Pro: 1 BSFL.

Fig. 2 shows the synergy in the Pro-BSFL combination in producing protease activity. Protease activity increased consistently as the BSFL ratio increased, with the highest activity achieved at T2 (1 Pro: 2 BSFL). This indicated that the addition of BSFL predominantly supported the increase in enzyme activity. This is because the protease activity of BSFL is individually greater than that of probiotics. Protease activity in probiotics was 1.21U/mL, while BSFL was 1.48U/mL (Table 1). This high protease activity was due to the high crude protein content in BSFL of 44.54%. In accordance with Choudhury (2023), protease activity is influenced by several factors, including substrate concentration, where enzyme activity will increase as the substrate concentration increases until saturation is reached.

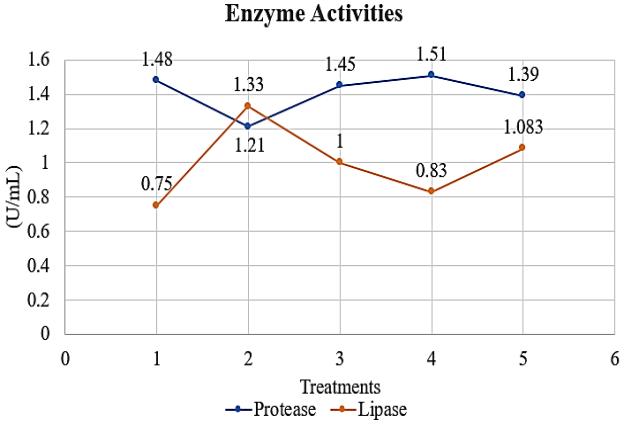

Fig. 2: Protease and Lipase Activity of Pro-BSFL Combination. Treatments are (1) BSFL, (2) Probiotic, (3) T1=1 Pro: 1 BSFL, (4) T2=1 Pro: 2 BSFL, and (5) T3=2 Pro: 1 BSFL.

T1 (1 Pro: 1 BSFL) and T3 (2 Pro: 1 BSFL) showed low protease activity of the Pro-BSFL combination compared to the protease activity of BSFL individually. The equal ratio in T1 may not support optimal protease enzyme synthesis. At the same time, too many probiotics in T3 could lead to an imbalance that interferes with the efficiency of enzyme action. However, the combination of Pro-BSFL in protease activity provides more benefits to the livestock body than individually. Increased protease activity, such as in T2, can help digest proteins more efficiently. Proteases catalyze protein hydrolysis, increasing the availability of amino acids for absorption (Peddie 2023). When included in diets, BSFL can modulate intestinal protein metabolism, promoting the expression of protease-related genes, which may lead to increased protease activity in the host (Fang et al. 2023). Meanwhile, probiotics *B. subtilis* and *B. licheniformis* in laying hens can regulate the balance of intestinal microflora, stimulate the activity of digestive enzymes in the host, and produce exoenzymes that play a role in protein digestion (Wang and Ji 2018). Increased protease activity facilitates better assimilation of nutrients. The combination of Pro-BSFL can significantly affect overall digestive function more than when used individually. The synergy effect is likely to result from the interaction between the exoenzyme activity of probiotics and potential BSFL gene modulation of digestive enzymes, which creates intestinal environmental conditions more conducive to protein degradation. For example, in the study by Storelli et al. (2018), the inoculation of the larvae diet with the symbiont *L. plantarum* up-regulates the expression of intestinal protease genes in gnotobiotic Drosophila larvae, thus increasing the activity of protease in the intestine. Temiraev et al. (2020) show that a combination of probiotics and enzyme preparations increased digestive enzyme activity and nutrient digestibility in growing and laying hens. Tajudeen et al. (2024) study showed that a multi-protease supplement can enhance hen-day egg production, egg mass, and eggshell thickness during peak laying, improving crude protein and amino acid digestibility.

Lipase activity on combination Pro-BSFL

Table 3 shows that lipase activity varied among three treatments, ranging from 0.83 to 1.083U/mL. Treatment T3 had the highest lipase activity (1.083U/mL) and the lowest T2 (0.83U/mL). Statistical analysis showed that lipase activity in T3 was significantly higher (P<0.05) than in T1 and T2 (Fig. 3).

Table 3: Enzyme activities of combination Pro-BSFL

Treatments	Protease	Lipase
T1	1.45±0.003a	1.00±0.12a
T2	1.51±0.007b	0.833±0.03a
T3	1.39±0.007a	1.083±0.00b
P-value	0.00	0.10

T1=1 Pro: 1 BSFL, T2=1 Pro: 2 BSFL, T3=2 Pro: 1 BSFL; P<0.05; Values (mean+SE) with different alphabets within the same column differ significantly.

Fig. 3 shows an increasing pattern (increasing trend) with small fluctuations reflecting the synergy in the Pro-BSFL combination. Lipase activity increased consistently as the probiotic ratio increased, with the highest activity

achieved at T3 (2 Pro: 1 BSFL). This was because the lipase activity of probiotics was higher than that of BSFL, with probiotics at 1.33U/mL and BSFL at 0.75U/mL (Table 1). In contrast, the twice BSFL ratio at T2 (1 Pro: 2 BSFL) resulted in the lowest lipase activity. However, all treatments showed lower lipase activity than Pro individually. This could be possible because environmental conditions such as pH or temperature in the combination treatment may not be optimal for lipase activity, where this study is not concerned with the optimal pH.

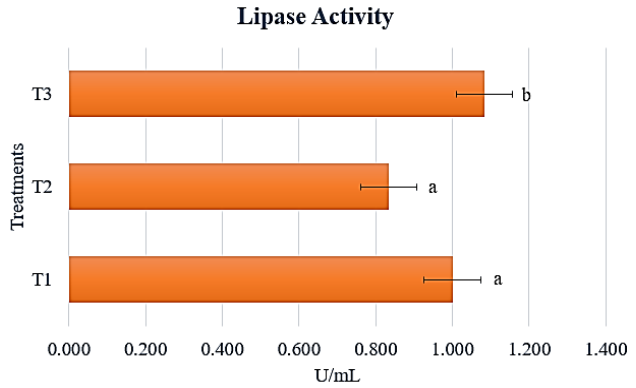

Fig. 3: Lipase Activity of Pro-BSFL Combination. Treatments are (T1) 1 Pro: 1 BSFL, (T2) 1 Pro: 2 BSFL, and (T3) 2 Pro: 1 BSFL.

However, the combination of Pro-BSFL may have other benefits, such as increasing enzyme stability or environmental adaptation. The presence of probiotics, such as *B. subtilis* and *B. licheniformis*, which can form endospores and are resistant to extreme conditions, including acidic environments, can protect enzymes from degradation in the digestive tract (Andriani et al. 2017). With better stability, lipase can remain active longer, thereby increasing the effectiveness of lipid digestion. In addition, in late-phase laying hens, gut integrity, digestive enzyme activity, and nutrient utilization efficiency decrease, ultimately decreasing egg quality (Gu et al. 2021). Supplementing the Pro-BSFL combination, especially at T3, shows potential application in laying hen diets to improve fat digestion efficiency, positively impacting growth and performance.

Conclusion

This study showed that the combination of probiotics and BSFL in various ratios had different effects on antimicrobial, protease, and lipase activities. T3 (2 Pro: 1 BSFL) had higher antimicrobial and lipase activities, while T2 (2 Pro: 1 BSFL) excelled in protease activity. The higher protease activity in T2 could support optimal protein digestion, which is highly relevant for supporting performance in late-phase laying hens. T2 (2 Pro: 1 BSFL) is recommended for supplementation tests in late-phase laying hens based on the balance of enzyme activities, especially the higher protease activity. Thus, combining probiotics and BSFL supplementation can improve the productivity of late-phase laying hens.

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Conflict of Interest: The authors declare that the present study had no conflict of interest.

Author's Contribution: C.K. designed and performed the experiments, analyzing data, writing the original paper, and editing. I.Y.A. Asmara conceptualization, writing original paper, and supervision. L.A. and N.N. conceptualization and supervision. All authors contributed to this manuscript.

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