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Effect of Sorghum and Indigofera Leaf Flour on Egg Quality, Daily Protein Intake, Phosphorus Availability and Total Colonies of *Bacillus* sp in Small Intestines of Laying Hens

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Abstract | An experiment was performed to investigate the effects of sorghum and indigofera leaf flour as substitutes for corn in laying hen rations on egg quality, daily protein intake, phosphorus availability, and total colonies of *Bacillus* sp. in the small intestines of laying hens. A completely randomized study design was used in this research, with 4 treatments, and each treatment was repeated 5 times: A (0% sorghum + 0% indigofera); B (20% sorghum + 4% indigofera); C (30% sorghum + 5% indigofera); and D (40% sorghum + 6% indigofera). The phosphorus availability, daily protein intake, eggshell thickness, eggshell strength, egg yolk color, egg yolk fat, egg yolk cholesterol, and total colony count of *Bacillus* sp. in the small intestines were measured. The results showed that the use of sorghum and indigofera leaf flour did not significantly ($P > 0.05$) affect the daily protein intake, phosphorus availability, eggshell thickness, eggshell strength, egg yolk color, egg yolk fat, and total colonies of *Bacillus* sp. in the small intestine. However, there was a highly significant ($P < 0.01$) effect on egg yolk cholesterol. In conclusion, 40% sorghum and 6% indigofera leaf flour can be used instead of 100% corn in laying hens ration and can reduce egg yolk cholesterol up to 26.29%.

Keywords | Egg yolk cholesterol, Egg quality, Indigofera leaf flour, Laying hens, Sorghum

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INTRODUCTION

The availability of feed ingredients in the poultry farming business has been affected by imports of cereal feed ingredients such as corn, as sources of protein and energy. Thus, the price of corn on the world market largely determines the price of corn on the domestic market. One way to reduce the use of corn is to look for alternative feed ingredients, such as sorghum [*Sorghum bicolor* (L.) Moench]. Sorghum has been used as a feed ingredient in poultry rations (Ochieng et al., 2018; Selle et

al., 2019). Giving sorghum to poultry diet can harm egg yolk color as poultry that consumes sorghum may produce a paler egg yolk color. According to Freitas et al. (2014), quails fed containing sorghum without pigment added in a paler egg yolk color compared to quails fed containing sorghum that pigment added. In this study, the addition of indigofera leaf flour, up to 6% in the ration can maintain egg yolk color, caused by β -carotene content in indigofera leaf flour. Indigofera leaves contain 507.6 mg/kg β -carotene, which functions as a source of antioxidants and egg yolk pigmentation (Palupi et al., 2014a). The use of carotenoids



in rations can improve the egg yolk color of laying hens (Kotrbaček et al., 2013). Carotenoid content in egg yolk is influenced by the consumed ration (Nys and Guyot, 2011). Some researchers have previously reported the ability of carotenoids to improve the egg yolk color. Hammershoj et al. (2010), stated carotenoid content in rations can increase the brightness of egg yolk color. Furthermore, Sangeetha and Baskara (2010) also report that laying hens can not turn all carotenoids into vitamin A, however, the remaining is used to color the yolk.

Furthermore, sorghum contains a phytic acid which range from 0.2 to 2.4 mg/g (Abdelhalim et al., 2019). Phytic acid cannot be digested by monogastric and has anti-nutritional properties that inhibit the absorption of various minerals, including calcium and phosphorus. Phosphorus is one of the essential nutrients for livestock, most of the phosphorus in plant seeds is in the form of phytic acid (Shanmugam, 2018). Phosphorus utilization can be increased by generally adding phytase enzymes in poultry diets (Dersjan et al., 2015). In the present study, a probiotic waretha (probiotic waretha contains *Bacillus amyloliquefaciens* bacteria) is added. *Bacillus amyloliquefaciens* can produce the phytase enzyme (Idriss et al., 2020). Phytase enzymes can degrade phytate in the digestive tract of laying hens. Thus the use of calcium and phosphorus in the digestive tract of laying hens is more optimal. Calcium and phosphorus significantly affect eggshell quality, such as egg strength and thickness. According to Ahmed et al. (2013), eggshell quality is influenced by calcium content in the diet. Furthermore, Jiang et al. (2013) reported that chickens fed with calcium levels of 2.62% in ration have weaker eggshells than those fed with calcium levels ranging from 3.70 to 4.40%. Additionally, *Bacillus amyloliquefaciens* also produces various types of enzymes that help digest food substances in the small intestine of laying hens, including cellulase, protease, and amylase enzymes (Ye et al., 2017; Nassar et al., 2015; Abd-Elhalem et al., 2015). Furthermore, *Bacillus amyloliquefaciens* has been reported as a bacterium that can be used as a probiotic and can increase ration efficiency in pitalah ducks (Zurmiati et al., 2017). Based on the above findings, a study was conducted to investigate the egg quality, daily protein intake, phosphorus availability, and total colonies of *Bacillus* sp in small intestines of laying hens fed sorghum and Indigofera leaf flour.

MATERIALS AND METHODS

BIRDS

Two hundred ISA Brown laying hens aged 63 weeks were used, with egg production at 80%.

Preparation of sorghum and Indigofera leaf flour: a). Sorghum: sorghum was harvested and dried to obtain 10-

15% moisture. after drying, the Sorghum was used as a feed ingredient for laying hens ration. b). Indigofera leaf flour: Indigofera leaf was collected and dried to obtain 10-15% moisture. Then the milling was carried out. After grinding the Indigofera leaf flour, it was ready to be used as a feed ingredient to prepare the ration for laying hens.

Ration: The ration used in this study consisted of concentrate for laying hens, corn, sorghum seeds, Indigofera leaf flour, coconut oil, vitamin B12, and rice bran (composition of the ration is showed in Table 1). This study was conducted for six weeks with an adaptation period of 10 days. Treatment rations are given twice a day i.e., at 08.00 PM and 15.00 AM as much as 125 g/bird/day, and drinking water was given ad-libitum. Probiotic waretha contains *Bacillus amyloliquefaciens* as much as $(43 \times 10^{12}$ CFU/mL) in all treatments. Administration of probiotic waretha through drinking water. Before giving the probiotic chicken was satisfied for two hours. During the research, waretha probiotic was given once.

EXPERIMENTAL DESIGN AND TREATMENT

A completely randomized study design was used in this study. There were four treatments A (0% sorghum + 0% indigofera); B (20% sorghum + 4% indigofera); C (30% sorghum + 5% indigofera); and D (40% sorghum + 6% indigofera), and all treatment was repeated five times. 10 laying hens were placed in battery cages for each treatment and replication.

PARAMETERS MEASURED

Phosphorus availability: Phosphorus availability calculated according to Sibbald (1982).

P= Phosphorus

Daily protein intake: Daily protein intake is the amount of protein consumed by the chickens. Protein consumption is expressed in grams, calculated according to Tillman et al. (1998). Daily protein intake (g) = feed consumption (g) x crude protein of the rations (%).

Eggshell thickness: The eggs were cleaned and then broken on a glass plate. The shells were separated from the egg contents. The thickness of the shell was measured by using a shell thickness micrometer on the pointed end, the blunt end, and the middle part. The measurement was recorded in mm (Aydin et al., 2008). The number of eggs used to measure the eggshell thickness was 20, with four eggs for each treatment.

Eggshell strength: The measurement of eggshell strength was performed by weighing the eggs, then placing them in a vertical position on an Instron plate. The eggs were com

Table 1: Ration composition, nutrient content (%) and metabolism energy (kcal/kg) content of the experimental ration

Feedstuff	Experimental diets Sorghum and indigofera leaf flour in rations (%)			
	A	B	C	D
Concentrate for laying hens	26.00	23.00	22.00	21.00
Corn	40.00	20.00	10.00	00.00
Sorghum seeds	0.00	20.00	30.00	40.00
Indigofera leaf flour	0.00	4.00	5.00	6.00
Palm oil	0.00	1.00	1.00	2.00
Vitamin B12	1.00	1.00	1.00	1.00
Rice bran	33.00	31.00	31.00	30.00
Total	100.00	100.00	100.00	100.00
	Calculated analysis			
Crude protein (%)	17,65	16,95	17,10	17,15
Crude fat (%)	3,79	4,27	4,46	4,59
Crude fiber (%)	6,73	6,89	6,57	6,13
Calcium (%)	3,28	3,24	3,21	3,17
Phosphorus (%)	0,58	0,59	0,61	0,63
Metabolism energy (kcal/kg)	2653.1	2692.6	2678.1	2659.3
Probiotic waretha (contain the <i>Bacillus amyloliquefaciens</i> bacterium) (CFU/mL)	43x10 ¹²	43x10 ¹²	43x10 ¹²	43x10 ¹²

Table 2: Average phosphorus availability, daily protein intake, eggshell thickness, and eggshell strength of laying hens fed sorghum supplemented with Indigofera leaf flour

Sorghum and Indigofera leaf flour in rations (%)	Phosphorus availability (%)	Daily protein intake (g/bird/d)	Eggshell thickness (mm)	Eggshell strength (kg/mm ²)
A (0% sorghum + 0% Indigofera)	77.40	18.72	0.47	4.86
B (20% sorghum + 4% Indigofera)	60.30	18.71	0.46	4.42
C (30% sorghum + 5% Indigofera)	86.78	18.71	0.43	4.04
D (40% sorghum + 6% Indigofera)	72.67	18.70	0.45	4.33
SE	5.09	0.15	0.004	0.11

SE: Standard Error

Parameter values in the same column showed no significant differences (P>0.05).

pressed until they broke. Reference graphs can be used to calculate the strength of the eggshells (kg/mm); the higher the value in kg/mm² for each measured egg, the higher the eggshell strength (Ranggana, 1986). The number of eggs used to measure the eggshell strength was 20, with four eggs for each treatment.

Egg Yolk color: The eggs were broken and placed on a glass table. The quality of the egg yolk color was determined visually by comparing the obtained egg yolk color with various egg yolk colors on the Roche Yolk Fan standard score on a scale of 1-15 from pale to dark orange (concentrated) (Vuilletjmier, 1968). The number of eggs used to measure the egg yolk color was 20, with four eggs for each treatment.

Egg Yolk fat: preparation of egg yolk fat test sample: 1 g

sample of egg yolk was weighed, then wrapped in oil paper, and dried in an oven at 105-110 °C. After that, the sample was extracted with diethyl ether by using Soxhlet until the liquid was clear. The extraction was stopped, and the samples were aerated to dry, then oven-dried for 6 hours at 105-110 °C. Furthermore, all samples were put into a desiccator for 15 minutes, and then the samples were weighed. The fat content was determined by using the Soxhlet method. Fat content = $a - b/c \times 100\%$. Description: a = sample weight after the extraction process; b = sample weight before the extraction process; and c = sample weight (AOAC, 2005).

Egg yolk cholesterol: preparation of egg yolk cholesterol test sample: egg yolk cholesterol was measured using spectrophotometry. 1 g of egg yolk sample is weighed and put into a centrifuge tube containing 10 ml of acetone al

Table 3: Average egg yolk color, egg yolk fat, egg yolk cholesterol, and total colony of *Bacillus* sp. in the small intestines of laying hens fed sorghum supplemented with Indigofera leaf flour

Sorghum and Indigofera leaf flour in ration (%)	Egg yolk color	Egg yolk fat (%)	Egg yolk cholesterol (mg/100g)	Total colony of <i>Bacillus</i> sp (log CFU/ml)
A (0% sorghum + 0% Indigofera)	8.50	26.66	903.96 ^a	8.22
B (20% sorghum + 4% Indigofera)	8.75	26.37	981.49 ^a	8.49
C (30% sorghum + 5% Indigofera)	8.50	26.10	881.66 ^a	7.98
D (40% sorghum + 6% Indigofera)	8.25	25.84	509.79 ^b	8.11
SE	0.26	26.08	726.70	0.12

SE: Standard Error

Different superscript lowercase letters in the same column indicate a significant difference (P<0.01).

cohol solution (1: 1), then boiled until the solution boils. After chilling, centrifuged at 3000 rpm for 15 minutes, the supernatant was taken and evaporated in a boiling water bath until the residue remained after that dilution was carried out with chloroform readings at 680 nm after turning green (\pm 5 minutes). Cholesterol level is calculated by comparing the absorbance with a standard cholesterol curve. The analysis of egg yolk cholesterol was performed by the method of *Salkowski* and *Liebermann-Burchard* (*Schunack et al.*, 1990).

$$Y = 2.354X + 0.005$$

Y = Absorbance of cholesterol sample
X = level of cholesterol (mg / 100ml)

Total colony of *Bacillus* sp. in the small intestines: The population of *Bacillus* sp bacteria was observed after six weeks of treatment by taking one chicken per unit. The small intestine fluid is taken as much as 1 gram, then diluted from 10^{-1} to 10^{-7} . 1 ml of each 10^{-7} dilution of the sample was inserted into the Petri dish, which had been filled with selective media of *Bacillus* sp then incubated at room temperature for 24 hours. The colonies grown in the Petri dish were then counted using a colony counter (*Cappucino and Sherman*, 1987; *Hadioetomo*, 1991).

STATISTICAL ANALYSIS

All the data were analyzed by analysis of variance (ANOVA), and Duncan's multiple range tests were used for the determine of differences in among means (*Steel and Torrie*, 1995).

RESULTS AND DISCUSSION

The use of sorghum and indigofera leaf flour in the rations of laying hens did not affect (P>0.05) the daily protein intake (Table 2). Over time, no difference in phosphorus availability, thickness, and the eggshell strength of laying hens from the five treatments was observed (P>0.05; Table 2). The use of sorghum and indigofera leaf flour in the rations of laying hens did not affect (P>0.05) egg yolk color, egg yolk fat, and *Bacillus* sp. total or colony count in the

small intestines (Table 3); however, it had a highly significant effect (P<0.01) on the egg yolk cholesterol of the laying hens (Table 3).

The sorghum and indigofera leaf flour are as palatable as the corn in the rations. The uniformity of the values of daily protein intake and phosphorus availability indicate that there were no negative effects from using 40% sorghum and 6% indigofera leaf flour in the rations of the laying hens. This was due to the nutritional quality, especially the ration protein content in all treatment rations that were equal in amount so that the inclusion of each level of sorghum and Indigofera leaf flour in rations had the same ration quality. This results in the same amount of protein consumed and utilized by layer chickens so that the resulting daily protein consumption is the same. *Leke et al* (2018), stated that protein intake affects egg production and that a decrease in protein consumption can cause a decrease in egg production. The substitution of corn with up to 40% sorghum with the addition of 6% indigofera flour does not affect the performance of laying hens (*Sriagtula et al.*, 2019).

The average daily protein intake in this study ranged from 18.70 to 18.72 g/bird/day. These averages were higher than those reported by *Traineau et al.* (2015), in which the average daily protein intake of laying hens ranged from 16.70 to 17.20 g/bird/day.

The use of up to 40% sorghum and 6% indigofera leaf flour also does not affect phosphorus availability. Sorghum contains a phytic acid ranging from 0.2 to 2.4 mg/g (*Abdelhalim et al.*, 2019). The utilization of phytic acid as a source of phosphorus in chicken rations through the hydrolysis of phytate-bound phosphate can increase the efficiency of the use of phosphorus in rations. The use of feed ingredients that contain high phytic acid levels hurts poultry because they cannot break down phytic acid in their digestive tract. In this study, the use of warethra probiotic (containing *Bacillus amyloliquefaciens*) can produce phytase enzymes, which will help degrade phytate in sorghum in the small

intestine of poultry. *Bacillus amyloliquefaciens* can produce the phytase enzyme (Idriss et al., 2020), which can degrade the phytate in the digestive tract of laying hens. Thus the use of calcium, phosphorus, and protein in the digestive tract of laying hens is more optimal. The addition of the phytase enzyme to poultry rations increases the hydrolysis of phytic acid, and the availability of minerals, amino acids, and energy for the poultry and therefore increasing their growth (Khan et al., 2013). Besides that, *Bacillus amyloliquefaciens* also produces various types of enzymes that help digest food substances in the small intestine, including cellulase, protease, and amylase enzymes (Ye et al., 2017; Nassar et al., 2015; Abd-Elhalem et al., 2015). Furthermore, *Bacillus amyloliquefaciens* has been reported as a bacterium that can be used as a probiotic and can increase ration efficiency on pitalah ducks (Zurmiati et al., 2017).

Furthermore, the use of sorghum and indigofera leaf flour in the rations of laying hens does not affect the thickness and strength of eggshells. Sorghum contains a phytic acid range from 0.2 to 2.4 mg/g (Abdelhalim et al., 2019). Phytic acid cannot be digested by monogastric and has anti-nutritional properties that inhibit the absorption of various minerals, including calcium and phosphorus. Phosphorus utilization can be increased by generally adding phytase enzymes in poultry diets (Dersjan et al., 2015). In this study, a probiotic waretha was added (probiotic waretha is containing *Bacillus amyloliquefaciens* bacteria) *Bacillus amyloliquefaciens* can produce the phytase enzyme (Idriss et al., 2020), which can degrade phytate in the digestive tract of laying hens. Thus the use of calcium and phosphorus in the digestive tract of laying hens is more optimal. Calcium and phosphorus significantly affect eggshell quality, such as egg strength and thickness. The primary nutrients that affect eggshell quality are calcium, phosphorus, and vitamin D3 (Neijat et al., 2011; Xiao et al., 2014). According to Ahmed et al. (2013), eggshell quality is influenced by calcium content in the diet. Furthermore, Jiang et al. (2013) reported that chickens fed with calcium levels of 2.62% in the ration had weaker eggshells than those fed with calcium levels of 3.70 to 4.40%. Besides that, the inclusion of sorghum and Indigofera leaf flour in the ration of laying hens does not affect the thickness and the strength of eggshell due to the almost equal presence of calcium (ranging from 2.47 to 3.01%) and phosphorus (ranging from 0.41 to 0.43%) in each treatment.

The use of sorghum and indigofera leaf flour in the rations of laying hens can maintain the egg yolk color. It is feared that the replacement of corn with sorghum up to 40% in the ration will harm the egg yolk color because sorghum does not contain carotenoids as does corn. It is believed that giving sorghum to poultry may harm egg yolk color. Chicken consuming sorghum produce a paler

egg yolk color. According to Freitas et al. (2014), quails fed sorghum without added pigment have in a paler egg yolk color compared to those fed with pigment added sorghum. In this study, the addition of Indigofera leaf flour, up to 6% in the ration can maintain egg yolk color due to the β -carotene contained in Indigofera leaf flour. Indigofera leaves contain 507.6 mg/kg of β -carotene, which functions as a source of antioxidants and egg yolk pigmentation (Palupi et al., 2014a). The use of carotenoids in rations can improve the egg yolk color of laying hens (Kotrbackek et al., 2013). The carotenoid content in egg yolk is influenced by the ration consumed (Nys and Guyot, 2011). Some researchers have previously suggested that carotenoids can improve the egg yolk color. Hammershoj et al. (2010) suggest that carotenoid contained in rations can increase the brightness of egg yolk color. Furthermore, Sangeetha and Baskara (2010) also suggest that although laying hens cannot turn all carotenoids into vitamin A, the remaining is used as colorant to their yolk.

Furthermore, the use of sorghum and indigofera leaf flour in the rations of the laying hens does not affect the egg yolk fat content because the fat contents of sorghum and corn are almost the same i.e., 2.71 and 2.66%, respectively (Non-Ruminant Nutrition Laboratory, Universitas Andalas, 2013). The fat content of rations affects the egg yolk fat content (Yamamoto et al., 2007). According to Schreiber et al. (2013), β -carotene is a potential natural antioxidant. In this study, the use of sorghum and indigofera leaf flour in the rations of laying hens was able to reduce egg yolk cholesterol. The use of 30% sorghum and 5% indigofera leaf flour, and 40% sorghum and 6% indigofera leaf flour in rations of laying hens significantly reduced the egg yolk cholesterol content. Increased carotenoid content in the rations of laying hens can reduce egg yolk cholesterol (Akdemir et al., 2012). Researchers have previously reported the effect of using indigofera on cholesterol contain. Palupi et al. (2014b), reported the use of indigofera sp., top leaf meal in rations of laying hens, reduces egg yolk cholesterol as much as 54.1%. Furthermore, Palupi et al. (2018), reported that the duck fed indigofera zollingeriana top leaf meal have lower cholesterol content compared to duck that not fed indigofera zollingeriana top leaf meal.

The use of sorghum and indigofera leaf flour in the ration does not affect the total colony count of *Bacillus* sp in the small intestines of the laying hens due to the inclusion of probiotic waretha that contain the bacterium *Bacillus amyloliquefaciens* at concentrations as high as 43×10^{12} CFU/mL. In this study, the total colony count of *Bacillus* sp in the small intestines of laying hens found ranged from 7.98 to 8.49 log CFU mL⁻¹. *Bacillus amyloliquefaciens* increase the total colony count of nonpathogenic bacteria and decrease the total colony count of pathogenic bacteria such

as *Escherichia coli* and *Salmonella* spp. (Tang et al., 2017). *Bacillus amyloliquefaciens* increase the efficiency of Pitalah duck rations by reducing feed conversion by as much as 15%, and it was found that the total colony count of *Bacillus* sp. in the small intestine of Pitalah ducks is as high as 14.84 log CFU/mL (Zurmiati et al., 2017a). The inclusion of up to 2000 ppm of the probiotic *Bacillus amyloliquefaciens* increase the efficiency of Pitalah duck rations by reducing the energy requirements of the rations by as much as 3.57% and reduce the protein requirements by as much as 5.56% (Zurmiati et al., 2017b).

CONCLUSION

In conclusion, the use of 40% sorghum with the addition of 6% indigofera leaf flour can replace the use of 100% corn in laying hen rations without disrupting phosphorus availability, daily protein intake, eggshell thickness, eggshell strength, egg yolk color, egg yolk fat, and total colony count of *Bacillus* sp in the small intestines of laying hens, and can reduce their egg yolk cholesterol up to 26.29%. The use of 40% sorghum and adding 6% indigofera leaf flour obtained 72.67% phosphorus availability, 18.70 g / bird/d daily protein intake, 0.45 mm eggshell thickness, 4.33 kg / mm² eggshell strength, 8.25 egg yolk color, 25.84% egg yolk fat, 509.79 mg/100g egg yolk cholesterol, 8.11 log CFU/ml total colony of *Bacillus* sp.

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CONFLICTS OF INTEREST

The authors declare that no conflicts of interest are involved in this study.

AUTHOR CONTRIBUTIONS

Ade Djulardi and Wizna participated in all stages of the research, namely the research design, the conduct of the experiment, sample analysis, data analysis, writing, and editing of articles. Ryesi Sriagtula participated in conducting the investigation, design, interpretation, Ahadiyah Yuniza was responsible for data analysis, interpretation, design, Zurmiati participated in writing, conception, and editing of articles. All authors participated in writing the article.

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