

Research Article



Effects of *Bacillus subtilis* Inoculum Dose and Fermentation Duration on the Nutrient Composition and Biological Value of Cocoa Pod Husk (*Theobroma cacao* L.) as a Poultry Feed Ingredient

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Abstract | Cocoa pod husk (*Theobroma cacao* L.) is an agricultural by product that contains valuable nutrients and has potential as an alternative poultry feed ingredient. This study aimed to evaluate the effects of *Bacillus subtilis* inoculum dose and fermentation duration on the nutrient composition and biological value of cocoa pod husk as poultry feed. A completely randomized design with a factorial arrangement was employed, consisting of two factors: inoculum dose (2%, 4%, and 6%) and fermentation duration (2, 4, and 6 days), with three replications per treatment. Differences among treatments were analyzed using Duncan's Multiple Range Test (DMRT). The parameters observed were dry matter, crude protein, crude fat, nitrogen retention, crude fiber digestibility, and metabolizable energy. The results showed that the interaction between inoculum dose and fermentation duration significantly ($P < 0.05$) affected crude protein, crude fat, nitrogen retention, crude fiber digestibility, and metabolizable energy, but had no significant effect ($P > 0.05$) on dry matter content. The optimal fermentation treatment was obtained at a *B. subtilis* inoculum dose of 4% and a fermentation duration of 4 days. Under this condition, fermentation improved the nutrient composition and biological value of cocoa pod husk, indicating its potential as a more suitable poultry feed ingredient.

Keywords | *Bacillus subtilis*, Cocoa pod husk, Fermentation duration, Nutrient composition, Biological value, Poultry feed

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INTRODUCTION

Feed cost remains the largest component of poultry production expenses; therefore, the utilization of locally available feed resources is an important strategy to reduce production costs without compromising animal performance (Wizna *et al.*, 2025). One potential local

feed resource is cocoa pod husk (CPH), which constitutes approximately 67–76% of the total weight of the cocoa fruit (Campos-Vega *et al.*, 2018). Its abundant availability in cocoa-producing regions makes CPH a low-cost and sustainable alternative feed ingredient. The potential use of CPH as poultry feed is supported by its nutritional composition, which includes 11.27% crude protein, 11.37%

ash, 9.60% crude fiber, 7.15% crude fat, 60.61% nitrogen-free extract, 3153.52 kcal/kg metabolizable energy, 1.32 g/100 g theobromine, 0.50 g/100 g tannin, and 30.49 mg/100 g phytate (Olugosi *et al.*, 2019).

However, the utilization of CPH in poultry diets is still limited due to its high structural fiber content and the presence of theobromine. Theobromine is a methylxanthine alkaloid naturally found in cocoa (Goya *et al.*, 2022) and acts as an antinutritional compound in poultry. High concentrations of theobromine may reduce feed intake, impair growth performance, and interfere with nutrient utilization, thereby limiting the inclusion of cocoa pod husk in poultry feed formulations (Mercogliano *et al.*, 2025). One approach to overcome these limitations is biological processing through fermentation. Fermentation has been reported to effectively reduce the theobromine content of CPH, thereby improving its safety and suitability as a poultry feed ingredient (Oduro-Mensah *et al.*, 2018).

Solid-state fermentation (SSF) is a relatively simple and cost-effective method for improving the nutritional value of fibrous feed ingredients. Feed processing through SSF generally enhances nutrient quality and protein digestibility while reducing antinutritional factors, thereby positively affecting poultry performance and gut health (Olukomaiya *et al.*, 2019). One microorganism with considerable potential for this process is *Bacillus subtilis*. This bacterium is capable of forming highly stable spores and producing various extracellular enzymes, including proteases, xylanases, and cellulases, which contribute to fiber degradation and improved nutrient availability (Su *et al.*, 2020). Several studies have demonstrated that fermentation using *B. subtilis* can improve the nutritional quality of feed ingredients and reduce antinutritional factors (Shi *et al.*, 2017). Furthermore, dietary supplementation with *B. subtilis* has been shown to enhance ileal protein and energy digestibility in broilers, indicating its role in improving nutrient utilization efficiency (Borojeni *et al.*, 2018).

The success of fermentation is largely determined by inoculum dosage and fermentation duration, as these factors influence microbial growth, enzyme activity, and changes in the nutrient composition of the substrate. Optimal growth and fermentative activity of *Bacillus* may vary depending on the strain and substrate used. Some studies have reported maximum activity at relatively low inoculum levels (approximately 2%) and short fermentation periods (around 48 hours) (Irfan *et al.*, 2016). Other studies have shown that peak metabolic activity and enzyme production can be achieved at longer fermentation periods, ranging from approximately 96 hours (4 days) to 6 days, depending on the fermentation objective and substrate characteristics (Sun *et al.*, 2023; Moharam *et al.*, 2023).

Numerous studies have been conducted to improve the nutritional quality of cocoa pod husk through fermentation. Fermentation of CPH using *Phanerochaete chrysosporium* and *Ganoderma lucidum* has been reported to reduce fiber content and improve feed quality (Wahyudi *et al.*, 2023). Other studies have employed *Aspergillus oryzae* (Rakhmani and Puastuti, 2021), *Ganoderma lucidum* (Samadi *et al.*, 2022), and *Talaromyces verruculosus* (Oduro-Mensah *et al.*, 2020) to enhance the nutritional value of cocoa pod husk. However, most of these studies have focused on the use of fungi as fermentation agents, whereas information regarding the use of bacteria, particularly *B. subtilis*, remains limited.

Previous studies have generally focused on changes in the chemical composition of cocoa pod husk, such as crude protein and crude fiber content, without comprehensively evaluating its biological value as a poultry feed ingredient, including nitrogen retention, crude fiber digestibility, and metabolizable energy. Moreover, information on the optimal combination of inoculum dosage and fermentation duration to improve both the nutritional composition and biological value of cocoa pod husk is still lacking. Therefore, this study was conducted to evaluate the effects of *B. subtilis* inoculum dosage and fermentation duration on the nutrient composition and biological value of cocoa pod husk as a poultry feed ingredient.

MATERIALS AND METHODS

MATERIALS

Cocoa pod husks were obtained from smallholder cocoa plantations in Medan City, Indonesia. The microorganism used in this study was *B. subtilis* ATCC 6051, which was rejuvenated at the Feed Nutrition and Technology Laboratory prior to its use as a fermentation inoculum. Nutrient Agar (NA) was used as the bacterial growth medium. Other materials included rice bran, rice husk ash, and distilled water.

A total of 33 five-week-old broiler chickens were used for the biological evaluation. Twenty-seven birds were allocated to the nine experimental treatment combinations, with three birds per treatment. Three additional birds were used for endogenous nitrogen correction, while three birds received the basal diet as the control group. Chemicals required for proximate analysis and reagents used for determining nitrogen retention, crude fiber digestibility, and metabolizable energy were also used in this study.

METHODS

The experiment was conducted using a 3 × 3 factorial Completely Randomized Design (CRD) with three replications.

Factor A was the inoculum dose of *Bacillus subtilis* (% w/w), consisting of three levels:

A1 = 2%

A2 = 4%

A3 = 6%

Factor B was the fermentation duration, consisting of three levels:

B1 = 2 days

B2 = 4 days

B3 = 6 days

The combination of these factors resulted in nine treatment combinations, with a total of 27 experimental units.

PREPARATION OF RICE HUSK ASH FILTRATE

Rice husk ash filtrate was prepared by completely combusting rice husks to obtain ash, which was subsequently dissolved in clean water. A 20% rice husk ash solution was prepared by mixing 2 kg of rice husk ash with 8 L of clean water. The mixture was allowed to stand for 24 h and was then filtered to obtain the filtrate.

SUBSTRATE PREPARATION

Cocoa pod husks were collected from cocoa plantations in Medan, Indonesia. The husks were chopped into small pieces and soaked in rice husk ash filtrate for 48 h. After soaking, the cocoa pod husks were drained and oven-dried, then ground into meal for use as the fermentation substrate.

REJUVENATION OF *BACILLUS SUBTILIS*

Bacillus subtilis was rejuvenated on Nutrient Agar (NA) medium. The NA medium was dissolved in sterile distilled water and heated until completely homogenized. Approximately 5 mL of the medium was dispensed into each test tube, sealed with cotton plugs and aluminum foil, and sterilized in an autoclave at 121°C for 15 min. After sterilization, the tubes were placed at an angle of approximately 20° to form agar slants. The medium was inoculated with *B. subtilis* using a sterile inoculating loop and incubated at 37 °C for 24 h. The resulting culture was used as the inoculum source for the subsequent stage.

PREPARATION OF *BACILLUS SUBTILIS* INOCULUM

A total of 100 g of rice bran was placed in a heat-resistant container and sterilized in an autoclave at 121°C and 15 psi for 15 min, then cooled to room temperature under sterile conditions. Colonies of *B. subtilis* from the rejuvenated agar slants were collected using a sterile inoculating loop and suspended in 10 mL of sterile distilled water to obtain a homogeneous suspension.

The suspension was further diluted with sterile distilled water to achieve an inoculum density of 10⁸ CFU/mL, as determined by the total plate count method. The inoculum

suspension was mixed with the sterilized rice bran, and sterile water was added until the moisture content reached approximately 50%. The mixture was incubated aerobically at 37 °C for 24 h. After incubation, the material was dried in an oven at 40 °C until a powdered form was obtained and subsequently used as a dry inoculum.

FERMENTATION OF COCOA POD HUSK WITH *BACILLUS SUBTILIS*

For each treatment, 200 g of cocoa pod husk substrate was weighed and sterilized in an autoclave at 121°C and 15 psi for 15 min, then cooled to room temperature under sterile conditions. The moisture content of the substrate was adjusted to approximately 50% prior to fermentation. The sterilized substrate was inoculated with *B. subtilis* at the respective treatment levels (2%, 4%, and 6% w/w of substrate weight) and thoroughly mixed to ensure uniform distribution.

Fermentation was carried out aerobically at 37°C for 2, 4, and 6 days according to the treatment. Upon completion of fermentation, the product was dried at 80°C for 2 h to terminate microbial activity, followed by further drying at 60°C for 6 h until a stable moisture content was achieved. The dried product was then ground to obtain a homogeneous sample and stored for subsequent analyses.

BIOLOGICAL EVALUATION

The biological evaluation was conducted to determine nitrogen retention, crude fiber digestibility, and metabolizable energy of the fermented cocoa pod husk. The birds underwent a 3-day adaptation period to allow adjustment to the experimental diets and experimental conditions, followed by a 5-day total excreta collection period. During the collection period, feed intake and excreta output were recorded daily. Excreta were collected quantitatively, processed, and analyzed for nitrogen and crude fiber contents, while feed and excreta samples were analyzed for gross energy. The resulting data were used to calculate nitrogen retention, crude fiber digestibility, and metabolizable energy. The same evaluation procedures were applied to all experimental groups.

PARAMETERS

LABORATORY ANALYSIS

Proximate analysis was conducted according to AOAC (2016) to determine dry matter, crude protein, and crude fiber contents. Dry matter was determined by oven drying to constant weight, crude protein by the Kjeldahl method based on nitrogen determination, and crude fiber by acid-alkali digestion followed by drying and ashing.

BIOLOGICAL EVALUATION

The biological parameters evaluated were nitrogen

retention, crude fiber digestibility (McDonald *et al.*, 2011), and metabolizable energy determination. The biological evaluation consisted of nitrogen retention, crude fiber digestibility, and metabolizable energy determination. Following a 3-day adaptation period, total excreta were collected for 5 consecutive days. Feed intake and total excreta output were recorded during the collection period. Excreta samples were collected daily, weighed, and processed for subsequent chemical analysis. Nitrogen retention was calculated from nitrogen intake and nitrogen excretion. Crude fiber digestibility was calculated from the intake and excretion of crude fiber. Metabolizable energy was determined using the Sibbald (1976) method.

DATA ANALYSIS

All data were analyzed using analysis of variance (ANOVA) based on a 3 × 3 factorial Completely Randomized Design (CRD), with *B. subtilis* inoculum dose, fermentation duration, and their interaction included in the statistical model. When significant treatment effects were detected ($P < 0.05$), Duncan's Multiple Range Test (DMRT) was applied for mean separation (Steel and Torrie, 1991).

RESULTS AND DISCUSSION

The effects of *B. subtilis* inoculum dose and fermentation duration on the chemical composition of cocoa pod husk are presented in Table 1. The analysis of variance showed that the interaction between inoculum dose and fermentation duration did not significantly affect dry matter content ($P > 0.05$). Likewise, neither inoculum dose nor fermentation

duration had a significant individual effect on dry matter. The absence of significant effects indicates that fermentation did not substantially change the total dry matter fraction of cocoa pod husk. During fermentation, some organic matter may be utilized by microorganisms, while microbial biomass and other fermentation products may be formed, resulting in relatively small changes in the final dry matter content (Wang *et al.*, 2020; Olagunju *et al.*, 2023). Thus, the main nutritional changes observed in this study were more apparent in the protein, fiber, and biological value parameters than in total dry matter.

Crude protein and crude fiber showed a significant interaction between inoculum dose and fermentation duration ($P < 0.05$), indicating that the response of these nutrients depended on the combination of inoculum level and fermentation time. The highest crude protein content was obtained at A2B2 (4% inoculum and 4 days of fermentation), reaching 8.91%, and was significantly different from the other treatments. This response may be associated with favorable fermentation conditions that supported microbial biomass formation and nutrient transformation. The increase in crude protein can partly be related to the incorporation of nitrogen into microbial biomass, while degradation of other organic fractions may also increase the relative proportion of protein in the fermented substrate (Maulana *et al.*, 2021; Thakaew *et al.*, 2024). Proteolytic activity may additionally contribute to the transformation of protein compounds into simpler forms during fermentation (Andika *et al.*, 2025).

Table 1: Effects of *Bacillus subtilis* inoculum dose and fermentation duration on the chemical composition of cocoa pod husk.

Factor A (Inoculum dose)	Factor B (Fermentation duration)	Dry matter (%)	Crude protein (%)	Crude fiber (%)
A1 (2%)	B1 (2 days)	42.52 ± 1.19	7.20 ± 0.33 ^c	31.53 ± 1.28 ^a
	B2 (4 days)	42.69 ± 1.95	7.75 ± 0.57 ^d	28.41 ± 1.39 ^b
	B3 (6 days)	42.99 ± 3.65	7.78 ± 0.23 ^{cd}	27.78 ± 0.23 ^b
	Average	42.73	7.58 ^b	29.24 ^a
A2 (4%)	B1 (2 days)	41.74 ± 2.57	7.41 ± 0.42 ^{de}	30.75 ± 0.58 ^a
	B2 (4 days)	42.51 ± 1.60	8.91 ± 0.04 ^a	22.90 ± 1.75 ^d
	B3 (6 days)	41.38 ± 2.30	8.44 ± 0.12 ^{ab}	25.48 ± 1.14 ^c
	Average	41.88	8.25 ^a	26.37 ^b
A3 (6%)	B1 (2 days)	41.21 ± 2.49	7.96 ± 0.06 ^{bc}	28.63 ± 1.17 ^b
	B2 (4 days)	42.38 ± 3.44	8.35 ± 0.26 ^b	25.69 ± 0.81 ^c
	B3 (6 days)	42.51 ± 3.07	8.37 ± 0.17 ^b	25.50 ± 1.43 ^c
	Average	42.03	8.25 ^a	26.60 ^b
Average	B1	41.82	7.52 ^b	30.30 ^a
	B2	42.53	8.34 ^a	25.67 ^b
	B3	42.29	8.20 ^a	26.25 ^b

Note: Means within the same column bearing different superscripts differ significantly ($P < 0.05$). No significant interaction between inoculum dose and fermentation duration was observed for dry matter (DM) content ($P > 0.05$), whereas significant interactions were detected for crude protein (CP) and crude fiber (CF) contents ($P < 0.05$).

The response at longer fermentation duration suggests that extending fermentation did not continuously improve crude protein content. At 6 days, crude protein decreased to 8.44% in A2B3 and 8.37% in A3B3. This decline may indicate that the beneficial effect of fermentation had reached a point beyond which further fermentation provided limited nutritional advantage. Changes in substrate availability and fermentation conditions may alter microbial activity and nutrient utilization during prolonged fermentation. Therefore, the higher protein content at A2B2 should be interpreted as the result of a favorable balance between inoculum dose and fermentation duration rather than simply an effect of increasing either factor.

A similar interaction was observed for crude fiber. The lowest crude fiber content was obtained at A2B2, with a value of 22.90%, indicating that 4% inoculum combined with 4 days of fermentation provided the most effective reduction under the conditions of this study. The reduction in crude fiber may be associated with the ability of *B. subtilis* to produce hydrolytic enzymes that contribute to the breakdown of structural carbohydrate components. Such degradation can improve the accessibility of nutrients within the plant matrix and is particularly relevant for cocoa pod husk because its relatively high fiber content limits its direct utilization as a poultry feed ingredient. Fermentation by enzyme-producing microorganisms has been reported to improve the availability of nutrients from fibrous plant substrates (Maulana *et al.*, 2021; Vivares *et al.*, 2025).

However, increasing the inoculum dose to 6% or extending fermentation to 6 days did not produce a proportional improvement in crude fiber reduction. This suggests that the effectiveness of fermentation depended on the balance between microbial activity and substrate availability. During prolonged fermentation, readily available substrates may become depleted, potentially limiting further degradation of the remaining structural fractions (Li *et al.*, 2025). The simultaneous increase in crude protein and reduction in crude fiber at A2B2 is therefore important from a feed-quality perspective, because these changes indicate an improvement in the nutritional profile of cocoa pod husk rather than a simple change in a single nutrient fraction.

The interaction between inoculum dose and fermentation duration also significantly affected nitrogen retention (NR), crude fiber digestibility (CFD), and metabolizable energy (ME) ($P < 0.05$; Table 2). These parameters provide a biological indication of whether the compositional changes observed after fermentation were accompanied by improved nutrient utilization. Nitrogen retention increased from 55.62% at A1B1 to 68.40% at A2B2, representing an improvement of approximately 23.0%. The increase suggests that the fermentation treatment improved the utilization of dietary nitrogen. This response may be related to changes in protein accessibility and microbial transformation during fermentation. The reduction in fiber may also facilitate greater access of digestive enzymes to nutrients within the substrate, potentially contributing to improved nitrogen utilization (Ali *et al.*, 2024).

Table 2: Effects of *Bacillus subtilis* inoculum dose and fermentation duration on the biological value of cocoa pod husk.

Factor A (Inoculum dose)	Factor B (Fermentation duration)	Nitrogen retention (%)	Crude fiber digestibility (%)	Metabolizable energy (Kcal/kg)
A1 (2%)	B1 (2 days)	55.62 ± 0.57 ^d	56.62 ± 2.47 ^d	1601.59 ± 1.28 ^f
	B2 (4 days)	58.87 ± 0.15 ^c	59.86 ± 1.11 ^c	1745.64 ± 1.39 ^{de}
	B3 (6 days)	59.03 ± 1.68 ^c	60.30 ± 1.13 ^c	1808.80 ± 0.23 ^{de}
	Average	57.84 ^b	58.93 ^b	1718.67 ^b
A2 (4%)	B1 (2 days)	58.78 ± 0.92 ^c	59.58 ± 1.05 ^c	1720.29 ± 0.58 ^{ef}
	B2 (4 days)	68.40 ± 0.53 ^a	67.97 ± 0.06 ^a	2263.95 ± 1.75 ^a
	B3 (6 days)	65.01 ± 1.83 ^b	64.47 ± 1.34 ^b	2078.22 ± 1.14 ^b
	Average	64.06 ^a	64.01 ^a	2020.82 ^a
A3 (6%)	B1 (2 days)	59.29 ± 0.59 ^c	62.96 ± 2.04 ^c	1861.94 ± 1.17 ^{cd}
	B2 (4 days)	63.40 ± 0.53 ^b	64.04 ± 0.15 ^b	1959.25 ± 0.81 ^{bc}
	B3 (6 days)	63.42 ± 1.23 ^b	64.31 ± 0.55 ^b	1999.69 ± 1.43 ^b
	Average	62.04 ^a	63.77 ^a	1940.29 ^a
Average	B1	57.90 ^b	59.72 ^b	1727.94 ^b
	B2	63.56 ^a	63.96 ^a	1989.61 ^a
	B3	62.49 ^a	63.03 ^a	1962.24 ^a

Note: Different superscripts within the same column indicate significant differences ($P < 0.05$). A significant interaction was observed between inoculum dose and fermentation duration on nitrogen retention (NR), crude fiber digestibility (CFD), and metabolizable energy (ME) ($P < 0.05$).

Nitrogen retention decreased to 65.01% at A2B3 after 6 days of fermentation. Thus, the improvement was not linear with increasing fermentation duration. The lower value at prolonged fermentation may reflect changes in substrate availability or fermentation conditions that limited further nutritional improvement. Although microbial growth dynamics may contribute to this response, microbial growth phases were not directly measured in the present study; therefore, this explanation should be considered a possible mechanism rather than a confirmed process. Crude fiber digestibility increased from 56.62% at A1B1 to 67.97% at A2B2, corresponding to an improvement of approximately 20.0%.

The increase in digestibility is consistent with the reduction in crude fiber observed at the same treatment combination. Fermentation by *B. subtilis* may facilitate the breakdown of structural carbohydrate components through its enzyme-producing capacity, resulting in a substrate that is more accessible to digestive processes (Maulana *et al.*, 2021). The highest CFD at A2B2 further indicates that the 4% inoculum dose and 4-day fermentation provided a favorable condition for improving the biological utilization of the fermented substrate. Increasing the inoculum dose or extending fermentation duration beyond this condition did not result in a proportional increase in CFD. This response indicates that a higher inoculum dose does not necessarily produce greater nutritional improvement when substrate availability or other fermentation conditions become limiting. The effectiveness of microbial fermentation depends on the balance between microbial activity, available substrate, and the duration required for nutrient transformation (Wei and Huang, 2025).

Metabolizable energy showed a similar response. ME increased from 1601.59 kcal/kg at A1B1 to 2263.95 kcal/kg at A2B2, representing an increase of approximately 41.4% (Table 2). The higher ME at A2B2 is consistent with the improvement in crude fiber digestibility, as greater degradation of fibrous components may increase the availability of nutrients for energy utilization. However, the relationship between fiber reduction and energy value is not necessarily linear because the response is also influenced by the type of substrate, its digestibility, and the extent of microbial utilization during fermentation (Zhang *et al.*, 2013). At 6 days of fermentation, ME decreased to 2078.22 kcal/kg in A2B3. This decline indicates that extending fermentation beyond 4 days did not provide additional benefits in energy value. One possible explanation is that microorganisms may utilize part of the available substrate as an energy source during prolonged fermentation, although this process was not directly measured in the present study (Folch *et al.*, 2021). Therefore, the lower ME at 6 days should be interpreted

cautiously as an indication that prolonged fermentation may reduce the efficiency of nutrient conversion rather than as direct evidence of microbial energy consumption.

Taken together, the results indicate that the nutritional and biological responses of cocoa pod husk were strongly dependent on the combination of inoculum dose and fermentation duration. The A2B2 treatment (4% *B. subtilis* and 4 days) consistently provided the most favorable response, particularly in crude protein, crude fiber, nitrogen retention, crude fiber digestibility, and metabolizable energy. These results suggest that an appropriate fermentation intensity is more important than simply increasing inoculum dose or extending fermentation duration. From a practical feed perspective, the improvement in protein content, fiber digestibility, nitrogen retention, and energy value at A2B2 indicates that fermentation can improve the potential nutritional value of cocoa pod husk for poultry feed utilization.

CONCLUSIONS AND RECOMMENDATIONS

It can be concluded that fermentation of cocoa pod husk with *Bacillus subtilis* at a 4% inoculum dose for 4 days provided the most favorable overall response for improving its nutritional and biological value. The improvement in protein utilization, crude fiber digestibility, nitrogen retention, and metabolizable energy, together with the reduction in crude fiber, indicates that fermentation can enhance the feeding value of cocoa pod husk. Therefore, *B. subtilis*-fermented cocoa pod husk has potential to be further developed as an alternative poultry feed ingredient, although further studies are needed to evaluate its application in practical poultry diets.

Based on the results of this study, it is recommended that fermentation of cocoa pod husk (*Theobroma cacao* L.) using *B. subtilis* at a 4% inoculum dose and 4 days of fermentation be used as an optimal reference condition to improve its nutritional composition and biological value as a poultry feed ingredient. However, prior to its wider application in feed formulation, further in vivo studies on poultry are required to evaluate its effects on production performance, such as feed intake, body weight gain, feed conversion ratio, and overall animal health.

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- This study advances the biological utilization of cocoa pod husk (*Theobroma cacao* L.) as a poultry feed resource by applying *Bacillus subtilis* fermentation to improve its nutritional quality and feeding value.
- The study provides new insight into the combined influence of *Bacillus subtilis* inoculum level and fermentation duration on the nutritional transformation of cocoa pod husk, contributing to the development of a more suitable fermentation approach for feed utilization.
- Beyond evaluating changes in chemical composition, this study integrates nitrogen retention, crude fiber digestibility, and metabolizable energy to characterize the biological feeding value of fermented cocoa pod husk, providing a more comprehensive basis for its potential use as a poultry feed ingredient.

AUTHOR'S CONTRIBUTION

MA was responsible for conceptualization, methodology, supervision, investigation, and overall coordination of the study. AT contributed to methodology development and validation. LY coordinated project administration and provided the research resources. FM performed the formal analysis and prepared the original draft of the manuscript. SU contributed to data curation and laboratory investigation. HSP assisted with data collection and experimental procedures. FH contributed to manuscript review, editing, and visualization. All authors have read, reviewed, and approved the final manuscript.

ETHICAL APPROVAL

This research complied with the ethical principles for the use of experimental animals as outlined in the Decree of the Minister of Agriculture of the Republic of Indonesia No. 306/KPTS/TN.330/4/1994 and in Law No. 18 of 2009 concerning Animal Husbandry and Animal Health, as amended by Law No. 41 of 2014.

GENERATIVE AI AND AI-ASSISTED TECHNOLOGY STATEMENT

The authors declare that ChatGPT (OpenAI, San Francisco, CA, USA) was used to assist in the initial translation of the manuscript from Indonesian to English. All outputs were reviewed, edited, and verified by the authors to ensure accuracy and originality.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest.

- Ali SA, Saeed SMG, Sohail M, Alzahrani AA, Yehia HM (2024). Gelatinization and fermentation synergy: Investigating the protein digestibility, mineral bioaccessibility and microstructural transformations of black mash beans through *Saccharomyces cerevisiae* and *Lactobacillus* spp. *Int. J. Food Prop.*, 27: 674–688. <https://doi.org/10.3390/fermentation10020077>
- Andika R, Wizna, Mirzah, Marlida Y (2025). Fermentation of corn tumpi with *Bacillus amyloliquefaciens*: Optimization of fermentation duration and inoculum dosage for superior poultry feed. *J. Anim. Health Prod.*, 13(1): 160–165. <https://doi.org/10.17582/journal.jahp/2025/13.1.160.165>
- AOAC (2016). Official methods of analysis of the association of official analytical chemists, 20th Ed. AOAC International, Gaithersburg, Maryland, USA.
- Boroojeni FG, Vahjen W, Männer K, Blanch A, Sandvang D, Zentek J (2018). *Bacillus subtilis* in broiler diets with different levels of energy and protein. *Poult. Sci.*, 97(11): 3967–3976. <https://doi.org/10.3382/ps/pey265>.
- Campos-Vega R, Nieto-Figueroa KH, Oomah BD (2018). Cocoa (*Theobroma cacao* L.) pod husk: Renewable source of bioactive compounds. *Trends Food Sci. Technol.*, 81: 172–184. <https://doi.org/10.1016/j.tifs.2018.09.022>
- Dworkin J, Harwood C (2022). Metabolic reprogramming and longevity in quiescence. *Annu. Rev. Microbiol.*, 76: 1–22. <https://doi.org/10.1146/annurev-micro-041320-111014>
- Folch PL, Bisschops M, Weusthuis R (2021). Metabolic energy conservation for fermentative product formation. *Microb. Biotechnol.*, 14: 829–858. <https://doi.org/10.1111/1751-7915.13746>
- Goya L, Kongor JE, De Pascual-Teresa S (2022). From cocoa to chocolate: Effect of processing on flavanols and methylxanthines and their mechanisms of action. *Int. J. Mol. Sci.*, 23: 14365. <https://doi.org/10.3390/ijms232214365>
- Irfan M, Asghar U, Nadeem M, Nelofer R, Syed Q (2016). Optimization of process parameters for xylanase production by *Bacillus* sp. in submerged fermentation. *J. Radiat. Res. Appl. Sci.*, 9(2): 139–147. <https://doi.org/10.1016/j.jrras.2015.10.008>
- Li J, Li K, Tang L, Hua C, Chen N, Yang C, Xin Y, Chen F (2025). Study on the modification of dietary fiber and degradation of zearalenone in corn germ meal by solid-state fermentation with *Bacillus subtilis* K6. *Foods*, 14: 2680. <https://doi.org/10.3390/foods14152680>
- Maulana F, Nuraini, Mirzah (2021). Kandungan dan kualitas nutrisi limbah sawit fermentasi dengan *Lentinus edodes*. *J. Peternak. Indones.*, 23(2): 174–182. <https://doi.org/10.25077/jpi.23.2.174-182.2021>
- McDonald P, Edwards RA, Greenhalgh JFD, Morgan CA, Sinclair LA, Wilkinson RG (2011). *Animal Nutrition*, 7th Ed. Pearson Education, Harlow, United Kingdom.
- Mercogliano F, Bani C, Tretola M, Landolfi C, Ottoboni M, Cheli F, Restani P, Pinotti L, Di Lorenzo C (2025). Preliminary assessment of alkaloid content in cocoa (*Theobroma cacao* L.) hulls for safe consumption as a feed ingredient. *Toxins*, 17: 441. <https://doi.org/10.3390/toxins17090441>
- Moharam ME, El-Bendary MA, Abo Elsoud MM, El-Beih F, Hassnin SM, Salama A, Omara EA, Elgamal NN (2023). Modeling and *in-vivo* evaluation of fibrinolytic enzyme produced by *Bacillus subtilis* Egy under solid state fermentation. *Heliyon*, 9(5): e16254. <https://doi.org/10.1016/j.heliyon.2023.16254>

- [org/10.1016/j.heliyon.2023.e16254](https://doi.org/10.1016/j.heliyon.2023.e16254)
- Oduro-Mensah D, Ocloo A, Nortey T, Antwi S, Okine LK, Adamafo NA (2020). Nutritional value and safety of animal feed supplemented with *Talaromyces verruculosus*-treated cocoa pod husks. *Sci. Rep.*, 10: 13163. <https://doi.org/10.1038/s41598-020-69763-9>
- Oduro-Mensah D, Ocloo A, Osei R, Addy F, Amoako EE, Dzogbefia VP (2018). Bio-dettheobromination of cocoa pod husks: Reduction of theobromine content by a newly isolated methylxanthine degrading bacterium and its potential for poultry feed production. *Microb. Cell Fact.*, 17: 79. <https://doi.org/10.1186/s12934-018-0931-x>
- Olagunju LK, Isikhuemhen O, Dele P, Anike F, Ike K, Shaw Y, Brice RM, Orimaye OE, Wuaku M, Essick BG, Holt NL, Udombang NS, Enemudo JO, Subedi K, Anele U (2023). Effects of the incubation period of *Pleurotus ostreatus* on the chemical composition and nutrient availability of solid-state-fermented corn stover. *Animals*, 13: 2587. <https://doi.org/10.3390/ani13162587>
- Olugosi OA, Agbede JO, Adebayo IA, Onibi GE, Ayeni OA (2019). Nutritional enhancement of cocoa pod husk meal through fermentation using *Rhizopus stolonifer*. *Afr. J. Biotechnol.*, 18(30): 901–908. <https://doi.org/10.5897/AJB2019.16940>
- Olukomaiya O, Fernando C, Mereddy R, Li X, Sultanbawa Y (2019). Solid-state fermented plant protein sources in the diets of broiler chickens: A review. *Anim. Nutr.*, 5(4): 319–330. <https://doi.org/10.1016/j.aninu.2019.05.005>
- Rakhmani SIW, Puastuti W (2021). Utilization of fermented cocoa pod husk (CPH) as feed ingredient for sheep. *Adv. Biol. Sci. Res.*, 18(1): 26–30. <https://doi.org/10.2991/absr.k.220207.006>
- Samadi, Nasution RW, Wajizah S, Zulfahrizal Z, Munawar A (2022). Optical characteristics of fermented cocoa pod husk based on near infrared reflectance spectroscopy and evaluation of its quality as animal feed material. *IOP Conf. Ser. Earth Environ. Sci.*, 1116: 012014. <https://doi.org/10.1088/1755-1315/1116/1/012014>
- Shi C, Zhang Y, Lu Z, Wang Y (2017). Solid-state fermentation of corn-soybean meal mixed feed with *Bacillus subtilis* and *Enterococcus faecium* for degrading antinutritional factors and enhancing nutritional value. *J. Anim. Sci. Biotechnol.*, 8: 50. <https://doi.org/10.1186/s40104-017-0184-2>
- Sibbald IR (1976). A bioassay for true metabolizable energy in feedstuffs. *Poult. Sci.*, 55(1): 303–308. <https://doi.org/10.3382/ps.0550303>
- Steel RGD, Torrie JH (1995). Statistical principles and procedures of a biometric approach, 2nd Ed. Translated by Bambang Sumatri. PT. Gramedia Pustaka Utama, Jakarta, Indonesia.
- Su Y, Liu C, Fang H, Zhang D (2020). *Bacillus subtilis*: A universal cell factory for industry, agriculture, biomaterials and medicine. *Microb. Cell Fact.*, 19: 173. <https://doi.org/10.1186/s12934-020-01436-8>
- Sun B, Zou K, Zhao Y, Tang Y, Zhang F, Chen W, Tang X, Chang C, Zheng Y (2023). The fermentation optimization for alkaline protease production by *Bacillus subtilis* BS-QR-052. *Front. Microbiol.*, 14: 1301065. <https://doi.org/10.3389/fmicb.2023.1301065>
- Thakaew R, Jaiwongsa S, Pumas C, Chaiklangmuang S (2024). Protein enhancement in low-grade maize by fermentation with yeast and bacteria. *J. Food Nutr. Res.*, 12(5): 312–318. <https://doi.org/10.12691/jfnr-12-5-3>
- Vivares G, De Nanclares MP, Conde-Aguilera A, De Vries S (2025). Quantifying the interactions between dietary fibers and macronutrient digestibility in broiler chickens: The importance of considering fiber solubility. *Anim. Feed Sci. Technol.*, 321: 116241. <https://doi.org/10.1016/j.anifeeds.2025.116241>
- Wahyudi I, Munawar AA, Yu P, Samadi S (2023). Optical characterization of NIR spectra for chemometric model of cocoa pod husk fermented for animal feed. *IOP Conf. Ser.: Earth Environ. Sci.*, 1183: 012003. <https://doi.org/10.1088/1755-1315/1183/1/012003>
- Wang C, Shi C, Su W, Jin M, Xu B, Hao L, Lu Z, Wang F, Wang Y, Du H (2020). Dynamics of the physicochemical characteristics, microbiota, and metabolic functions of soybean meal and corn mixed substrates during two-stage solid-state fermentation. *mSystems*, 5: e00501-19. <https://doi.org/10.1128/msystems.00501-19>
- Wei J, Huang Y (2025). Microbial function in food fermentations: Current research and future directions. *Curr. Res. Microb. Sci.*, 9: 100491. <https://doi.org/10.1039/D5FO01485A>
- Wizna, Rusfidra, Heryandi Y, Andika R, Maulana F, Agasi SY, Wulandari M, Safitri R (2025). Application of *Bacillus amyloliquefaciens* in fermenting water lettuce (*Pistia stratiotes*) as a feed source for Mojosari ducks. *J. Anim. Health Prod.*, 13(3): 555–564. <https://doi.org/10.17582/journal.jahp/2025/13.3.555.564>
- Zhang W, Li D, Liu L, Zang J, Duan Q, Yang W, Zhang L (2013). The effects of dietary fiber level on nutrient digestibility in growing pigs. *J. Anim. Sci. Biotechnol.*, 4: 17. <https://doi.org/10.1186/2049-1891-4-17>