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# Evaluation of intestinal health and caecal microbial populations in Javanese super chickens supplemented with fermented soybean meal

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**ABSTRACT**

The research protocol aimed to assess the intestinal health of the Javanese super chicken fed with fermented soybean meal using *Bacillus subtilis* and *Aspergillus niger*. A total of 36 healthy male one-day-old Javanese Super Chickens were categorized into three dietary treatments: control diet with unfermented SBM (CON), fermented SBM with *Bacillus subtilis* (B-SBM), and fermented SBM with *Aspergillus niger* (A-SBM), in a completely randomized design. Intestinal allometric measurements, histomorphometric and histopathological analyses, and caecal microbial populations were evaluated following fermented soybean meal (FSBM) supplementation. The study demonstrated that all dietary treatments led to substantial improvements in all parameters without causing any detrimental effects on overall gut health. Both treatment groups exhibited a significant increase in intestinal length and relative weight compared to the control group ($P<0.05$). An enhancement in intestinal histomorphology of birds fed with FSBM, characterized by increased villus height, a higher villus height-to-crypt depth (VH:CD) ratio, and larger villus absorptive surface areas. The treatment groups also exhibited significantly lower histopathological scores than the control group ($P<0.05$). FSBM supplementation also positively altered caecal microbial composition by increasing LAB counts while reducing *Coliform* and *E. coli* populations. In conclusion, FSBM supplementation in Javanese super chicken significantly enhances intestinal morphology and caecal microbial composition, leading to an improved intestinal health index.

## Introduction

The chicken digestive system is essential for growth performance due to its role in nutrient absorption and act as a protective barrier. The gut ecosystem, consisting of the microbial population, intestinal morphology, and immune system, is influenced by factors such as genetics, diet, housing, litter, and age of birds (Pourabedin and Zhao, 2015; Cowieson *et al*., 2017). A balanced gut ecosystem ensures efficient nutrient absorption and supports overall health. As a key component of this system, the small intestine plays a pivotal role in processing nutrients, making it a reliable indicator of growth and performance (Awad *et al*., 2006).

In Indonesia, the demand for chicken meat continues to rise each year, with Javanese super chickens becoming increasingly popular in the livestock industry. This hybrid, a cross between a broiler-laying hen and a male Kampoeng chicken, combines the traits of both breeds (Mulyono and Raharjo, 2005; Hidayat and Asmarasari, 2015). Given the crucial role of the digestive system in growth performance, the productivity of Javanese super chickens depends, among other factors, on high-quality feed. Furthermore, as a native Indonesian slow-growing breed, Javanese super chickens are often associated with poor feeding management and efficiency (Irmaya *et al*., 2021).

Soybean meal (SBM) is a primary source of protein and essential amino acids in chicken feed. However, it contains antinutritional factors (ANFs), such as trypsin inhibitors, β-conglycinin, glycinin, and phytate, which hinder nutrient digestion and absorption (Yan *et al*., 2022). Fermented soybean meal (FSBM) is a nutritionally enhanced feed ingredient known to support intestinal health and overall productivity in animals. Fermentation of SBM effectively reduces ANFs and increases bioavailable peptides (Feng *et al*., 2007). SBM fermented with microorganisms such as *Bacillus subtilis*, *Aspergillus oryzae*, *Aspergillus niger*, or *Aspergillus usamii* has been demonstrated to significantly improve the average daily gain in broilers, quails, and laying hens (Shi *et al*., 2017; Li *et al*., 2020; Xu *et al*., 2020; Obianwuna *et al*., 2024). This improvement is attributed to the ability of these microorganisms to enhance the nutrient profile of SBM by increasing the availability of soluble proteins and essential amino acids while reducing ANFs. *Bacillus subtilis* and *Aspergillus niger* are among the most effective and widely used microorganisms in SBM fermentation, playing a crucial role in optimizing poultry performance (Irawan *et al*., 2022; Akhirini *et al*., 2024).

Studies have demonstrated that substituting SBM with FSBM improves digestive enzyme activity, enhances gut morphology, and boosts growth performance in broiler chickens (Jazi *et al*., 2019; Soumeh *et al*., 2019). Specifically, extensive research highlights the positive impacts of FSBM on intestinal morphology, gut integrity, and gut microbiota in various birds (Feng *et al*., 2007; Abeddargahi *et al*., 2022; Supriya *et al*., 2022; Ncube and Mawere, 2024; Obianwuna *et al*., 2024). However, data on its impact on Javanese super chickens remain scarce. The aim of this study was to evaluate the effects of incorporating FSBM into the diet of Javanese super chickens on intestinal morphology and caecal microbial population.

## Materials and methods

### *Ethical approval*

This study followed a standard procedure certified by the Ethical Board of the Faculty of Animal Husbandry, Universitas Sebelas Maret, Surakarta, Indonesia, under certification number 172/UN27.14.1/PT/2023.

### *Study period and location*

The experimental study was carried out for three months, from November 2022 to January 2023, and was conducted in the poultry house experimental units at the Faculty of Animal Science, Universitas Sebelas


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Maret, Surakarta, Indonesia. All facilities were provided to ensure animal welfare, including sufficient space, ventilation, lighting, security, and access to feed and water.

*Preparation of FSBM*

Pure cultures of *Bacillus subtilis* and *Aspergillus niger* were obtained from the Laboratory of Microbiology, Universitas Gadjah Mada. *Aspergillus niger* was isolated from Indonesian fermented soybeans, and *Bacillus subtilis* was sourced from the intestinal tract of a healthy broiler chicken on a corn-SBM diet. A solid-state fermentation method was adapted from Suprayogi *et al*. (2022), using sterilized soybean meal (SBM) inoculated with either *Bacillus subtilis* (B-SBM) or *Aspergillus niger* (A-SBM). Ten grams of sterilized SBM was mixed with 10 mL of distilled water containing the respective inoculum (1:10 dilution), achieving $1\times10^6$ CFU/g of *Aspergillus niger* or $1\times10^8$ CFU/g of *Bacillus subtilis*. The inoculated samples were incubated at 25°C for 24 hours (B-SBM) or 48 hours (A-SBM), then autoclaved at 60°C for 30 minutes and sun-dried for 60 minutes. The fermentation was performed under static aerobic conditions.

*Birds, diets and experimental design*

A total of 36 healthy male one-day-old Javanese Super Chickens, with an average body weight of 48 g, were obtained from a local hatchery. The birds were reared under standard environmental conditions in a colony system. For the first 10 days, the chicks were housed in brooder boxes. All birds received a Gumboro disease vaccine via mouth-drop on day 14, followed by a Newcastle disease vaccine (LaSota strain) administered through drinking water on day 18. Vaccination and medical care were administered under the supervision of a veterinarian. Rice hulls served as bedding in the floor cages. The chickens were raised on an untreated corn-SBM-based diet until 20 days of age, with ad libitum access to drinking water. Starting on day 21, the birds were divided into three dietary treatment groups, with six replicate pens per group, each housing two chickens. The 60-day study utilized 18 pens, each with dimensions of 45cm × 35cm × 60cm. The dietary treatments consisted of CON (unfermented soybean meal), B-SBM (soybean meal fermented with *Bacillus subtilis*), and A-SBM (soybean meal fermented with *Aspergillus niger*). The details of the composition and calculated chemical analysis of the experimental diets for Javanese super chicken are presented in Table 1.

*Intestinal relative weight and length measurements*

On day 60, 10 birds from each treatment group were weighed and slaughtered. The measurements were performed by weighing (g) all segments of the intestine and each part of the intestine (duodenum, jejunum, ileum, caecum, and colorectum), on an analytical scale with 0.001-g precision. According to Assis *et al*. (2021), the weights of the birds and each organ were used to calculate the relative weight of the organs (%) by applying the following formula: Relative organ weight = (organ weight/live weight) × 100. The intestinal segments were also measured (cm) separately, using a measuring tape. The duodenum was collected from the gizzard outlet to the distal portion of the duodenal loop, jejunum from the distal portion of the duodenal loop to Meckel's diverticulum, ileum from the portion anterior to the caecum, caecum from the junction between ileum and colon, and colorectum from the caecum to the cloaca.

*Histomorphometric and histopathological examinations*

Organ Sampling

Two-centimeter tissue samples from the small intestine were collected from the upper (beginning of the organ), middle (central section), and lower (end of the organ) portions of the duodenum, jejunum, and ileum for histological slides, following Giannenas *et al*. (2010). In brief, the samples were fixed in 10% neutral buffered formaldehyde (NBF) for 72 hours, followed by dehydration, clearing, and embedding in wax. Histological analysis was conducted on 5 µm thick transverse sections (cut with a microtome), which were mounted on slides and stained with hematoxylin and eosin. The tissue sections were examined using an Olympus CX-31 microscope (Olympus Corp.) with 40X-400X magnification, and images were captured with an Optilab Advanced Plus camera (Miconos Corp.).

Table 1. Composition and calculated chemical analysis of the experimental diets for Javanese super chicken.

| Items | CON | B-SBM | A-SBM |
|---|---|---|---|
| Ingredients, % | | | |
| Yellow corn | 47.62 | 48.85 | 48.64 |
| Bran | 20 | 20 | 20 |
| Palm oil | 2.64 | 2.44 | 2.47 |
| SBM (unfermented) | 21.54 | 0 | 0 |
| B-SBM | 0 | 20.52 | 0 |
| A-SBM | 0 | 0 | 20.69 |
| Fish Meal | 5 | 5 | 5 |
| DL-Methionine 99% | 0.15 | 0.15 | 0.15 |
| L-Lysin | 0.3 | 0.3 | 0.3 |
| Premix[a] | 0.5 | 0.5 | 0.5 |
| Mineral B12 | 2 | 2 | 2 |
| Salt | 0.25 | 0.25 | 0.25 |
| Total | 100 | 100 | 100 |
| Calculated and determined chemical composition[b] | | | |
| Metabolism energy (kcal/kg) | 2950 | 2950 | 2950 |
| Crude protein, % | 19 | 19 | 19 |
| Crude fiber, % | 2.18 | 2.16 | 2.16 |
| Crude fat, % | 7.43 | 7.22 | 7.26 |
| Crude ash, % | 4.06 | 3.06 | 3.06 |
| Calcium, % | 1.49 | 1.49 | 1.49 |
| Phosphorus, % | 1.12 | 1.12 | 1.12 |
| Lysin, % | 1.23 | 1.37 | 1.31 |
| Methionine, % | 0.50 | 0.49 | 0.49 |

SBM = Soybean meal, CON = control group, B-SBM = SBM fermented with *Bacillus subtilis*, A-SBM = SBM fermented with *Aspergillus niger*, [a]Premix provided the following per kilogram of diet: vitamin A, 1200,000 IU; vitamin D3, 200,000 IU; vitamin E, 800 IU; vitamin $K_3$, 200 mg; vitamin $B_1$, 200 mg; vitamin $B_2$, 500 mg; vitamin $B_6$, 50 mg; vitamin B12, 1,200 µg; vitamin C, 2,500 mg; calcium-D-pantothenate, 600 mg; niacin, 4,000 mg; choline chloride, 1,000 mg; methionine, 3,000 mg; lysine, 3,000 mg; Mn, 12,000 mg; Fe, 2,000 mg; I, 20 mg; Zn, 10,000 mg; Co, 20 mg, Cu, 400 mg; Santoquin, 1,000 mg. [b]Based on the analysis result in the Laboratory of Animal Feedstuffs, Animal Science Faculty Universitas Gadjah Mada (2022).

Histomorphometric assessment

Villus height, villus width, and crypt depth in the duodenum, jejunum, and ileum were measured using a computer with the OptiLab Viewer 4 program. Villus height was measured from the tip (including the lamina propria) to the base (villus-crypt junction), while villus width was measured at the midpoint of the villus. Crypt depth was determined from the villus-crypt junction to the distal end of the crypt. Three sections from each part (upper, middle, and lower) of the duodenum, jejunum, and ileum were measured, with two villi per section per segment per bird. A total of eighteen villi were counted from nine different sections across each segment (duodenum, jejunum, and ileum) for each bird, and the average was calculated for the mean villus height, villus width, and crypt depth (Yamauchi *et al*., 2006; Rajput *et al*., 2013). The absorptive surface area of the small intestine villus was estimated by considering a villus as a cylindrical structure. Villus absorptive surface area was calculated using the formula: Villus absorptive surface area = 2π × (average villus width/2)

× villus height (Prakatur *et al*., 2019).

Histopathological evaluation

The histopathological evaluation method in this study was adapted from the ISI (I See Inside) methodology applied for microscopy, according to Kraieski *et al*. (2017). The ISI methodology is based on a numeric score of histopathological lesions with the evaluated parameters listed in Table 2. The Impact Factor (IF) is assigned to each lesion based on microscopic analysis. It is determined according to the degree of functional impairment in an organ, using existing literature and research as a reference. The IF ranges from 1 to 3, with 3 representing the highest impact on organ function. Additionally, the extent or intensity of each lesion, or the frequency of its occurrence compared to unaffected organs, is evaluated in each organ/tissue. The score (S) ranges from 0 to 3, where score 0 indicates no lesion or frequency; score 1 indicates an alteration affecting up to 25% of the area or frequency; score 2 represents alterations affecting 25.1% to 50% of the area or frequency; and score 3 indicates alterations extending over 50% of the area or frequency.

To calculate the final ISI index, known as the ISI total score, the impact factor (IF) of each alteration is multiplied by its corresponding score number, and the results of all alterations are summed using the formula: ISI = Σ(IF × S). For instance, an increase in lamina propria thickness has an IF of 2. This value is multiplied by the observed score (ranging from 1 to 3). If the score (S) is 3 (the maximum score), the final ISI value for this parameter in the villus would be ISI = (2 × 3) = 6. The ISI score for the intestine ranges from 0 to 45. The final ISI value for each bird is determined by averaging the ISI values of 20 villi in each part of the small intestine.

Cecal microbiota evaluation

Immediately after slaughter, cecal contents were randomly collected from four birds in each group for microbiological examination. The selected caecal microbial population were analyzed using a culture method adapted from Shang *et al*. (2016) with slight modifications. Dilutions of cecal content were plated in duplicate on selective agars: Brilliance *E. coli*/*Coliform* Selective agar (Oxoid, Basingstoke, Hampshire, UK) for *Escherichia coli*, incubated at 37°C for 24 hours; and Peptone Glucose Yeast (PGY) agar (Difco, USA) for Lactic Acid Bacteria, incubated at 37°C for 48 hours. Microbial populations were expressed as $\log_{10}$ colony-forming units (CFU) per gram of wet digesta for each cecal sample.

*Statistical analysis*

Data were analyzed with SPSS version 27.0 (SPSS Inc.) using a completely randomized design. Results are expressed as Mean±SEM. Prior to analysis, the normality of the data was assessed using the Shapiro-Wilk test. One-way ANOVA was used to compare parametric data, followed by Tukey's post-hoc test. Probability values less than 0.05 ($P < 0.05$) were considered statistically significant.

## Results

*The relative weight and length of the small intestine*

The measurements of the intestine, including intestinal relative weight and intestinal length, are summarized in Table 3. The data reveal a marked increase in total intestinal weight, particularly in the duodenum, ileum, and cecum segments, in the treatment groups (B-SBM and A-SBM) compared to the CON group ($P<0.05$). Additionally, a considerable increase in intestinal length, specifically in the ileum, was observed in both treatment groups compared to the control.

*The small intestine histomorphometry*

The histomorphometric data, including villus height, width, crypt depth, and absorptive surface area in the duodenum, jejunum, and ileum of Javanese super chickens, are presented in Table 4 and Fig. 1. The morphometric assessment method for villus-crypt units is shown in Fig. 3A. The results revealed that both treatment groups (B-SBM and A-SBM) had improved gut morphology, with increased villus height, reduced crypt depth, and a higher VH:CD ratio compared to the control group. Additionally, the villus absorptive surface areas in the duodenum, jejunum, and ileum were significantly larger in the B-SBM and A-SBM groups than in the control group ($P<0.05$).

*Histopathological evaluation using ISI methodology*

To better assess the impact of feeding FSBM to Javanese super chickens, evaluating the total histopathological score of the small intestine using the ISI method is crucial. Data total histopathological score of small intestine alteration is illustrated in Fig. 2. Lower ISI scores indicate better intestinal health. Results showed that birds fed with FSBM had significantly improved intestinal health indices. Both treatment groups exhibited significantly lower ISI scores compared to the control across all intestinal segments ($P<0.05$), except for the A-SBM group in the duodenum, which showed no significant difference from the control. In contrast, the control group had the highest ISI scores, attributed to histological lesions including increased lamina propria thickness, epithelial thickness, enterocyte proliferation, epithelial plasma cell infiltration, lamina propria inflammatory infiltration, and goblet cell proliferation (Fig. 3B, 3C).

*Caecal microbial population*

The selected caecal microbial population of Javanese super chickens fed with fermented soybean meal (FSBM) is shown in Table 5. The results

Table 2. ISI histological alterations evaluated in the intestine.

| Alterations | Impact Factor (IF) | | Score (S) | Final score | Maximum score[1] |
|---|---|---|---|---|---|
| Lamina propria thickness | 2 | X | 3 | 6 | 45 |
| Epithelial thickness | 1 | X | 3 | 3 | |
| Enterocytes proliferation | 1 | X | 3 | 3 | |
| Epithelial plasma cell infiltration | 1 | X | 3 | 3 | |
| Lamina propria inflammatory infiltration | 3 | X | 3 | 9 | |
| Goblet cells proliferation | 2 | X | 3 | 6 | |
| Congestion | 2 | X | 3 | 6 | |
| Presence of oocysts | 3 | X | 3 | 9 | |

[1]The maximum score is calculated as the sum of all alterations based on the formula ISI = Σ(IF×S), where IF = impact factor (previous fixed) and S = Score (observed) considering the maximum observed S. For instance, if the lamina propria thickness has IF = 2 and a score of S = 3 (maximum observed) is recorded for this parameter in a villus, the ISI for the lamina propria thickness in that villus will be ISI = (2×3) = 6. The final ISI value for each bird is determined by averaging the ISI values of 20 villi in each part of the small intestine.

Table 3. Relative weight and length of the intestine in Javanese super chickens fed fermented soybean meal

| Parameter | Treatment | | | |
|---|---|---|---|---|
| | CON | B-SBM | A-SBM | *P*-value |
| Relative weight (%) | | | | |
| Total intestine | 0.41 ± 0.01[a] | 0.51 ± 0.01[b] | 0.54 ± 0.02[b] | 0 |
| Duodenum | 0.09 ± 0.003[a] | 0.10 ± 0.006[a] | 0.12 ± 0.007[b] | 0.00 |
| Jejunum | 0.13 ± 0.005 | 0.16 ± 0.006 | 0.16 ± 0.009 | 0.05 |
| Ileum | 0.10 ± 0.005[a] | 0.15 ± 0.005[b] | 0.16 ± 0.009[b] | 0 |
| Caecum | 0.05 ± 0.003[a] | 0.06 ± 0.002[ab] | 0.07 ± 0.005[b] | 0.06 |
| Colorectum | 0.02 ± 0.001 | 0.02 ± 0.004 | 0.02 ± 0.002 | 0.40 |
| Length (cm) | | | | |
| Total intestine | 157.58 ± 2.89[a] | 168.58 ± 2.11[b] | 169.61 ± 2.59[b] | 0.00 |
| Duodenum | 25.08 ± 0.81 | 26.33 ± 0.84 | 27.08 ± 0.43 | 0.16 |
| Jejunum | 50.33 ± 1.25 | 53.25 ± 1.42 | 53.50 ± 1.31 | 0.19 |
| Ileum | 48.58 ± 1.13[a] | 53.42 ± 1.06[b] | 54.00 ± 1.03[b] | 0.00 |
| Caecum | 26.67 ± 0.82 | 28.25 ± 0.93 | 28.08 ± 0.90 | 0.39 |
| Colorectum | 6.92 ± 0.19 | 7.33 ± 0.39 | 7.25 ± 0.25 | 0.57 |

CON = control group, B-SBM = SBM fermented with *Bacillus subtilis*, A-SBM = SBM fermented with *Aspergillus niger*. Mean±SEM, [a-b]different letters on values in the same row indicate a significance (P<0.05).

Table 4. Histomorphometric measurements of the small intestine of Javanese super chicken fed with fermented soybean meal.

| Variable | Treatment | | |
|---|---|---|---|
| | CON | B-SBM | A-SBM |
| Duodenum | | | |
| Villus height (µm) | 904.29 ± 14.37[a] | 1129.06 ± 50.36[b] | 1254.95 ± 31.65[c] |
| Crypt depth (µm) | 396.27 ± 6.82[c] | 346.33 ± 13.21[b] | 305.33 ± 11.58[a] |
| VH:CD ratio | 2.30 ± 0.06[a] | 3.33 ± 0.18[b] | 4.21 ± 0.18[c] |
| Villus width (µm) | 213.16 ± 5.26[b] | 214.35 ± 3.31[b] | 199.28 ± 5.30[a] |
| Villus absorptive surface area ($mm^2$) | 0.60 ± 0.01[a] | 0.76 ± 0.04[b] | 0.78 ± 0.03[b] |
| Jejunum | | | |
| Villus height (µm) | 815.81 ± 14.61[a] | 1005.86 ± 50.49[b] | 1107.29 ± 18.80[c] |
| Crypt depth (µm) | 311.95 ± 11.87[c] | 272.18 ± 13.02[b] | 216.38 ± 2.20[a] |
| VH:CD ratio | 2.69 ± 0.11[a] | 3.71 ± 0.09[b] | 5.13 ± 0.11[c] |
| Villus width (µm) | 142.68 ± 2.09[a] | 159.61 ± 6.21[b] | 156.00 ± 2.18[b] |
| Villus absorptive surface area ($mm^2$) | 0.36 ± 0.01[a] | 0.51 ± 0.04[b] | 0.54 ± 0.01[b] |
| Ileum | | | |
| Villus height (µm) | 525.33 ± 7.25[a] | 766.13 ± 7.14[b] | 886.98 ±8.61[c] |
| Crypt depth (µm) | 252.59 ± 4.85[c] | 215.23 ± 9.28[b] | 153.27 ± 2.61[a] |
| VH:CD ratio | 2.09 ± 0.04[a] | 3.71 ± 0.18[b] | 5.82 ± 0.13[c] |
| Villus width (µm) | 163.03 ± 5.36[b] | 174.40 ± 3.53[c] | 150.12 ± 1.08[a] |
| Villus absorptive surface area ($mm^2$) | 0.27 ± 0.01[a] | 0.42 ± 0.01[b] | 0.41 ± 0.00[b] |

CON = Control group, B-SBM = SBM fermented with *Bacillus subtilis*, A-SBM = SBM fermented with *Aspergillus niger*. Mean±SEM, [a-c]different letters on values in the same row indicate a significant (P<0.05). VH= Villus Height CD = Crypt Depth.

Table 5. The selected caecal microbial population of Javanese super chickens fed with fermented soybean meal.

| Species ($Log_{10}$ CFU/g) | Treatments | | | *P*-value |
|---|---|---|---|---|
| | CON | B-SBM | A-SBM | |
| LAB | 8.22 ± 0.27 | 8.39 ± 0.22 | 8.29 ± 0.17 | 0.87 |
| *Coliform* | 5.79 ± 0.33[b] | 4.38 ± 0.24[a] | 5.24 ± 0.21[b] | 0.01 |
| *E. coli* | 5.35 ± 0.41[b] | 4.23 ± 0.26[a] | 5.03 ± 0.21[ab] | 0.08 |
| LAB/*Coliform* ratio | 1.41 ± 0.11[a] | 1.93 ± 0.14[b] | 1.59 ± 0.08[ab] | 0.03 |

CON = control group, B-SBM = SBM fermented with *Bacillus subtilis*, A-SBM = SBM fermented with *Aspergillus niger*. Mean±SEM. [a-b]different letters on values in the same row indicate a significant (P<0.05). LAB = Lactic Acid Bacteria, CFU = Colony-Forming Unit.

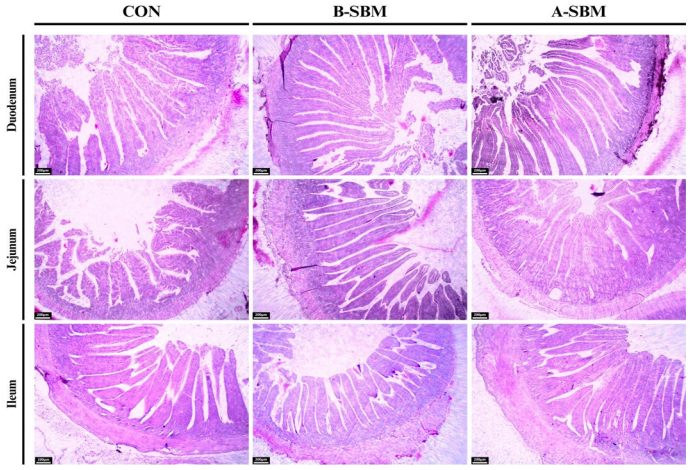


Fig 1. Photomicrographs of the duodenum, jejunum, and ileum of Javanese super chickens fed with fermented soybean meal (H&E; 40X).

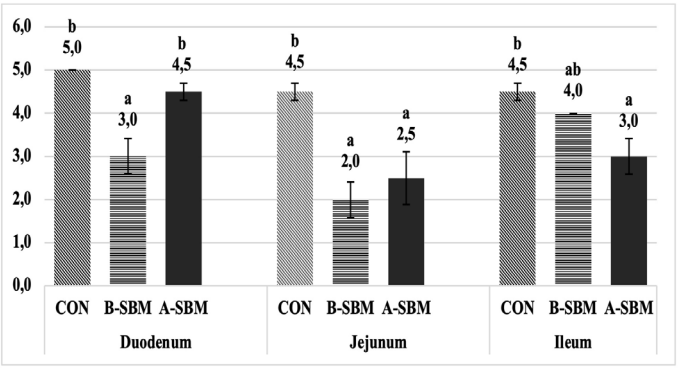


Fig. 2. ISI total histopathological score of small intestine lesions. Error bars represent the standard error of the mean. Different superscript letters indicate a significant difference (P<0.05). CON = control group, B-SBM = SBM fermented with *Bacillus subtilis*, A-SBM = SBM fermented with *Aspergillus niger*.

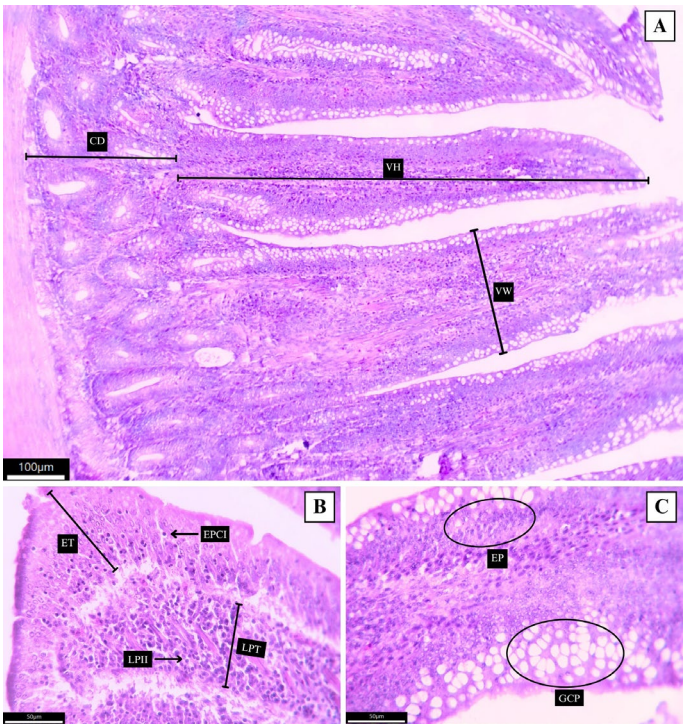


Fig 3. Photomicrographs of small intestine sections of Javanese super chickens fed with fermented soybean meal. (A) Methodology for morphometric assessment of villus-crypt unit (H&E; 100X), (B-C) Histological alterations along with visible pathological lesions contributed to the highest ISI total score (P<0.05) (H&E; 400X). VH=Villus Height, VW =Villus Width, CD=Crypt Depth, LPT=Lamina Propria Thickness, ET=Epithelial Thickness, EP=Enterocytes Proliferation, EPCI=Epithelial Plasma Cell Infiltration, LPII=Lamina Propria Inflammatory Infiltration, GCP=Goblet Cells Proliferation.

indicate that FSBM altered caecal microbial counts, significantly reducing *Coliform* and *E. coli* populations in the B-SBM group compared to the control group (P<0.05). Although not statistically significant, LAB counts showed an increase in all treatment groups than in the control group.

## Discussion

FSBM using *Bacillus subtilis* and *Aspergillus niger* had a positive effect on the intestinal morphology. In the current study, allometric analysis of intestinal relative weight and length showed significant differences between the control and treatment groups (B-SBM and A-SBM), particularly in the ileum length and the relative weight of the total intestine, ileum, and caecum. The A-SBM group exhibited higher values than the B-SBM group, although not statistically significant. This effect is likely due to structural and functional changes in the intestine that enhance nutrient digestion and absorption. According to Assis *et al.* (2021), a larger proportion of digestive organs enhances feed efficiency, as the size and functionality of the gastrointestinal tract are crucial for nutrient breakdown and absorption, ultimately improving nutrient utilization.

To assess the impact of FSBM supplementation on intestinal structural changes, this study conducted histomorphometric analysis, focusing on villus height, villus width, crypt depth, villus height-to-crypt depth (VH:CD) ratio, and villus absorptive surface area. The results demonstrated that both treatment groups exhibited improved gut morphology, characterized by increased villus height, reduced crypt depth, and a higher VH:CD ratio compared to the control group. Previous studies have demonstrated that longer intestinal villi indicate enhanced nutrient absorption capacity (Caspary, 1992; Awad *et al.*, 2006). This is attributed to active mitosis within villi, which increases their absorptive potential for various nutrients (Samaya and Yamauchi *et al.*, 2002; Onderci *et al.*, 2006). An increase in crypt depth supports the rapid regeneration of villi, as crypt cells divide and migrate upwards to form new epithelial cells (Rebolé *et al.*, 2010; Xia *et al.*, 2004), a normal mechanism to maintain the balance and integrity of the intestinal tissue. However, excessively deep crypts may also indicate issues such as tissue exfoliation, inflammation, or a response to pathogen-derived toxins (Rajput *et al.*, 2013).

The current study found a significant increase in the villus height-to-crypt depth (VH:CD) ratio in chickens fed with FSBM (B-SBM and A-SBM) compared to the control, indicating improved intestinal absorptive capacity. A higher VH:CD ratio reflects better nutrient digestion and absorption (Silva *et al.*, 2009). Conversely, a lower ratio with shorter villi and deeper crypts suggests impaired nutrient absorption and increased mucin production, raising the energy demand for intestinal function (Langhout *et al.*, 1999). This study also confirmed that the B-SBM and A-SBM groups had significantly larger absorptive surface areas in the duodenum, jejunum, and ileum compared to the control group.

Birds fed diets supplemented with FSBM in the present study showed better intestinal health, as indicated by histopathological evaluation using the ISI method. Notably, lower ISI scores, which reflect better tissue integrity and fewer histological lesions, signify improved intestinal health. Lesions, such as thickened lamina propria and epithelium, increased enterocyte and goblet cell proliferation, and infiltration of epithelial plasma and inflammatory cells, although not in large values, were found in birds fed with unfermented SBM (control group), whilst it significantly reduced in birds fed FSBM (B-SBM and A-SBM group). Some studies have suggested that the morphological changes in the intestine observed in young animals are due to transient hypersensitivity to antigenic components of soybean meal (Lalles *et al.*, 1993; Hong *et al.*, 2004). Antigenic materials in soybean proteins are associated with villus atrophy, increased crypt cell mitosis, and crypt hyperplasia, and thereby cause a malabsorption syndrome (Kenworthy and Allen, 1966). Meanwhile, the improvement of intestinal morphology in the present study may be associated

with the degradation of antigenic materials after fermentation. Previous studies have reported that fermentation could degrade large-size proteins to small-size peptides (Kiers *et al*., 2000; Hong *et al*., 2004), which mitigates morphological alterations. In addition, the reduction of trypsin inhibitors and lectins in FSBM enhances its interaction with the intestinal epithelial brush border, supporting cell viability, crypt functionality, and tissue development (Liener, 1994).

The gastrointestinal (GI) tract of poultry harbors a diverse microbial community, with its composition and activity significantly influencing metabolism, immune function, gut integrity, and growth performance (Pan and Yu, 2013). In the current research, the cecal lactic acid bacteria (LAB) count was higher in chickens fed fermented soybean meal (FSBM) diets (B-SBM and A-SBM groups) compared to the control group. Although the increase in LAB was not statistically significant, the counts of *Coliforms* and *E. coli* were notably lower in chickens fed FSBM fermented with *Bacillus subtilis*. Furthermore, the LAB-to-*Coliform* ratio was highest in the B-SBM group among all groups. These findings align with previous research demonstrating that dietary FSBM supplementation in broilers increases LAB counts while reducing *Coliform* levels in the intestinal tract (Jazi *et al*., 2017; Soumeh *et al*., 2019; Supriya *et al*., 2022). Jazi *et al*. (2018) further highlighted that the biological activity of *Bacillus subtilis* creates favorable conditions for LAB growth and proliferation. LAB activity, in turn, lowers the feed's pH through lactic acid production, thereby establishing a protective environment that inhibits the growth and viability of pathogenic organisms, including members of the *Enterobacteriaceae* family. In summary, the findings of this study on gut health in Javanese super chickens indicate that FSBM supplementation positively influences intestinal morphology and caecal microbial composition.

## Conclusion

Dietary inclusion of FSBM with *Bacillus subtilis* or *Aspergillus niger* improves intestinal morphology and the caecal microbial population in Javanese Super Chickens by increasing LAB counts, reducing *Coliform* and *E. coli* populations while positively altering villus structure, epithelial thickness, and proliferation of enterocytes and goblet cells, leading to a better intestinal health index.

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## Conflict of interest

The authors have no conflict of interest to declare.

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