

# Fermentation of blood

*by* Wizna Wizna

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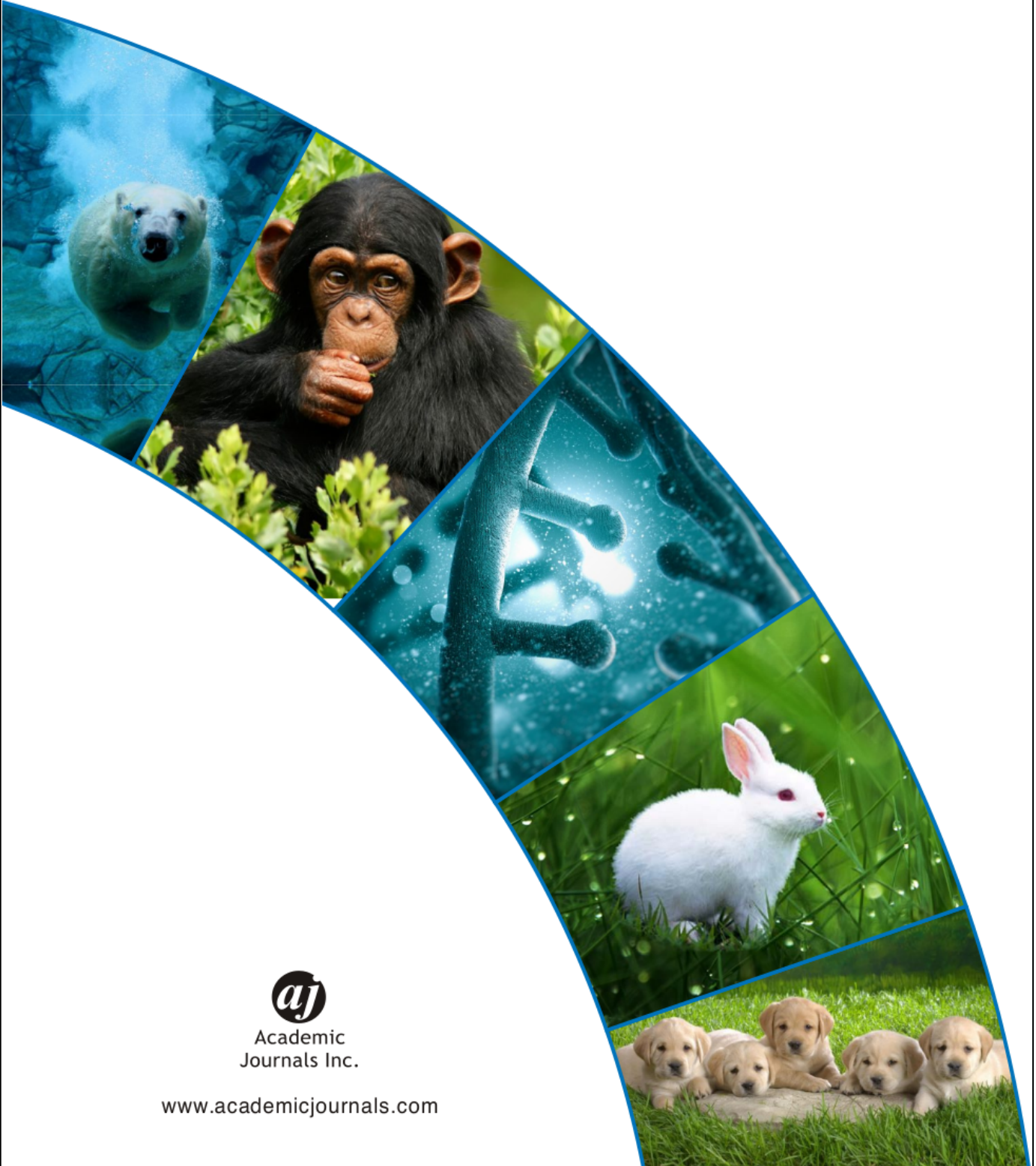
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## Research Article

# Fermentation of Blood Meal with *Bacillus amyloliquefaciens* as Broiler Feeding

R.F. Ramadhan, Wizna, Yetti Marlida and Mirzah

1

Department of Animal Nutrition and Feed Technology, Faculty of Animal Science, Andalas University, West Sumatera, Indonesia

## Abstract

2

**Objective:** The aim of this study was to find out the best combination of bovine blood and agri-industrial waste which fermented by *Bacillus amyloliquefaciens* with different fermentation time to increase the quality of blood mixtures such as protein content, fiber content, nitrogen retention, amino acid composition and enzyme activity. **Methodology:** Bovine blood and agri-industrial waste obtained from a slaughterhouse and traditional markets. The design used in this study was a Completely Randomized Design (CRD) factorial using three factors. **Results:** The results showed that there was a highly significant interaction ( $p < 0.01$ ) between blood concentrations, types of agricultural waste and fermentation time on crude protein, nitrogen retention and energy metabolism which the best results was mixtures blood (300/250 mL) and coconut pulp with fermentation time 120 h whereas, the crude protein content, nitrogen retention and enzyme activity (protease and mannanase) were 50.70 and 55.60%, protease  $37.34 \text{ U g}^{-1}$  and mannanase  $0.992 \text{ U g}^{-1}$ , respectively. Meanwhile, the mixtures of blood (300/250 mL) and PKC had the best amino acid composition. **Conclusion:** The study can be concluded that the best results were mixture of blood meal (300 mL) with coconut pulp and fermentation time 120 h.

**Key words:** Bovine blood, agri-industrial waste, nutrient content, amino acids composition

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**Corresponding Author:** R.F. Ramadhan, Department of Animal Nutrition and Feed Technology, Faculty of Animal Science, Andalas University, West Sumatera, Indonesia Tel: +6282169091298

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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

Bovine blood is a slaughterhouse waste that have a high protein and abundant availability that can be utilized as feedstuff. In 2013, the number of cattle slaughtering<sup>1</sup> in Indonesia reached 1.3 million. Bovine blood can be utilized in the form of blood meal. Rasyaf<sup>2</sup> reported blood meal containing 80% crude protein, 1.6% fat, crude fiber 1% but poor in calcium and phosphorus. Blood meal is richest source of lysine, arginine, methionine, cystine and leucine but very poor in isoleucine and contains less glycine than fishmeal<sup>3</sup>.

Meanwhile, agricultural wastes that have a nutritional value and abundant availability can also be used as animal feed such as coconut pulp, palm kernel cake and coconut cake. They have a high nutrient content, crude protein and crude fiber of coconut pulp<sup>4</sup> is CP 4.89% and CF 28.72%, palm kernel cake<sup>5</sup> is CP 15.43% and CF 21.7% and coconut cake<sup>6</sup> is CP 20% and CF 25.5%.

Utilization of blood meal and coconut pulp in poultry rations is very limited. Blood meal is not recommended for use exceeding 5% in poultry rations<sup>7</sup>, this was caused the digestibility of blood meal not efficient than fishmeal<sup>8</sup>. Crawshaw<sup>9</sup> reported that limitations of blood meal in broiler ration due to amino acid imbalances that led to the decline in broiler performance. Blood meal also contains poor isoleucine which led to a decrease in production of carcass<sup>10</sup>. Meanwhile, coconut pulp contains high fiber and poultry have not the digestive enzymes to digest crude fibre. Tilman *et al.*<sup>11</sup> said that the crude fibers can affect feed intake so it will affect the absorption of other nutrients.

The methods to improve the quality of blood meal has been widely performed. Fermentation is one of method the processing blood meal which can improve the quality of blood meal. Fermentation requires a low cost and can improved bioavailability of nutrient by increasing the digestibility of nutrients<sup>12</sup>. Fermentation is requires microorganism to digest complex compounds into simpler compound. *Bacillus amyloliquefaciens* is a microorganism that can be used as inoculum in the fermentation. It has produced many enzyme such as  $\alpha$ -amylase,  $\alpha$ -acetolactase, decarboxylase,  $\beta$ -glucanase, hemicellulase, maltogenic amylase, protease, xylanase<sup>13</sup>, phytase<sup>14</sup>, mannanase<sup>15</sup>,  $\beta$ -mannanase<sup>16</sup>, chitinase<sup>17</sup>, lipase<sup>18</sup> and endoglucanase<sup>19</sup>. The present study is designed to improved the quality of blood meal through the adsorbation of bovine blood to agricultural waste and fermented with *Bacillus amyloliquefaciens* for broiler feeding.

## MATERIALS AND METHODS

**Bovine blood:** Fresh bovine blood was collected from slaughtering house in Padang, West Sumatera, Indonesia.

**Agricultural wastes:** Agricultural wastes such as Coconut Pulp (CP), Palm Kernel Cake (PKC) and Coconut Cake (CC) collected from agriculture industry and traditional market in Padang, West Sumatera, Indonesia.

**Research methods:** The method used in this study was a Completely Randomized Design (CRD) factorial using three factors, factor A consists of three type of agricultural waste A1: Coconut pulp, A2: Palm kernel cake and A3: Coconut cake. Factor B is the concentration of blood B1: 200/250 mL, B2: 250/250 mL and B3: 300/250 mL of agricultural waste. Factor C is time fermentation, C1: 24 h, C2: 72 h and C3: 120 h. All the treatments were three replicate. Differences between treatments were tested by Duncan's Multiple Range Test (DMRT).

**Research procedures:** The fresh blood was weighed according to the desired ratio and mixed with agricultural waste (coconut pulp, palm kernel cake and coconut cake) and then inoculated *Bacillus myloliquefaciens* (3%). The mixture is fermented for 24, 72 and 120 h. After fermented that was dried in a oven at low temperature 60°C for 2-3 days and then ground into flour. The mixture of bovine blood and agricultural waste at the following ratios:

- |                  |   |
|------------------|---|
| Coconut pulp     | = 250 mL of coconut pulp and 200 mL of bovine blood     |
|                  | = 250 mL of coconut pulp and 250 mL of bovine blood     |
|                  | = 250 mL of coconut pulp and 300 mL of bovine blood     |
| Palm kernel cake | = 250 mL of palm kernel cake and 200 mL of bovine blood |
|                  | = 250 mL of palm kernel cake and 250 mL of bovine blood |
|                  | = 250 mL of palm kernel cake and 300 mL of bovine blood |
| Coconut cake     | = 250 mL of coconut cake and 200 mL of bovine blood     |
|                  | = 250 mL of coconut cake and 250 mL of bovine blood     |
|                  | = 250 mL of coconut cake and 300 mL of bovine blood     |



## Parameters

**Chemical analysis:** Blood meal sample was analyzed in laboratory for proximate constituents<sup>20</sup>. Proximate analyzed including crude protein and crude fibre.

**Nitrogen balance assay:** To measure the nitrogen retained by the broiler using the method of Sibbald<sup>21</sup>, 6 weeks old chickens were placed in metabolic cages. Feces was collected, weighed and dried at 60°C for 24 h and then ground into flour. The faecal sample was analyzed in laboratory for proximate constituents such crude nitrogen.

**Amino acid analysis:** Analysis of amino acid from blood meal used High Performance Liquid Chromatography (HPLC) method.

## Enzyme activity

**Protease:** To measure protease activity using the method of Anson<sup>22</sup>. Approximately 2.5 mL casein 1% put into test tube and added 1.5 mL buffer phosphate pH 7, incubation for 10 min at 37°C. Then, enter 1 mL crude enzyme and continue incubation 10 min at 37°C. Stop the reaction of the enzyme activity by adding 5 mL TCA 20% while shaking. Chill in ice water for 30 min until the protein coagulates. Then, centrifuge for 10 min at a speed of 5,000 rpm, take 2 mL of supernatant and added 5 mL of 0.5 N NaOH and 0.5 mL of folin reagent ciocalteau allow 10 min and measure the absorbance at a wavelength of 440 nm.

**Mannanase:** To measure mannanase activity using the method of Nelson<sup>23</sup>. Test of enzyme activity mananase carried out by taking 1 mL of crude enzyme and added 1 mL substrate (0.1% CMC in buffer phosphate 0:01 M pH 7) and then mixed using vortex, reacted in a shaker water bath for 30 min at 60°C. Furthermore, the reaction is stopped by placing in boiling water for 5 min. Then, the hydrolysis products centrifuge with speed 5000 rpm for 30 min. About 1 mL hydrolyzate, added reagent Nelson A and Nelson B (1 mL), cooked in boiling water for 5 min, add the reagent phosphomolibidate (1 mL), add distilled water (7 mL) then glucose released is read using a spectrophotometer at a wavelength of 575 nm.

## RESULTS AND DISCUSSION

**Chemical composition:** The chemical composition of the mixtures bovine blood and agricultural waste which fermented by *Bacillus amyloliquefaciens* for 24, 72 and 120 h shown in Table 1 and 2.

Table 1: Crude protein of the mixture of bovine blood and agricultural waste

Agricultural waste	Fermentation time (h)	Level of blood (mL)		
		200	250	300
CP	24	37.79 <sup>a</sup>	44.85 <sup>d</sup>	39.42 <sup>c</sup>
	72	39.26 <sup>c</sup>	43.75 <sup>de</sup>	45.31 <sup>d</sup>
	120	43.79 <sup>d</sup>	48.40 <sup>e</sup>	50.70 <sup>a</sup>
PKC	24	32.63 <sup>a</sup>	40.09 <sup>c</sup>	44.39 <sup>d</sup>
	72	34.85 <sup>f</sup>	41.57 <sup>c</sup>	45.56 <sup>cd</sup>
	120	40.40 <sup>c</sup>	44.25 <sup>d</sup>	47.48 <sup>bc</sup>
CC	24	36.70 <sup>c</sup>	37.98 <sup>e</sup>	39.75 <sup>e</sup>
	72	39.69 <sup>c</sup>	39.17 <sup>c</sup>	42.82 <sup>de</sup>
	120	40.76 <sup>c</sup>	41.63 <sup>c</sup>	44.90 <sup>d</sup>

Different letters in the same row and column indicated significant differences ( $p < 0.05$ )

Table 2: Crude fibre of the mixture of bovine blood and agricultural waste

Agricultural waste	Level of blood (mL)	Fermentation time		
		1	3	5
CP	200	14.72 <sup>bc</sup>	11.78 <sup>f</sup>	9.48 <sup>j</sup>
	250	9.29 <sup>ij</sup>	8.59	8.28 <sup>j</sup>
	300	8.29 <sup>j</sup>	7.45	6.78 <sup>j</sup>
PKC	200	7.67 <sup>i</sup>	6.99	5.55 <sup>j</sup>
	250	6.85 <sup>i</sup>	5.75	5.33 <sup>j</sup>
	300	6.61 <sup>i</sup>	6.26	5.30 <sup>j</sup>
CC	200	17.86 <sup>a</sup>	15.05 <sup>b</sup>	14.05 <sup>c</sup>
	250	13.87 <sup>cd</sup>	11.13 <sup>g</sup>	10.42 <sup>h</sup>
	300	12.00 <sup>e</sup>	9.44 <sup>j</sup>	8.44 <sup>j</sup>

Different letters in the same row and column indicated significant differences ( $p < 0.01$ )

Table 1 shows that there were interaction between types of agricultural waste, blood concentration and fermentation time. The DMRT test results shows that mixtures blood (300 mL) with CP and fermentation time for 120 h had significant ( $p < 0.05$ ) increased crude protein from 43.79-50.70% compared to other treatments. This crude protein of the mixture increased with increasing level of blood and fermentation time. Onyimonyi and Ugwu<sup>24</sup> reported that increasing the proportion of blood meal in diet will be increase crude protein. That was caused the bovine blood contains protein<sup>25</sup> more than 80%. The increase in crude protein can also be caused by the activities of *Bacillus amyloliquefaciens*. Fermentation with bacteria, fungi or yeast which are optimal sources of protein could be increase in crude protein<sup>26</sup>. Fazi *et al.*<sup>27</sup> also reported that increase in protein could be decreased carbohydrate content after fermentation.

Table 2 shows that the crude fibre in the mixture bovine blood and agricultural waste. The DMRT test results show that crude fiber from mixture of bovine blood (300 mL) with CP and PKC not significant ( $p > 0.05$ ) different. Meanwhile, there was highly significant ( $p < 0.01$ ) difference compared CC. However, coconut pulp was decreased crude fiber more high from 14.72-6.78% than palm kernel cake and coconut cake.

Table 3: Amino acid composition

Amino acid	Blood+CP	Blood+PKC	Blood+CC	Blood meal	Fishmeal
Histidine	2.72	1.43	1.73	5.14	1.45
Threonine	2.54	1.51	1.78	3.76	2.31
Arginine	2.24	2.09	2.36	3.75	3.82
Isoleucine	0.65	0.67	0.69	0.97	2.66
Leucine	7.25	4.24	4.85	10.82	4.48
Lysine	5.10	2.73	3.04	7.45	4.72

The crude fibre component was decreased that if the level of blood was increased (300 mL). The crude fiber component decreased when the bovine blood fraction increased. It was caused blood meal contains 1% crude fibre<sup>2</sup>. It also caused to the enzyme activity of *Bacillus amyloliquefaciens*. Wizna *et al.*<sup>28</sup> reported *Bacillus amyloliquefaciens* is cellulolytic bacteria and be able to degrade crude fiber and produce some extracellular enzyme that is cellulolytic and hemicellulase.

**Amino acid composition:** Table 3 shows that the ratio of isoleucine:leucine and lysine:arginine from a mixtures of bovine blood and agri-industrial waste.

Ratio of leucine:isoleucine from BBPKC had a ratio approaching fishmeal compared to other mixtures. The DMRT test results show that ratio of leucine:isoleucine from BBPKC was 6:1, there was highly significant ( $p < 0.01$ ) difference compared ratio of the BBCP and BBCC was 11:1 and 7:1, respectively. Blood meal contains less isoleucine and excess leucine. The excess of leucine can be inhibit the absorption of isoleucine which resulted less isoleucine. National Research Council<sup>29</sup> reported a ratio of leucine:isoleucine from fishmeal was 1.6:1. The ratio of leucine:isoleucine from fishmeal is as a comparison, this is caused the blood meal being fed to livestock as a replacement for fishmeal. The ratio between the amino acids that are interrelated affect the growth of livestock as leucine and isoleucine. Widodo<sup>30</sup> reported that amino acid deficiency can be corrected by an amino acid that is the antagonism of the amino acid itself, when leucine increased and lead to growth inhibition can be neutralized by increasing isoleucine and valine.

Meanwhile, ratio of lysine:arginine from BBPKC and BBCC had the same value was 1.3:1. This ratio is approaching ratio of lysine:arginine from fishmeal. Ratio of lysine:arginine from fishmeal<sup>29</sup> was 1.2:1. The DMRT test results shows that ratio of lysine:arginine from BBPKC was 1.3:1, there was highly significant ( $p < 0.01$ ) difference compared ratio of BBCP and non-significant ( $p > 0.05$ ) difference compared BBCC was 2.2:1 and 1.3:1, respectively. Excess lysine will inhibit the absorption of arginine and can cause poisoning. Poisoning occurs when one of the amino acid exceeds of needs<sup>30</sup>.

Harper<sup>31</sup> defined amino acid imbalances as resulting from additions to a low protein diet of one or more amino acids, other than the one that is growth limiting in amounts that individually are not toxic. They cause depressions in food intake and growth that are readily prevented by a supplement of the growth-limiting amino acid. Harper<sup>32</sup> categorized the adverse interaction of disproportionate balance of amino acids as imbalances, antagonisms and toxicities. Cieslak and Benevenga<sup>33</sup> said that amino acid imbalance can result in reduced feed consumption which resulting in lower performance of livestock, this is caused the amino acids in the plasma is reduced that amino acids carried to the brain slightly. Hier *et al.*<sup>34</sup> observed growth depression of rats fed a diet containing excessive concentrations of individual amino acids and attributed the effects to amino acid imbalances. They also suggested that an amino acid imbalance induced by excessive amounts of other amino acids in the diet increased the requirement for the most limiting amino acid.

Samadi<sup>35</sup> described formulation of animal feed should also be noted the balance of amino acids, especially the essential amino acids. Samadi<sup>35</sup> also reported that formulation of essential amino acids that are not appropriate either excess or less will result in an imbalance of amino acids, antagonists and also be toxic to livestock. Harper<sup>32</sup> also categorized the adverse interaction of disproportionate balance of amino acids as imbalances, antagonisms and toxicities. The maximum productivity of poultry will be achieved when the poultry got feed which a balanced amino acids. Amino acids balanced can be obtained by way mixing various of protein sources in the rations. Increased of amino acids balanced from BBPKC is caused donation of protein from palm kernel cake and microba protein from *Bacillus amyloliquefaciens*.

**Nitrogen retention:** The nitrogen retention of mixtures bovine blood and agricultural waste by fermentation with *Bacillus amyloliquefaciens* are shown in Table 4.

Table 3 shows that there were interaction between types of agricultural waste, blood concentration and fermentation time. The DMRT test results shows that the mixture of bovine blood (300 mL) with coconut pulp and fermentation time 120 h have significant ( $p < 0.01$ ) difference than other mixtures. Mixture of bovine blood (300 mL) with coconut pulp and fermentation time 120 h gave the best nitrogen retention compared other mixtures. Balogun<sup>36</sup> reported that addition of blood meal to pig diet resulted in higher percentage apparent nitrogen retention. Meanwhile, Seifdavati *et al.*<sup>37</sup> reported that nitrogen retention in broiler chickens didn't influence by substitution ratio of broiler feed.

Table 4: Nitrogen retention

Agricultural waste	Level of blood (mL)	Fermentation time (h)		
		24	72	120
CP	200	28.21 <sup>m</sup>	24.84 <sup>n</sup>	30.75 <sup>m</sup>
	250	34.50 <sup>k</sup>	45.42 <sup>de</sup>	46.84 <sup>cd</sup>
	300	24.24 <sup>n</sup>	44.12 <sup>def</sup>	55.60 <sup>a</sup>
PKC	200	30.75 <sup>m</sup>	34.10 <sup>k</sup>	38.59 <sup>hi</sup>
	250	41.58 <sup>gh</sup>	43.23 <sup>efg</sup>	46.26 <sup>cde</sup>
	300	33.02 <sup>kl</sup>	40.39 <sup>gh</sup>	49.25 <sup>c</sup>
CC	200	34.26 <sup>k</sup>	36.68 <sup>i</sup>	40.25 <sup>ghi</sup>
	250	45.41 <sup>de</sup>	46.80 <sup>d</sup>	48.67 <sup>c</sup>
	300	30.19 <sup>m</sup>	44.58 <sup>def</sup>	52.59 <sup>b</sup>

Different letters in the same row and column indicated significant differences (p<0.01)

Table 5: Protease activity

Agricultural waste	Fermentation time (h)	Level of blood (mL)		
		200	250	300
CP	24	18.30 <sup>a</sup>	19.02 <sup>ik</sup>	18.70 <sup>ikm</sup>
	72	21.43 <sup>de</sup>	21.76 <sup>de</sup>	20.64 <sup>efg</sup>
	120	20.23 <sup>gh</sup>	34.73 <sup>b</sup>	37.34 <sup>a</sup>
PKC	24	12.51 <sup>n</sup>	22.45 <sup>d</sup>	27.56 <sup>c</sup>
	72	21.33 <sup>def</sup>	21.02 <sup>ef</sup>	21.20 <sup>ef</sup>
	120	18.84 <sup>k</sup>	19.60 <sup>ghj</sup>	18.97 <sup>ik</sup>
CC	24	9.74 <sup>o</sup>	16.22 <sup>m</sup>	20.58 <sup>efg</sup>
	72	20.77 <sup>efg</sup>	21.11 <sup>ef</sup>	20.13 <sup>ghi</sup>
	120	19.14 <sup>hik</sup>	18.60 <sup>hkm</sup>	18.79 <sup>ik</sup>

Different letters in the same row and column indicated significant differences (p<0.01)

**Enzyme activity:** The protease activity of mixtures bovine blood and agricultural waste by fermentation with *Bacillus amyloliquefaciens* are shown in Table 5.

Table 5 shows that the best protease activity on the mixture of bovine blood (300 mL<sup>-1</sup>) with coconut pulp and fermentation time 120 h (37.34 U mL<sup>-1</sup>) which was much higher than the protease activities from other mixtures. In this study, the use of waste blood of cattle slaughtering and coconut pulp from agro-industrial waste provides the highest protease activity. Mussatto *et al.*<sup>38</sup> reported that the agro-industrial wastes can be used in processes of fermentation as solid support, carbon, nitrogen and/or mineral sources which would allow obtaining more economical fermentation processes avoiding the use of expensive chemical components in the media fermentation.

The mannanase activity of mixtures bovine blood and agricultural waste by fermentation with *Bacillus amyloliquefaciens* are shown in Table 6.

Table 6 shows that mannanase activity on the mixture of bovine blood and agro-industrial waste with different fermentation time. In this study, the use of waste blood (300 mL) of cattle slaughtering and coconut pulp from agro-industrial waste provides the highest protease activity (0.992 U mL<sup>-1</sup>). In this study, mannanase activity lower than protease activity, this was caused medium fermentation

Table 6: Mannanase activity

Agricultural waste	Fermentation time (h)	Level of blood (mL)		
		200	250	300
CP	24	0.909 <sup>no</sup>	0.943 <sup>hi</sup>	0.955 <sup>ef</sup>
	72	0.946 <sup>hi</sup>	0.955 <sup>ef</sup>	0.960 <sup>de</sup>
	120	0.937 <sup>gh</sup>	0.950 <sup>gh</sup>	0.992 <sup>a</sup>
	24	0.904 <sup>p</sup>	0.917 <sup>mn</sup>	0.943 <sup>hi</sup>
PKC	72	0.906 <sup>p</sup>	0.917 <sup>mn</sup>	0.929 <sup>k</sup>
	120	0.942 <sup>i</sup>	0.953 <sup>efg</sup>	0.964 <sup>cd</sup>
	24	0.925 <sup>s</sup>	0.923 <sup>kl</sup>	0.937 <sup>l</sup>
CC	72	0.905 <sup>p</sup>	0.915 <sup>mn</sup>	0.922 <sup>klm</sup>
	120	0.976 <sup>p</sup>	0.950	0.971 <sup>bc</sup>

Different letters in the same row and column indicated significant differences (p<0.01)

consist high protein and lower crude fiber. Microorganism produce a product according growth medium. Mabrouk and Ahwany<sup>16</sup> reported that potato peels were found to be the most suitable substrate for mannanase production. Agro-industrial waste are available in large amounts and they have been used for the production of several enzymes<sup>39</sup>. Mabrouk and El Ahwany<sup>16</sup> also reported that using agro-industrial waste that were potato peels and ammonium nitrate as carbon and nitrogen source whereas mannanase activity was 33.8 U mL<sup>-1</sup>.

Fermentation time 120 h gave the highest protease and mannanase enzyme, the longer fermentation time show high enzyme activity. Lazim *et al.*<sup>40</sup> reported protease activity was detected on 5th day of incubation. De Azeredo *et al.*<sup>41</sup> reported that maximal protease activity by *streptomyces* sp. 594 was attained after the 4th day, reaching the level of 15.50 U g<sup>-1</sup>. Meanwhile, Yang and Wang<sup>42</sup> in the case of *S. rimosus*, maximal protease activity (15.80 U g<sup>-1</sup>) was attained on 9th day. *Bacillus* produces extracellular protease during late exponential phase<sup>43</sup>. Sexena and Singh<sup>44</sup> reported that the maximum level of protease was produced after 72 h. After it, the enzyme activity decreased considerably.

**CONCLUSION**

The mixture of bovine blood (300 mL) with coconut pulp which fermented for 120 h were the best combination in crude protein, crude fiber, nitrogen retention, protease activity and mannanase activity.

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