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Fermented Feed to Improve the Performance and Immunity of Indigenous Chickens under Free-Range Rearing

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ABSTRACT

The purpose of this study was to evaluate the use of fermented feed on the growth performance and immunity of Thai indigenous chicken rearing under free-ranging. Sixty 35-day-old Thai indigenous chickens (*Pradu hangdum*) were divided into three groups with four replicates on five birds: a control group receiving a dry diet, and two experimental groups receiving 25% and 50% fermented feed, respectively. The fermented feed was prepared by adding 1% Thua-nao to a basal diet, then filling tap water at a ratio of 1:1.5 and incubating at 37°C for 24 hours. The chickens were raised in a free-range system until they reached 98 days of age. It was found that the feed conversion ratio of chickens fed fermented feed was better than the control group ($P<0.05$). The AST was decreased in chickens fed fermented feed ($P<0.05$). The serum IgY of chickens fed 50% fermented feed was the highest, while serum IgM was not significantly different among the group. The staining intensity of IgA using 3,3'-diaminobenzidine was highest in chickens fed 50% fermented feed ($P<0.05$). The defeathered and eviscerated carcasses of chickens fed fermented feed showed higher values than the control ($P<0.05$), while most of the gastrointestinal organ weights of chickens fed fermented feed were lower than the control ($P<0.05$). Therefore, feeding 25-50% fermented feed enhances the growth performance and immunity of Thai indigenous chickens under free-range rearing.

Key words: Fermented feed, Free-rang rearing, Immunity, Thai indigenous chicken, Thua-nao

INTRODUCTION

Nowadays, people are concerned about food safety. This includes not only the final product but also the raising of animals without antibiotics and minimizing stress. Free-ranging chicken farming provides access to outdoor rearing, allowing birds to express their normal behavior. However, there have been reports that chickens reared under free-ranging conditions may be susceptible to pathogenic bacteria and intestinal parasites (Sherwin et al. 2013; Tomza-Marciniak et al. 2014).

Fermented feed is considered acceptable and beneficial for animals. Several studies have reported the effectiveness of fermented feed, such as improved growth performance, enhanced carcass, and increased immunity in chickens (Engberg et al. 2009; Naji et al. 2016; Zhang et al. 2016; Zhu et al. 2020; Li et al. 2022; Palupi et al. 2023; Zhu et al. 2023). The quality of fermented feed is influenced by a variety of factors, including the microorganism population during the fermentation process (Missotten et al. 2015). Fermented feed containing *B. subtilis* is widely utilized in livestock and poultry production, recognized as a safe and effective method for enhancing the nutritional quality of animal feed. *B. subtilis* is a naturally occurring bacterium used extensively in the fermentation of various food and feed products.

Thua-nao is a local food in the northern region of Thailand. It has been reported that microbes playing an important role in its fermentation include *Bacillus sp*. (mainly *B.subtilis*) (Chukeatirote et al. 2010). These microbes produce protein-digesting enzymes, resulting in the formation of numerous peptides (Chukeatirote 2015). Adding Thua-nao with soybean meal before fermentation and use as a feed ingredient in the diet of tilapia enhances survival rates (Phinyo et al. 2024).

The IgA, IgY, and IgM comprise the main class of immunoglobulin and have the adaptive humoral immunity of chickens. They play a role in protecting the body from pathogenic invasion. There are several reports that the immunoglobulin increases in chickens fed fermented feed

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with *B.subtilis* (Guo et al. 2021; Abeddargahi et al. 2022; Chen et al. 2023; Wang et al. 2023). Previous research has demonstrated that fermented feed enhances intestinal health by balancing gut microbiota (Zhou et al. 2023). There was limited information about how fermented unconventional feed affected local gut immunity. Therefore, we examined the use of fermented feed with Thua-nao, a source of *B. subtilis*, on growth performance, blood profiles, and immunity of Thai Indigenous chickens (Pradu handgun) under free-range rearing.

MATERIALS AND METHODS

Ethical statement

The protocol for the feeding trials and sample collection was approved by the Ethics Committee of Maejo University, Thailand (MACUC 032P/2564).

Thua nao and fermented feed

Dried soybeans were soaked in tap water for 12h and then cooked until soft (approximately 2-3h). After removing excess water, the cooked soybeans were incubated at 37°C for 48h, resulting in Thua Nao. The fermented feed was prepared by combining one kilogram of basal diet with 1.5 liters of tap water and 1% Thua Nao. The mixture was then fermented at 37°C for 24h.

Chicken, diet, and experimental design

Initially, one hundred and four 1-day-old Thai native chicks (*Pradu hangdum*) were raised in a floor pen with an electrical brooder for the first 7 days, followed by continuous rearing until day 35. On day 35, chickens with similar body weights were divided into three groups: a control group receiving a dry diet and two experimental groups receiving 25 and 50% fermented feed. Each group comprised four replicates, with five birds per replicate. The feed formulation is displayed in Table 1. The chickens were raised in a free-range system, which included indoor housing (1 bird per 1.2m²) for feeders and water dispensers and an outdoor area (1 bird per 3m²). The chicks had continuous access to both feed and water throughout the study. Light was provided for 16 hours/day, while feed intake was measured daily, and body weight gain was measured weekly on a pen basis until they reached 98 days of age.

Table 1: Feed formulation in this experiment

Ingredient, %	35-42 days of age	43-98 days of age
Maize	61.00	66.00
Soybean meal (45%CP)	25.00	22.00
Fish meal (65%CP)	6.00	4.10
Palm oil	5.50	5.20
Dicalcium phosphate	1.55	1.95
Sodium chloride	0.25	0.12
DL-methionine	0.16	0.05
L-Lysine	0.05	0.08
Vitamin premix[1]	0.50	0.50
Calculated composition		
ME, kcal/kg	3200	3200.00
CP, %	20.00	18.00

[1]Provided per kg diet: vitamin A, 10000 IU; vitamin D3, 2000 IU; vitamin E, 11 IU; vitamin K3, 2mg; vitamin B1, 2.5mg; vitamin B2, 5mg; vitamin B6, 3mg; vitamin B12, 0.03mg; pantothenic acid, 10mg; nicothenic acid, 25mg; biotin, 0.12mg; folic acid, 1.5mg; vitamin C, 7.5mg.

Blood collection

At 98 days of age, three birds from each replicate (12 birds/group) were selected for blood collection. Blood was collected from the wing vein, put into anticoagulation tubes, and kept at 4°C. Subsequently, the blood was determined with complete blood count parameters such as packed cell volume (PCV), red blood cell count (RBC), hemoglobin, and white blood cell count (WBC). Additionally, blood was collected into activated coagulation tubes, then centrifuged to get the serum and kept in a freezer at -20°C.

Visceral organ weight and Carcass yield

Thirteen birds per group were randomly selected from each replication to determine visceral organ weight and carcass yield.

Immunoglobulin analysis

The concentrations of serum IgY and IgM were determined using commercial enzyme-linked immunosorbent assay kits, and the protocol was carried out following company guidelines (IgY, ab157693; IgM, ab157692). The sample was determined in duplicate using a microplate reader (BioTek Synergy H1M) at OD 450nm.

Immunohistochemistry

At 98 days of age, 4 birds from each replicate of each treatment were used for intestinal sampling. The birds were killed by decapitation, after which both carotid arteries and jugular veins were cut. Subsequently, the gross small intestine was removed and immersed with 10% paraformaldehyde in PBS (pH 7.4), then each segment was collected. The tissue segment was embedded in paraffin (paraplast®). The duodenal sections were cut at 5µm, deparaffinized, and dehydrated. Then, the slides were immersed in sodium citrate buffer and heated in a microwave for antigen retrieval. The sections were incubated with 3% H_2O_2 for 30min. After that, the tissue sections were incubated with 10% bovine serum albumin (A7906, Sigma-Aldrich) in PBS for 1h in a moist chamber at room temperature. The primary antibody (Goat Anti-chicken IgA H&L, HRP) (AB 112847, Abcam Plc, Cambridge, UK) was applied at 1:100 dilution in a moist chamber at room temperature for 1h. After washing two times with PBS, the sections were treated with 3,3'-diaminobenzidine (DAB) solution containing H_2O_2 (AB 64238, Abcam Plc, Cambridge, UK) for 30min and counterstained with hematoxylin. The samples were observed and photographed using a light microscope (Olympus BX53, Tokyo, Japan) with 100x magnification and the same light intensity (5 images/slides). The photographs were quantified to evaluate the DAB area using ImageJ/Fiji software. Briefly, the images were changed into 8-bit, and then thresholding was used to segment and measure the area of DAB staining.

Statistical analyses

The data, such as growth performance, blood profiles, and relative carcass and visceral organ weight, were analyzed by one-way analysis of variance (ANOVA). Statistical significance was determined at the $P<0.05$ level due to group differences and separated by Duncan using IBM SPSS Statistics.

RESULTS

Growth performance

The final body weight and weight gain of chickens fed with 25 and 50% fermented feed tended to increase compared to the control group (P=0.098, P=0.084), as shown in Table 2. In addition, the feed conversion ratio of chickens fed with fermented feed was significantly (P<0.05) greater than that of the control group (Table 2).

Hematology and serum biochemistry

The PCV and hemoglobin levels in chickens fed with 50% fermented feed tended to increase (P=0.069, 0.062) compared to the other groups (Table 3). At the same time, there were no significant differences in RBC and WBC levels among the groups. Chickens fed with 25% fermented feed exhibited the highest lymphocyte count (P<0.05), and there was a tendency (P=0.069) for monocytes to increase in the same group. Additionally, the H:L ratio in chickens fed with 25% fermented feed tended (P=0.077) to be the lowest among the groups. The chickens fed fermented feed had significantly lower AST compared to the control group (P<0.05), while there was no significant difference in ALT among the groups (Table 3).

Table 2: The growth performance of chicken fed different level of fermented feed

	Level of fermented feed			*SEM*	P-value
	0 (control)	25%	50%		
Initial body weight (g)	370.25	369.29	354.40	6.13	0.542
Final body weight (g)	1250.37	1406.89	1406.00	45.99	0.098
Feed intake (g)	3238.39	3140.47	3315.93	52.02	0.425
Body weight gain (g)	880.13	1037.60	1051.60	36.05	0.084
Feed conversion ratio	3.70[b]	3.11[a]	3.17[a]	0.11	0.045

SEM, Standard error of mean. [a–b] Values in row followed by different letters differ (P<0.05).

Table 3: The hematology and serum biochemistry of chicken fed different level of fermented feed

Parameter	Level of fermented feed			SEM	P-value
	0 (control)	25%	50%		
Hematology					
PCV ,%	29.14	28.14	30.53	0.43	0.069
RBC, 10^6/µL	2.42	2.36	2.55	0.04	0.144
Hemoglobin, g/dL	9.77	9.47	10.28	0.21	0.062
WBC, 10^3/µL	38.80	42.33	33.143	2.28	0.147
Heterophil, %	35.21	25.86	29.07	1.69	0.072
Heterophil,10^3/µL	14.48	10.70	9.91	1.10	0.199
Eosinophil, %	1.00	1.00	1.67	0.16	0.627
Eosinophil, 10^3/µL	0.44	0.45	0.53	0.05	0.824
Lymphocytes, %	62.86	71.57	68.67	1.64	0.089
Lymphocytes, 10^3/µL	23.62[b]	30.48[a]	22.48[b]	1.33	0.027
Monocytes, %	1.71	2.43	1.93	0.17	0.216
Monocytes, 10^3/µL	0.61	1.08	0.66	0.09	0.069
H:L ratio	0.63	0.37	0.47	0.05	0.077
Serum chemistry					
ALT, U/L	6.67	5.60	5.11	0.25	0.140
AST, U/L	212.33[a]	159.20[b]	159.56[b]	7.28	0.035

PCV, Packed cell volume; RBC, Red blood cell; WBC, White blood cell; H:L, Heterophil:Lymphocytes; ALT, Alanine transaminase; AST, Aspartate aminotransferase; SEM, Standard error of mean; [a–b] Values in row followed by different letters differ (P<0.05).

IgY, IgM and IgA

The IgY and IgM in serum are displayed in Fig. 1. The serum IgY of chickens fed 50% fermented feed showed the highest value (P<0.05). However, there was no significant difference in serum IgM among the groups. For the IgA, we used the immunohistochemistry technique and ImageJ/Fuji software to evaluate the duodenal area of IgA staining. It was found that chickens fed 50%FF had the highest value (Fig. 2).

Relative carcass and visceral organ weight

The de-feathered and eviscerated carcass percentage of chickens fed 25-50% fermented feed was significantly increased compared to the control. In contrast, the gastrointestinal weights of chickens, such as proventriculus, duodenum, and jejunum, decreased with increasing fermented feed (Table 4).

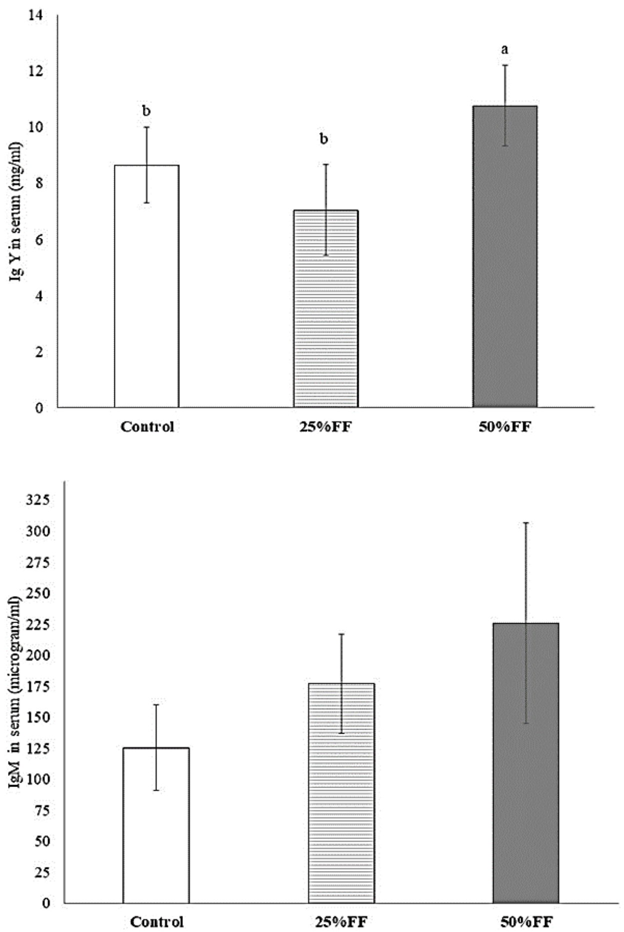

Fig. 1: The IgY (upper) and IgM (lower) levels varied in chickens fed fermented feed (FF) at different levels. Bars (mean±SD) marked with different letters are significantly different at P<0.05.

Table 4: The relative carcass and visceral organ weight of chicken fed different levels of fermented feed

Parameter	Level of fermented feed			SEM	P-value
	0 (control)	25%	50%		
%Defeathered and eviscerated carcass*	78.12[b]	81.58[a]	82.71[a]	0.71	0.020
Carcass (g/100g BW)					
Breast	13.74	15.41	14.85	0.31	0.130
Wings	9.30	9.57	9.82	0.16	0.619
Drum-sticks and thigh	24.24	24.36	24.27	0.33	0.988
Liver with gallbladder	2.20	2.01	1.99	0.05	0.114
Proventriculus	0.49[a]	0.38[b]	0.36[b]	0.02	0.044
Gizzard	3.15	2.69	2.93	0.09	0.111
Spleen	0.35	0.24	0.30	0.02	0.078
Duodenum	0.96[a]	0.68[b]	0.47[c]	0.05	0.000
Jejunum	1.25[a]	1.24[a]	0.84[b]	0.06	0.005
Ileum	0.87	0.93	0.72	0.07	0.187

BW, Body weight; SEM, Standard error of mean. [a–b] Values in row followed by different letters differ (P<0.05).

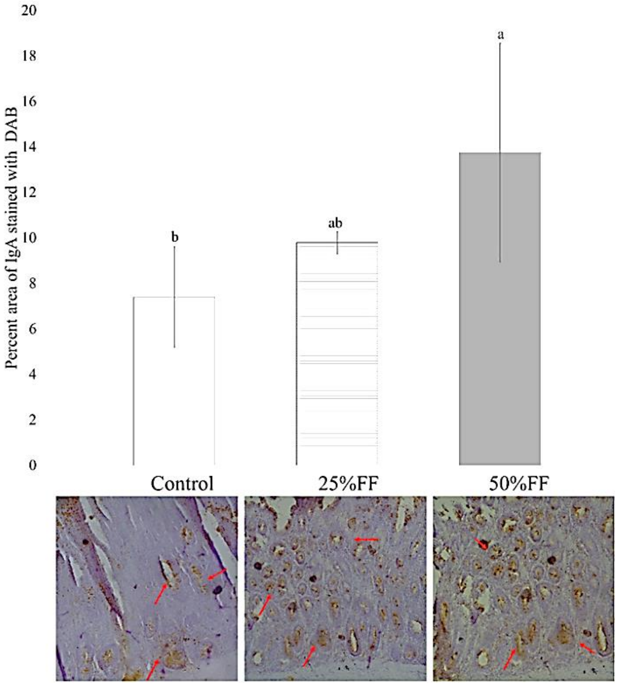

Fig. 2: Percent area of IgA stained with DAB in chickens fed fermented feed (FF) at varied levels and a representative picture of IgA in duodenal segment staining with DAB shown in brown color (red arrow). Data expressed as mean±SD. Bars marked with different letters are significantly different at P<0.05.

DISCUSSION

Currently, fermented feed improves performance, such as body weight gain and FCR, in Thai native chickens reared under a free-ranging system. These results agreed with previous studies that reported supplying fermented feed enhanced growth performance of animal (Li et al. 2022; Engberg et al. 2009; Missotten et al. 2015; Naji et al. 2016; Zhu et al. 2023). On the other hand, the broilers that consumed fermented feed showed no significance compared to the control group (Missotten et al. 2013). Fermented feed contains microorganisms that aid in breaking down large nutrients into smaller ones, resulting in improved digestion when fed to chickens. At present, the pH value of fermented feed ranges from 4.60-4.70, which decreases, and contains lactic acid as well as lactic acid bacteria or LAB (Khonyoung et al. 2024), thus promoting the growth of beneficial microorganisms in the gastrointestinal tract of chickens.

Hematology and serum biochemistry are indicators used to evaluate animal health. In the present study, the number of lymphocytes was higher in chickens fed 25% fermented feed than in those fed the control and 50% fermented feed. Furthermore, the H:L ratio of chickens fed fermented feed tended to be lower than that of chickens in the control group. Increased lymphocytes were found in chickens fed fermented red ginseng extract (Ao et al. 2011). The H:L ratio is an indicator of chicken stress (Gross et al. 1980). Vicuña et al. (2015) reported that an increased H:L ratio was found in chickens treated with dexamethasone, a synthetic glucocorticoid. Serum ALT and AST are enzymes that indicate liver and tissue injury. Values higher than normal indicate the presence of abnormalities in the tissues (Tokofai et al. 2021). Saleh et al. (2021) reported that the use of fermented feed with *B.licheniformis* did not affect ALT or AST values compared to chickens in the control group. However, the results of the present showed that chickens fed fermented feed had lower AST values than those in the control group. These findings suggest that fermented feed may protect the liver function of Thai native chickens. The IgA IgY and IgM have adaptive humoral immunity in chickens. IgA is mostly found in mucosal tissue, especially intestinal mucosa, and plays a role guarding against pathogens and maintaining homeostasis of the gut (Macpherson et al. 2018). IgY and IgM are largely detectable in serum (Dias da Silva and Tambourgi 2010). In the present study, the serum IgY values of chickens fed 50% fermented feed were the highest. These results are consistent with previous results that showed chickens fed fermented feed increased IgG and IgM in their serum (Xu et al. 2023; Zhu et al. 2023). However, the present IgM was not significant among the group. In the present study, we focused on the expression of IgA in duodenal mucosa using immunohistochemistry techniques. The production of intestinal IgA depended on the feed and gut microbiota (Hapfelmeier et al. 2010). The experiment by Wang et al. (2023) found that the bacteroides upregulated the intestinal IgA in chickens. In the current study, the chickens fed fermented feed showed a high reaction of duodenal IgA compared with chickens fed normal feed (control group). The serum IgA of broilers fed fermented feed increased compared to the control (Zhu et al. 2020; Zhu et al. 2023). The fermented feed consisted of LAB, which is a beneficial bacteria used to stimulate mucosal immunity (Perdigon et al. 1999; Kawashima et al. 2018).

In the present study, the defeathered and eviscerated carcass percentage of chickens fed fermented feed was increased. Similarly, broilers fed 10% fermented feed showed a higher percentage of carcasses than the group that did not receive fermented feed (Palupi et al. 2023). The present results may be related to the lighter body weight of chickens in the control group, resulting in a lower defeathered and eviscerated carcass percentage. In addition, the weights of the digestive organs of chickens fed fermented feed in the present study were lower than those of the control group. This may explain why chickens fed fermented feed showed a higher defeathered and eviscerated carcass percentage. Further, the decreased proventriculus, duodenum, and jejunum in chickens fed fermented feed may be attributed to the use of fermented feed, which facilitates microbial digestion of feed ingredients before feeding them to the animals, thereby reducing the workload of the digestive system, leading to reduced organ weights and potentially improved feed efficiency.

Conclusion

Feeding 25-50% fermented feed with 1% Thua-nao can enhance Thai indigenous chickens' feed efficiency and immunity under a free-ranging system.

Author's Contribution: Conceptualization, DK; methodology, DK, MW, WM; performing experiments, SY, PW, ST; data analysis, DK, WM; lab analyzed, DK, KP; manuscript preparation, DK; review and editing, WM. All authors have read and agreed to the published version of the manuscript.

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