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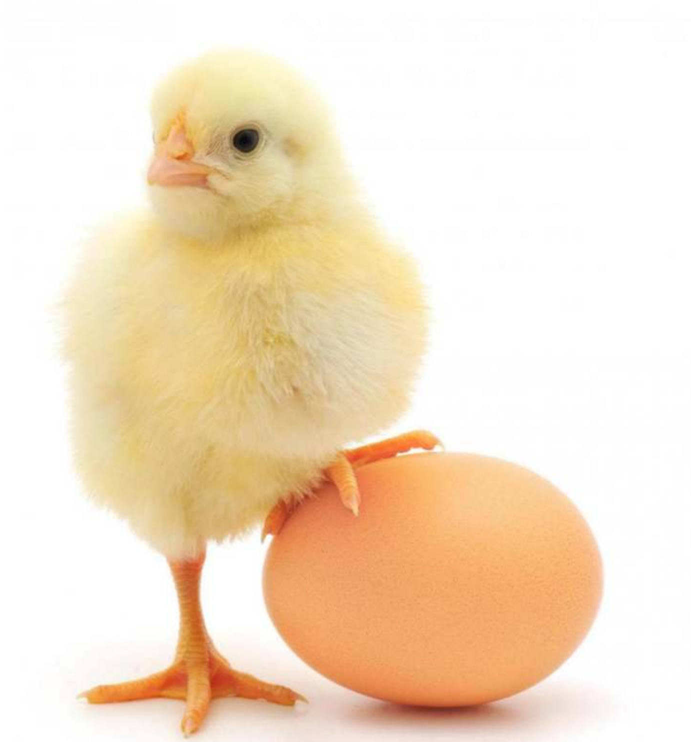

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Research Article

Effects of Amino Acid Composition in a *Bacillus amyloliquefaciens*-fermented Mixture of Bovine Blood and Coconut Pulp on Growth Performance, Blood Cholesterol of Broilers

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Abstract

Background and Background: Bovine blood is a livestock by-product that can be used as a protein source for livestock, particularly when incorporated in broiler rations. This study was conducted to investigate the effects of amino acids in *Bacillus amyloliquefaciens*-fermented mixtures of bovine blood and coconut pulp (blood meal) on growth performance, blood cholesterol and erythrocyte content in broilers. **Methodology:** A total of 100 six day-old CP 707 (Strain Cobb) broilers were divided into 5 experimental groups with 4 replicates of 5 broilers. The experimental groups received rations supplemented with 0, 5, 10, 15 and 20% blood meal that substituted for fish meal or soybean meal. The experimental period was 5 weeks. Treatment effects on broiler performance parameters such as final body weight, final weight gain, average daily gain, feed intake, average abdominal fat and Feed Conversion Ratio (FCR), total serum cholesterol and erythrocyte count, as well as the return on investment were determined. **Results:** The group fed rations supplemented with 10% blood meal showed final body weight, final weight gain and FCR of 1,172, 1,035 and 1.75 g, respectively, which was significantly higher ($p<0.05$) than that for the other treatments. Rations with 10% blood meal also had the best return on investment ($0.92) relative to the other groups. **Conclusion:** A mixture of 10% bovine blood and coconut pulp fermented with *Bacillus amyloliquefaciens* can be used in broiler feed without adversely affecting broiler performance and can replace 47% of total fishmeal and 53% of soybean meal in broiler rations.

Key words: Bovine blood, coconut pulp, fermentation, *Bacillus amyloliquefaciens*, broiler

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Poultry is a popular protein source in Indonesia. The 4.2% increase in the broiler population in Indonesia to 1.6 million birds between 2015 and 2016 reflects increased demand for poultry protein[1]. However, feed production has not kept pace with the increased population. In 2015, Indonesia imported 90% of the fish meal and 70% of the soybean meal that is commonly used for livestock rations[2]. For broiler chickens, these meals typically represent 15-20% of the ration.

Feedstuff is an important aspect of commercial poultry performance and represents the largest cost for poultry production. Renewable feed ingredients are one means for reducing the cost of poultry production and many researchers have explored various renewable feed ingredients that can be used as protein sources for poultry. Local resources such as livestock by-products and agri-industrial waste can be used to reduce production costs, as these highly abundant and economical materials are not in high demand for human consumption and can provide benefits for livestock growth and performance.

Bovine blood is a livestock by-product that can be used as a protein source for livestock, particularly broilers. Based on the Central Bureau of Statistics, the number of cattle slaughtered in Indonesia in 2013 was 1,326,395 head, which could yield 11.9 million kg of blood. Bovine blood contains 80% crude protein, 1% crude fiber, 1.6% crude fat, 1.6% calcium and 80% water, as well as 2,750 kcal kg^{-1} Metabolizable Energy (ME), 1.0% methionine, 1.4% cysteine, 6.9% lysine, 1.0% tryptophan, 0.8% isoleucine, 3.05% histidine, 5.2% valine, 10.3% leucine, 2.35% arginine and 4.4% glycine[3-5]. Since drying of bovine blood can be an extended process, adsorbents such as agri-industrial waste are often used to accelerate drying. Coconut pulp is an agri-industrial waste that is abundant and has good nutrient content with 4.89% crude protein and 28% crude fiber[6].

Khawaja *et al.*[7] reported that blood meal can be added to broiler rations to as much as 3% without adversely affecting performance. Meanwhile, Ekunseitan *et al.*[8] reported that the maximum amount of blood meal that can be used in broiler rations is 6%. There are some limitations to the use of blood meal in broiler rations because of its low biological value and amino acid imbalance. For example, blood meal is rich in the essential amino acid lysine but has a low isoleucine content[9]. Such amino acid imbalances can reduce feed consumption rates and diminish livestock performance due to lower amounts of amino acids in the plasma[10]. Gietzen[11] also showed that lower amino acid concentrations alter animal behavior, and, in particular, can reduce feed intake.

Fermentation is one of the methods to increase the nutrient content of feed ingredients. Fermentation changes the physicochemical and microbiological properties of feed in ways that can improve animal performance[12]. Rations that include a mixture of bovine blood and agri-industrial waste such as coconut pulp that is then fermented with *Bacillus amyloliquefaciens* can be used for poultry feed. *Bacillus amyloliquefaciens* can form endospores to survive under extreme conditions such as high heat and can survive until environmental conditions are more favorable, when the spores germinate to form unicellular bacteria[13]. This study was conducted to investigate how broiler performance is affected by rations supplemented with various percentages of an amino acid-balanced mixture of bovine blood and agriculture industrial waste fermented with by *Bacillus amyloliquefaciens*.

MATERIALS AND METHODS

Broilers and experimental treatments: A total of 100 six day-old CP 707 (Cobb) broilers were obtained from a local commercial hatchery. The broilers were randomly allocated to 5 experimental treatments that had 20 broilers arranged into 4 replicates of 5 birds each. Each replicate was assigned to a clean cage (1 m^2) that was 1 m high. Heat was provided by a lamp affixed to each cage. The experimental period was 4 weeks.

The experimental rations contained maize, rice bran, fish meal, soybean meal, bone meal and mineral mix as the basal diet. The fish and soybean meals were substituted with 0, 5, 10, 15 and 20% of a *Bacillus amyloliquefaciens*-fermented mixture of bovine blood and coconut pulp (blood meal) (Table 1) for 100% fish meal and 88% soybean meal. The experimental protocol was approved by the Animal Care and Use Committee at the Andalas University.

The *Bacillus amyloliquefaciens*-fermented mixtures of bovine blood and coconut pulp (blood meal) were made by first mixing fresh bovine blood and fresh coconut pulp. The mixtures were then fermented with *Bacillus amyloliquefaciens* as an inoculum. The nutrient medium for the inoculum was peptone (2%), glucose (15%), K_2HPO_4 (0.1), $MgSO_4 \cdot 7H_2O$ (0.05%), urea (1.5%) and $CaCO_3$ (1%) mixed with 500 mL water. The medium was autoclaved for 15 min at 121 °C before one inoculating loop full of *Bacillus amyloliquefaciens* was added. After 5 days, the broth can be used as inoculum. The medium was prepared to increase the isoleucine content using a method described by Chibata *et al.*[14]. The 1.2:1 (v/v)

Table 1: Composition of experimental diets containing varying amounts of *Bacillus amyloliquefaciens*-fermented bovine blood and coconut pulp (blood meal) mixtures

Components	Diet A 0% BM	Diet B % BM	Diet C 10% BM	Diet D 15% BM	Diet E 20% BM
Ingredients, % of feed					
Maize	63	63	63	63	63
Rice bran	3	5.5	8	11	11
Fish meal	15	11	7	3	-
Soybean meal	17	13	9	5	2
Bone meal	1	1.5	2	2	3
Blood meal	-	5	10	15	20
Mineral mix[1]	1	1	1	1	1
Analyzed nutrients,% DM basis					
Crude protein (%)	21.68	21.67	21.85	21.99	22.66
Crude fat (%)	3.68	4.13	4.59	5.11	5.29
Crude fiber (%)	2.44	3.00	3.56	4.22	4.33
Calcium (%)	1.01	1.02	1.03	0.91	1.09
Phosphorus (%)	0.55	0.55	0.55	0.48	0.56
ME (kcal kg^{-1})	3002.17	3004.52	3006.87	3017.57	3031.77
Essential amino acids					
Arg	1.3	1.2	1.1	1.0	0.9
His	0.5	0.6	0.6	0.7	0.8
Ile	1.1	0.9	0.8	0.6	0.5
Leu	2.0	2.0	2.1	2.1	2.2
Lys	1.1	1.0	1.1	1.1	1.2
Met	0.8	0.7	0.6	0.4	0.3
Phe	1.1	1.1	1.1	1.1	1.2
Thr	0.9	0.9	0.9	0.8	0.8
Val	1.2	1.2	1.2	1.2	1.3
Non-essential amino acids					
Ala	1.5	1.5	1.4	1.4	1.4
Asp	1.9	1.8	1.8	1.8	1.8
Glu	2.7	2.6	2.6	2.5	2.5
Gly	1.2	1.1	1.0	0.9	0.8
Ser	1.1	1.0	1.0	0.9	0.9
Tyr	1.0	0.9	0.8	0.7	0.7

[1]Mineral premix supplied per kilogram of diet: 1,200,000 IU vitamin A, 200,000 IU vitamin B3, 800 IU vitamin E, 0.2 g vitamin K, 0.2 g vitamin B1, 0.5 g vitamin B2, 0.05 g vitamin B6, 1,200 mg vitamin B12, 2.5 g vitamin C, 0.6 g Calcium-D-pantothenate, 4 g niacin, 1 g choline chloride, 3 g methionine, 3 g lysine, 12 g manganese, 2 g iron, 0.02 g iodine, 10 g zinc, 0.020 g cobalt, 0.4 g copper, 2.1 g zinc bacitracin, 1 g excipient q.s, BM: Blood meal, DM: Dry matter, ME: Metabolizable energy

Table 2: Nutrient composition nutrition and amino acid profile of *Bacillus amyloliquefaciens*-fermented bovine blood and coconut pulp (blood meal) mixtures

Nutrients	Composition (%/kcal kg^{-1})	Amino acid (g kg^{-1})
Crude protein	67.94	
Crude fiber	5.59	
Crude fat	6.77	
Calcium	1.10	
Phosphorous	0.2	
Metabolizable energy	3,500 kcal kg^{-1}	
Amino acid		
Aspartic acid		49.1
Glutamic acid		48.3
Serine		23.8
Histidine		26.8
Glycine		21.5
Threonine		24.8
Arginine		20.3
Alanine		39.7
Tyrosine		14.1
Methionine		6.4
Valine		42.2
Phenylalanine		36.8
Isoleucine		6.0

mixture of bovine blood and coconut pulp was fermented with *Bacillus amyloliquefaciens* for 5 days at 40°C. After fermentation, the mixtures were dried in a at 60°C in an oven for 2-3 days and then ground into flour. The composition of the blood meal is given in Table 2.

Amino acid composition: Analysis of amino acids in the blood meal was carried out using High-Performance Liquid Chromatography (HPLC) as previously described[15]. Prior to HPLC, the blood meal was subjected to liquid hydrolysis and derivatization in 5 mL 6 N HCl at 110°C for 22 h under a vacuum. After hydrolysis, the tube was cooled and the solution filtered using a 30, 0.45 µm pore size Spartan-HPLC syringe filter (GE Healthcare Life Sciences). The filtrate was diluted with water by 1:20 (v/v). HPLC was performed at 37°C with a mobile phase containing 60% acetonitrile–AccQ Tag Eluent A in a gradient system with a flow rate of 1.0 mL per minute. The fluorescence detector was set to 250 and 395 nM excitation and emission wavelengths, respectively. The injection volume was 5 mL.

Broiler growth performance: Broiler performance parameters, including final body weight (g), final weight gain (g), average daily gain (g), feed intake (kg per chick), average abdominal fat (g) and Feed Conversion Rate (FCR) were determined every week throughout the experimental period.

HDL AND LDL analysis: Blood samples for cholesterol analysis were collected without anticoagulant through the wing vena brachialis. Serum cholesterol was analyzed using an endpoint colorimetric method[16]. Briefly, cholesterol standards were prepared by diluting 20 μL of a 2 mg mL^{-1} cholesterol standard solution with 140 μL cholesterol assay buffer to prepare either a 0.25 mg mL^{-1} (colorimetric assays) or 25 μg mL^{-1} standard solution (fluorometric assays). Increasing amounts of the given standard solution (0, 4, 8, 12, 16 and 20 μL) were added to wells of a 96-well plate to produce 0 (blank), 1, 2, 3, 4 and 5 μg/well standards. For both colorimetric and fluorometric assays 50 μL of sample was used for each reaction. To separate HDL and LDL, 100 μL 2× precipitation buffer was mixed with 100 μL of the serum sample in a microcentrifuge tube and incubated for 10 min at room temperature before centrifuging at 2,000 rpm for 10 min. The resulting supernatant containing HDL was transferred to a new tube. The remaining precipitate contained the LDL fraction. To measure LDL, the samples were again centrifuged at 2,000 rpm for 10 min to remove any residual HDL supernatant. The pellet was resuspended in 200 μL PBS.

Return on investment: Return On Investment (ROI) is a gross margin concept and was obtained by subtracting the feed costs from the price of the final broiler weight.

Data analysis: Collected data were processed using Microsoft Excel. One-way single-factor analysis of variance (ANOVA) was performed using SPSS version 11[17]. Statistically significant effects were further analyzed and means were compared using Duncan's multiple range test. Statistical significance was determined at $p \leq 0.05$.

RESULTS

Amino acids in blood meal mixtures: The amino acid profiles of the rations containing different percentages of *Bacillus amyloliquefaciens*-fermented bovine blood and coconut pulp mixtures (blood meal) are shown in Table 2. Isoleucine was the first limiting amino acid followed by methionine as the second limiting amino acid.

Growth performance of broilers: Dietary substitution of blood meal for fish meal and soybean meal had significant effects ($p<0.05$) on broiler performance (Table 3). However, blood meal could only be used at up to 10% of the ration in substitution for fish meal (47%) and soybean meal (53%); negative effects on performance were seen with higher percentages of blood meal. The DMRT test results showed that, relative to control broilers, 10% blood meal was associated with a significant increase ($p<0.05$) in final body weight, final weight gain and FCR, which were 1,172, 1,035 and 1.75 g, respectively. Meanwhile, substituting 20% blood meal in the ration resulted in reduced feed intake relative to rations containing lower amounts of blood meal. Overall, 10% blood meal in the ration gave the best feed intake and showed better FCR compared to control and rations having other amounts of blood meal.

Effect of blood meal substitution on cholesterol levels: The HDL in serum cholesterol increased with increased levels of blood meal in the ration (Table 4). Meanwhile, DMRT test results showed that substitution of 10%

Table 3: Growth performance of broilers fed diets containing different percentages of blood meal

	Diets				
Growth performance parameters	A (control)	B (5%)	C (10%)	D (15%)	E (20%)
Final body weight (g)	1.103^{c}	1.163^{b}	1.172^{a}	896^{d}	618^{e}
Final weight gain (g)	962.0^{c}	1.025^{b}	1.035^{a}	757^{d}	480^{e}
Feed intake (kg per chick)	1.895^{a}	1.893^{a}	1.812^{b}	1.421^{c}	1.067^{d}
Average abdominal fat (%)	1.95^{c}	1.56^{b}	1.29^{a}	1.22^{a}	1.12^{a}
Feed convention rate (FCR)	1.97^{b}	1.85^{a}	1.75^{a}	1.90^{b}	2.23^{c}

$^{a-c}$Means with different superscripts within the same row differed significantly ($p \leq 0.05$)

Table 4: Effect of mixtures of bovine blood and coconut pulp fermented by *Bacillus amyloliquefaciens* on high density lipoprotein (HDL), low density lipoprotein (LDL) and Erythrocyte contents

	A (control)	B (5%)	C (10%	D (15%)	E (20%)
HDL (mg dL^{-1})	91.0^{d}	91.5^{cd}	92.3^{c}	95.0^{b}	102.5^{a}
LDL (mg dL^{-1})	33.5^{c}	33.0^{cd}	32.8^{d}	37.3^{b}	38.8^{a}
Erythrocyte (10^{6} mm^{-3})	2.12^{b}	2.01^{c}	2.14^{a}	1.93^{d}	1.90^{e}

$^{a-c}$Means with different superscripts within the same row differ significantly ($p \leq 0.05$)

Table 5: Return on investment relative to percentage of blood meal in broiler rations

Diet	Sale ($US)	Cost ration ($US)	ROI ($US)
A (0%)	1.63	1.12	0.51
B (5%)	1.72	0.98	0.74
C (10%)	1.74	0.81	0.92
D (15%)	1.33	0.53	0.79
E (20%)	0.92	0.40	0.51

blood meal resulted in a decrease in serum LDL from 33.5 to 32.8 mg dL^{-1} relative to control.

Return on investment: The ration containing 10% blood meal that substituted for 47% fish meal and 53% soybean meal gave the best return on investment ($0.92) that was nearly double that of the control and the highest amount of blood meal (20%) (Table 5).

DISCUSSION

Blood meal composed of a *Bacillus amyloliquefaciens*-fermented mixture of bovine blood and coconut pulp can be used as a protein source for broiler chickens. The blood meal contains 67.94% crude protein, 5.59% crude fiber, 1.10% calcium, 0.2% phosphorous, 4.41% lysine, 0.60% isoleucine, 5.90% leucine and 3,500 kcal kg^{-1}. In this study, the blood meal also contained 4.41% lysine, which was lower than that for studies by Onwudike[18] and Khawaja *et al.*[7] who reported blood meal lysine levels of 7-8%. The isoleucine content was also lower than that reported by Khawaja *et al.*[7] whose study involved blood meal containing 0.80% isoleucine. Notably, excess leucine can inhibit isoleucine absorption. The NRC[19] reported that the optimal leucine:isoleucine ratio is 1.6:1, which is in accordance with the ratio present in fishmeal. The rations used in the current study had a leucine: isoleucine ratio of 9.8:1, which is closer to fish meal than pure blood meal that has a ratio of 11:1.

Fermentation of the bovine blood and coconut pulp mixture by *Bacillus amyloliquefaciens* can produce enzymes that help balance the amino acid content. *Bacillus amyloliquefaciens* is a spore-forming, Gram-positive bacterium that produces several enzymes, including alpha-amylase, alpha acetolactate, decarboxylase, beta-glucanase, hemicellulase, maltogenic amylase, protease, xylanase, phytase, mannanase, β-mannanase, chitinase, lipase and endoglucanase[20-23]. During fermentation, these enzymes work to metabolize substrates to obtain the energy needed for bacterial metabolism and growth. In the process, the feed material is altered[24].

In the present research, substitution of 10% blood meal for fish meal and soybean meal significantly ($p<0.05$) increased the final body weight, weight gain and FCR compared to the control group. However, higher amounts of blood meal, (15 and 20%), were associated with decreased final body weight, weight gain and FCR. This result is in contrast to a previous study conducted by Onwudike[18], who reported that >3% blood meal reduced broiler weight gain, which they hypothesized was due to low sulfur and isoleucine levels. Meanwhile, Donkoh[4] also reported that weight gain in broiler chickens was reduced with higher concentrations of blood meal due to very low levels of several amino acids such isoleucine. The NRC[9] also reported that blood meal is rich in lysine but not isoleucine.

Here, increased amounts of blood meal in the rations caused decreased feed intake, likely due to an amino acid imbalance. Such imbalances were previously shown to lower feed consumption and affect livestock performance in a variety of ways, such as insufficient amounts of amino acids needed for brain function[10]. This possibility is supported by the study of Gietzen[11], showing who reported that decreased amino acid concentrations in the brain can alter animal behavior, particularly with respect to feed intake.

Serum cholesterol levels are correlated with the amount of abdominal fat, whereas, the present study showed that increased amounts of HDL and decreased LDL levels were associated with reduced abdominal fat. Rations containing 20% blood meal yielded higher HDL levels (102.5 mg dL^{-1}), whereas broilers fed the 10% blood meal ration had lower levels of both HDL and LDL (92.3 and 32.8 mg dL^{-1}, respectively). Broilers fed 10% blood meal rations also had significantly ($p<0.05$) decreased abdominal fat rather relative compare to control broilers and those fed rations with 5% blood meal. These results are consistent with a study conducted by Musa *et al.*[25] who reported a positive correlation between abdominal fat and total cholesterol and Low-Density Lipoprotein (LDL).

Dietary substitution with blood meal increased serum HDL, whereas for the 10% blood meal group, the LDL level was significantly lower than the control group. These changes may be due to the presence of *Bacillus amyloliquefaciens* acting as a probiotic. Indeed, Kalavathy *et al.*[26] reported that probiotic supplementation can increase HDL and decrease LDL, whereas Moghadam *et al.*[27] also found that supplementation of broiler diets with feed additives such probiotics decreased blood cholesterol and LDL.

Return on investment data clearly indicated that to achieve optimal broiler performance, 10% blood meal was more economical than control or other amounts of blood meal. Substitution of fish meal (47%) and soybean meal (53%) with 10% blood meal gave the best return on investment (0.92 USD) because blood meal is a less costly protein source than fish meal and soybean meal. Rations containing 10% blood meal have a balanced nutritional composition, whereas feeds that have nutrient imbalances can reduce productivity and profit. As such, reduced feed costs that do not compromise quality is an approach to increase profits[28].

This study can have implications for producing more economical broiler feed that reduces production costs. Subsequent research will be needed to achieve the optimal composition for use of blood meal in commercial broiler production.

CONCLUSION

It is concluded that rations containing 10% of a *Bacillus amyloliquefaciens* fermented mixture of bovine blood and coconut pulp can be used in place of 47% total fishmeal and 53% soybean meal to improve broiler growth performance and enhance returns on investments.

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