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RESEARCH ARTICLE
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Characteristics of *Bacillus subtilis* and *Bacillus licheniformis* Consortium as Probiotics for Late-Phase Laying Hens

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ABSTRACT

This study was carried out to characterize the consortium of *Bacillus (B.) subtilis* and *B. licheniformis* as potential probiotic supplements for late-phase laying hens by characteristics of their antimicrobial, protease, and lipase activities. Both bacteria were tested for their resistance to acidic conditions and bile salts to ensure the suitability of both bacteria as probiotics. The results of resistance tests to acid pH and bile salt indicate that *B. subtilis* and *B. licheniformis* are suitable as probiotics for laying hens. A Complete Randomized Design experimental design having three treatments and six replications was used during current study. The treatments included T1=1 *B. subtilis* : 1 *B. licheniformis*; T2=1 *B. subtilis*: 2 *B. licheniformis*; T3=2 *B. subtilis* : 1 *B. licheniformis*. The study used the agar well diffusion method to assess antimicrobial activity, lipase activity using the titrimetric method, and protease activity using the enzymatic method. Statistical analysis showed a significant difference ($P<0.05$) in the inhibition zones against *E. coli*, *S. aureus*, and *S. typhimurium*, as well as in protease and lipase activities. The results showed that the T1 consortium have excellent antimicrobial activity. Although the enzyme activity in T1 was not the highest, the difference was minimal compared to the other treatments. This study found the optimal ratio of *B. subtilis* and *B. licheniformis* (1:1) as a potential probiotic for late-phase laying hens, contributing to improved digestive health and function.

Keywords: Antimicrobial activity, *Bacillus licheniformis, Bacillus subtilis*, Bacterial resistance, Enzyme activity.

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INTRODUCTION

The productivity of laying hens is affected by age, nutrition, housing systems and health status (Vlckova et al., 2019). In accordance with the egg-laying cycle, the amount of egg production begins to decline slowly after reaching the peak phase. The laying productivity of 82 weeks-old decrease below 50% due to degradation of physiological system, especially digestive functions (Salang, 2015). The morphometric degeneration in the intestinal mucosa late-phase laying hens lead to changes in the intestinal environment including decreased moisture and pH of intestine. These changes can affect the growth of certain bacteria and disrupt the composition of bacteria in the intestine, leading to decreased nutrient absorption (Karcher et al., 2015; Yang et al., 2022). Excessive growth of pathogenic bacteria such as *Escherichia coli, Salmonella typhimurium,* and *Staphylococcus aureus* in the intestinal tract causes tissue oxidative stress and excessive immune response (Li et al., 2015). This results in reduction of laying productivity, contamination of poultry products, and increase in mortality (Cravens et al., 2015).

Efforts to improve the productivity of late-phase laying hens include providing probiotic feed supplements. When ingested in sufficient quantities, probiotics confer health benefits. Probiotics change the abundance and

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activity of microbes, eventually regulating the balance of the flora ecosystem in the small intestine and influencing host health. Probiotics can suppress pathogenic bacteria and support the development of non-pathogenic bacteria in chicken digestive tract. This condition will expand the surface of the intestinal villi and increase the number of goblet cells, thereby improving nutrient absorption (Adriani et al., 2019; Feng and Liu, 2022). Probiotics also modulate the immune system, produce antimicrobial metabolites, inhibit pathogen adhesion to the intestinal mucosal epithelium, and compete with pathogenic bacteria (Ahasan et al., 2015; Adriani et al., 2023).

Bacillus sp. is a stable, heat-resistant and spore-forming probiotic bacteria that can endure stomach acid and bile salt (Yang et al., 2022; Cappellozza et al., 2023; Kumalasari et al., 2023). *B. subtilis* and *B. licheniformis* are widely used bacteria, especially for the commercial production of lipase and protease enzymes. Both bacteria do not produce toxins, do not require expensive substrates, and can survive at high temperatures. These bacteria are found naturally in the digestive tract of laying hens (Andriani et al., 2017).

This study used multi-strain probiotics, specifically *B. subtilis* and *B. licheniformis*. Multi-strains probiotics are more effective than single strains because they can serve a variety of functions. A combination of *B. subtilis* and *B. licheniformis* was shown to strengthen intestinal barrier function and improve performance, led to enhanced eggshell strength and reduced cholesterol levels in egg yolks, improved histological structure of villi height, crypts, and villi-to-crypt (V/C) ratios in the duodenum, jejunum, and ileum (Ouwehand et al., 2018; Yang et al., 2020; Yang et al., 2022). Resistance to acids and bile salts is an important property of probiotics. It allows them to survive the stomach acidic environment and bile salt alkaline condition (Andriani et al., 2017). Probiotics must remain viable within the host, navigating the challenging conditions of the upper intestine, including bile salt exposure and acidic pH levels (Makete et al., 2016).

Previous studies by Yang et al. (2017) tested a consortium of *B. subtilis* and *B. licheniformis* in a 1:2 ratio, but did not evaluate the antimicrobial, lipase, and protease activities within the bacterial combination. In-vitro testing of different *B. subtilis* and *B. licheniformis* ratios are crucial, as each ratio may exhibit distinct effects on enzymatic activity and pathogen inhibition. Thus, present study aims to characterize a consortium of *B. subtilis* ATCC 19659 and *B. licheniformis* ATCC 12759 as a potential probiotic supplement for laying hens by evaluating its antimicrobial, protease, and lipase activities. This testing will assist in determining the optimal ratio, enabling the probiotics to function synergistically, maximize nutritional efficacy, and sustain bacterial viability within the diverse digestive conditions encountered by laying hens.

MATERIALS & METHODS

Ethical Approval

This study does not require ethical approval as it was not related to animal use.

Experimental Design

The experiment focuses on determining the ratio of consortium *B. subtilis* and *B. licheniformis* as probiotics through antimicrobial, lipase, and protease activities. The study was conducted from May to July 2024 at the Biotechnology Research and Testing Laboratory, Faculty of Animal Husbandry, Universitas Padjadjaran, Indonesia. The bacteria were *B. subtilis* ATCC 19659 and *B. licheniformis* ATCC 12759 obtained from IPB Culture Collection, Institut Pertanian Bogor University, Indonesia. This study used experimental with Complete Randomized Design had three treatments and six replications. The treatments include T1=1 *B. subtilis* : 1 *B. licheniformis;* T2=1 *B. subtilis:* 2 *B. licheniformis;* T3=2 *B. subtilis* : 1 *B. licheniformis*. *B. subtilis* and *B. licheniformis* were tested for resistance to acidic conditions and bile salt to ensure both bacteria suitability as probiotics. Each bacterium was individually tested for antimicrobial, lipase, and protease activities to establish a baseline for treatment comparisons.

Analysis of Resistance to Acidic pH and Bile Salt

Each bacterial culture was inserted into Nutrient Broth (Merck) with pH 2, 4, and 6 (corresponding to the pH of digestion). The Total Plate Count (TPC) method was used for total bacterial counts in 0 and 5 hours of incubation on Nutrient Agar (Merck). The end of incubation (5 hours) corresponds to the feed retention time in the digestive tract (Svihus and Itani, 2019). After incubation, the lowest number of cells indicates that the bacteria are less resistant to acidic pH.

Each bacterial culture was inserted into Nutrient Broth containing 0.3% and 0.5% bile salt, then incubated for 24 hours at 37^{o}C. At the end of the incubation, the total number of bacteria was determined using the TPC method on Nutrient Agar media with a dilution of 10^{-5} to 10^{-10}. After incubation, the lowest number of cells indicates that the bacteria are less resistant to bile salt (Andriani et al., 2017).

Antimicrobial Activity Test

The method used for antimicrobial activity testing is the agar well diffusion method (Atipairin et al., 2022). The pathogens in this study were Gram-positive (*S. aureus* ATCC 29213) and Gram-negative (*E. coli* ATCC 25922 and *S. enterica sv typhimurium* ATCC 14028) sourced from Agritama Sinergi Inovasi Corps, Indonesia. The positive control was a standard chloramphenicol solution at 500ppm (HiMedia). The pathogens were previously cultured in Nutrient Broth (Merck). Wells with a diameter of 5mm was created on the solid Nutrients Agar (Merck) plate, and a total of 40µl of treatments consortium isolate was inserted. The plates were incubated at 37°C for 8 hours. The clear zone that forms indicates the inhibition of test-pathogenic bacterial growth by the treatments consortium isolate. The clear zone around the well is measured using callipers.

Determinantion of Lipase Activity

Lipase activity was measured using the titrimetric method (Suci et al., 2018). The supernatant of isolate treatments was obtained by centrifugation at 6000rpm for 3min. 1mL of supernatant from each treatment was

combined with 2g of palm cooking oil and 4mL of 0.05M phosphate buffer solution in an Erlenmeyer flask. The mixture was then homogenized using a magnetic stirrer for 60min. Then add 10mL of acetone: alcohol solution (1:1) and stir until homogeneous. Then add 1% phenolphthalein indicator, as much as 2-3 drops. Then titrate with 0.05N KOH solution dissolved in alcohol. The titration is terminated when the solution turns pink, the color persists for 1 minute indicating that the endpoint has been reached. The volume of KOH used is then recorded. The blank solution is prepared similarly to the sample, except a mixture of acetone and alcohol (1:1) is added at the start before homogenizing with a magnetic stirrer for 60min.

Determination of Protease Activity

Protease activity was measured using the method of Bergmeyer et al. (1983). A total of 42µL of supernatant from each sample was placed in a microtube containing 42µL of distilled water and 42µL of Tris HCl buffer. The mixture was incubated at 37°C for 30min. TCA (Sigma-Aldrich, USA) 84µL, 1mL mixture of 50 $NaCO_3$: 1 $CuSO_{4.5}H_2O$, and 270µL Follin Ciocalteu (Merck) were added. The mixture was centrifuged (Sigma 1-16K, Sigma-Aldrich, Osterode am Harz Germany) at 13000rpm 4°C for 10min. Absorbance was measured with a spectrophotometer (Agilent Cary 60 UV-Vis Spectrophotometer, US) at 540nm. The blank solution was made in the same way as the sample, but the addition of 42µL sample was replaced by 42µL distilled water. Tyrosine solution (Sigma-Aldrich, USA) 5 mM as a standard. Prior to the spectrophotometer, tyrosine standard was made 500-6000µmol. One unit of activity is defined as the amount of enzyme that can produce 1µmol tyrosine per minute under test conditions.

Data Analysis

The antimicrobial, lipase and protease activities data were statistically analyzed using Analysis of Variance (ANOVA) and mean comparisons were performed using Duncan Multiple Distance Test with $P<0.05$ significance level. SPSS 25 was used to analyze data.

RESULTS & DISCUSSION

Resistance Test to Acidic pH and Bile Salt on *B. subtilis* and *B. licheniformis*

Table 1 shows *B. subtilis* is more resistant to acidic pH conditions than *B. licheniformis* as evident from TPC of *B. subtilis,* which was higher at various acidic pH levels. However, the differences are insignificant ($P>0.05$). *B. subtilis* forms endospores resistant to extreme environmental conditions, including acidic environments and can survive long. Both bacteria can maintain an intracellular pH that is more alkaline than the extracellular pH (Chen et al., 2019; Tran et al., 2024). *Bacillus sp.* has an efficient proton pump system to remove hydrogen ions from the cell, maintaining a stable intracellular pH (Wulandari, 2021). This study is similar to findings of Lee et al. (2017) and Zulkhaeri-Amin et al. (2020), who highlighted the capacity of *Bacillus sp.* to persist within the gastrointestinal tract. During current study, it is observed that *B. subtilis* and *B. licheniformis* can survive at pH 2, 4 and 6. The populations of both *B. subtilis* and *B. licheniformis* decreased progressively as the pH decreased. Probiotics exhibited a decrease in bacterial populations under pH 3 (simulating fed gastric conditions) and pH 2 (simulating fasted gastric conditions) as demonstrated by Wulandari (2021). As per findings of current investigation, it is assumed that *B. subtilis* and *B. licheniformis* can survive in the digestive tract of laying hens. According to Ravindran (2013), the pH range in the stomach is 2.5–3.5 and in the small intestine 5–7.5.

Table 1: Number of *B. subtilis* and *B. licheniformis* colonies to acidic pH

Bacteria	pH 2 (CFU/mL)		pH 4 (CFU/mL)		pH 6 (CFU/mL)	
	0 hour	5 hours	0 hour	5 hours	0 hour	5 hours
B. subtilis	1.83×10^6	3×10^4	8.6×10^6	1.6×10^6	$3{,}5 \times 10^8$	1.1×10^6
B. licheniformis	2×10^4	1×10^4	1.2×10^6	4×10^5	2.8×10^7	3×10^6

Table 2 shows that *B. subtilis* and *B. licheniformis* bacteria have high resistance to bile salt concentrations of 0.3 and 0.5%, indicated by the high number of probiotic colonies of 10^{11} to 10^{12} CFU/mL. *B. subtilis* and *B. licheniformis* were able to survive in 0.5% bile salt conditions although there was a decrease in numbers after 24 hours of incubation. Both bacteria in this study were able to withstand the bile salts pH (7-8), in line with the optimal pH for *Bacillus sp*. to grow (5-9) (Andriani et al., 2017). Based on current research, it is assumed that *B. subtilis* and *B. licheniformis* can survive in the digestive tract of late-phase laying hens.

Table 2: Number of *B. subtilis* and *B. licheniformis* colonies to Bile Salt

Bacteria	0.3%	0.5%
B subtilis	3.5×10^{11}	1×10^{11}
B licheniformis	3.2×10^{12}	2.8×10^{11}

Antimicrobial Activity on Ratio of Consortium *B. subtilis* and *B. licheniformis*

This study evaluated the antimicrobial activity against three selected pathogen bacteria, *S. aureus* (gram-positive), *E. coli,* and *S. typhimurium* (gram-negative). The antimicrobial activities were assessed by evaluating the inhibition zone shown in Fig. 2. The results showed that *B. subtilis* and *B. licheniformis* could inhibit the growth of all tested pathogenic bacteria. Fig. 1 shows that the consortium treatment had a significant against *E. coli*, *S. typhimurium* and *S. aureus* ($P<0.05$). *B. subtilis* and *B. licheniformis* only as the basis for the consortium treatments T1, T2, and T3. This study showed that the inhibition zone of *B. subtilis* was higher than *B. licheniformis.* In contrast to Andriani et al. (2017), the inhibition zone of *B. licheniformis* was higher than *B. subtilis.* This may be due to differences in strains and the number of antimicrobial compounds produced. T1 had a significantly sensitive inhibition zone ($P<0.05$) against *S. typhimurium* and *S. aureus*. When *B. subtilis* and *B. licheniformis* were tested singly, they produced a lower inhibition zone compared to the multi-strain test. Multi-strain probiotics generally demonstrate superior antagonistic effects compared to their single-strain counterparts, although results can vary based on the specific pathogens and methods used in testing. In line

with the research of Irkitova and Grebenshchikova (2018), a consortium of two *Bacillus sp.* strains showed improved inhibition of *E. coli* growth compared to individual strains. Multi-strain formulations may utilize diverse mechanisms of action, including producing bacteriocin-like substances, which are effective against antibiotic-resistant pathogens (Fugaban et al., 2021).

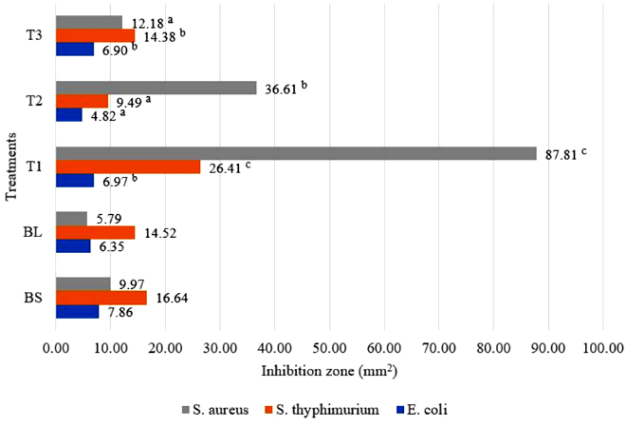

Fig. 1: Inhibition zone of *B. subtilis, B. licheniformis*, and the ratio of consortium; [a,b] Means with different superscripts within the same color bar chart are different in accordance with respective significance levels.

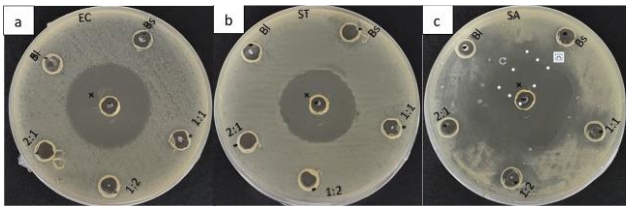

Fig. 2: Inhibition zone of *B. subtilis, B. licheniformis*, and the ratio of consortium against a) *E. coli,* b) *S. thyphimurium*, c) *S. aureus.* Bs = *B. subtilis,* Bl = *B. licheniformis,* 1:1 = 1 *B. subtilis* :1 *B. licheniformis* (T1), 1:2 = 1 *B. subtilis* : 2 *B. licheniformis* (T2), 2:1 = 2 *B. subtilis* :1 *B. licheniformis* (T3).

The antimicrobial activity of various compounds is significantly influenced by pH and environmental conditions in the digestive tract. Acidic pH impairs the synergistic activity of antimicrobial combinations (Cengiz and Hepbostanci, 2020). Modulating environmental pH can alter the structure and function of the gut microbiota community, with some changes occurring independently of the host (Firrman et al., 2022). Probiotics that colonize the surface of the digestive tract will create an acidic environment in the digestive tract up to pH 4-5, that inhibit growth of pathogen bacteria (Guo et al., 2017). This condition makes the activity of *B. subtilis* and *B. licheniformis* as supplementation for laying hens optimal in inhibiting pathogenic bacteria. Both bacteria produce subtilin-like antibiotics, such as sublichenin, demonstrating strong antibacterial activity against foodborne pathogens and antibiotic-resistant lactic acid bacteria (Halami, 2019).

Lipase Activity on Ratio of Consortium *B. subtilis* and *B. licheniformis*

Fig. 3 shows T1 and T3 were 12.5 and 23.02% lower than T2, respectively. The protease activity of *B. subtilis* and *B. licheniformis* served as the baseline for comparing the consortium treatments. The results showed that T2 significantly produced higher activity ($P<0.05$) than other consortiums, at 1.52IU/mL. This indicates that increasing the proportion of *B. licheniformis* has a positive effect on increasing lipase production. According to Daouadji et al. (2015), 60 bacterial strains fermented from the industrial rejection of gas stations showed that *B. licheniformis* had the highest lipase activity. *B. licheniformis* is more effective in producing lipase enzymes than *B. subtilis*. Previous studies have shown that *B. licheniformis* produces lipase at concentrations reaching up to 4.44IU/mL when cultivated under optimal conditions. Furthermore, the characterization of lipases from these two species shows that the specific activity and stability of the lipases from *B. licheniformis* are often superior (Zhao et al., 2022). Meanwhile, T3 had the lowest lipase activity ($P<0.05$), indicating that the high ratio of *B. subtilis* could not increase lipase activity. This result is in accordance with Patel and Shah (2018) and Santos (2024) that *B. subtilis* produces lipase at relatively low levels with maximum activity around 1.72IU/mL.

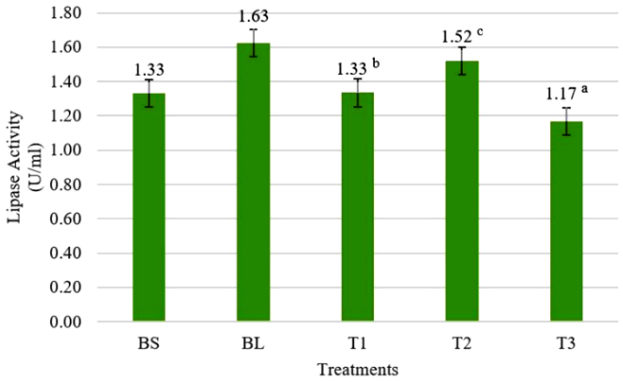

Fig. 3: Lipase Activity of *B. subtilis, B. licheniformis,* and the ratio of consortium; [a,b] Means with different superscripts within the same graph are different in accordance with respective significance levels.

Optimal lipase activity is crucial for enhancing fatty acid absorption, significantly boosting egg productivity. The efficiency of lipases in catalyzing the hydrolysis of fats directly influences the availability of fatty acids, essential for egg production. Lipases catalyze the hydrolysis of triglycerides into free fatty acids, vital for various biological processes, including egg formation (Cesario et al., 2021).

Protease Activity on Ratio of Consortium *B. subtilis* and *B. licheniformis*

The protease activity of *B. subtilis* (1.26 U/mL) and *B. licheniformis* (1.25U/mL) was almost same, indicating both have relatively balanced basic capabilities in producing protease when tested individually (Fig. 4). The protease activity of *B. subtilis* in the study was almost the same as reported by Alam et al. (2017). The protease activity of *B. subtilis* and *B. licheniformis* served as the baseline for comparing the consortium treatments. T3 showed significantly higher protease activity ($P<0.05$) compared to all other consortiums at 1.33U/mL. T1 and T2 were 9.92% and 10.83% lower than T3. The consortium with a higher ratio of *B. subtilis* enhances protease activity. In line with Vijayalakshmi et al. (2013) showed that *B. subtilis* can produce protease enzymes with higher and more stable production levels than *B. licheniformis*.

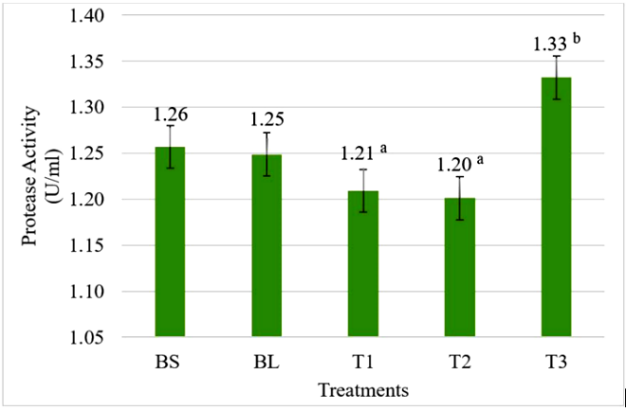

Fig. 4: Protease Activity of *B. subtilis, B. licheniformis,* and the ratio of consortium; [a,b] Means with different superscripts within the same graph are different in accordance with respective significance levels.

Protease plays a crucial role in the digestive system of chicken by breaking down complex proteins into absorbable amino acids, essential for growth, egg production, and overall health. Multi-protease also improved egg production and quality, significantly increasing the digestibility of key amino acids (Tajudeen et al., 2024). Research indicates that the supplementation probiotic consortium of *B. subtilis* and *B. licheniformis* may enhance amino acid digestibility and nutrient utilization in laying hens.

Conclusion

Based on the results of resistance tests to acidic pH and bile salt *B. subtilis* and *B. licheniformis* showed great potential as probiotic. The consortium with a ratio of 1 *B. subtilis*: 1 *B. licheniformis* (T1) was selected as the best supplement option for feeding trial for late-phase laying hens. This selection was based on superior antimicrobial activity, and minimal differences in protease (9%) and lipase (14%) activities compared to other treatments that produced the highest enzyme activities. These results indicate that the T1 consortium can provide optimal benefits for the digestive health of laying hens.

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

Author's Contribution: CK designed and performed the experiments, analyzing data, writing, reviewing and editing the article. IYA conceptualization, writing the original paper, and supervision. LA conceptualization, writing the article, and supervision, and NN conceptualization and supervision. All authors contributed to this manuscript.

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