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Spirulina Flour-based Feed Additive Improves the Physical and Chemical Eggs Quality of Tegal-Laying Duck

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Abstract. Spirulina is a species of blue green algae that contains many phenolic compounds, phytonutrients and pigments which have potential as a feed additives to improve the physical and chemical quality of eggs. The present study evaluated the effect of spirulina flour-based feed additive supplementation during the late growth to the early egg-laying period on Tegal-laying ducks' physical and chemical egg quality. A total of 25 ducks were randomly divided into 5 treatments; the control group treated with standard feed (P0), P1, P2, P3, and P4 were treated with standard feed supplemented by spirulina flour-based feed additive at concentrations of 0.3%, 0.6%,1.2%, and 2.4%, respectively for four weeks. Physical quality indicators included the Haugh unit, yolk index, weight measurements, eggshell thickness, and shape index. Chemical quality indicators assessed albumen protein content, egg yolk cholesterol, and β-carotene levels. This research revealed that spirulina flour, added at concentrations between 0.30% and 2.40%, significantly enhanced the egg quality of Tegal-laying ducks compared to the control group. This study concludes that spirulina flour can be used as a feed additive to improve the physical and chemical quality of Tegal-laying ducks' egg. The novelty of this research is spirulina flour has the potential to be used as a feed additive to increases the albumen protein levels, increases β-carotene, and reduces egg yolk cholesterol in eggs of Tegal-laying ducks. Spirulina flour can be a potential source of feed additive for various other local ducks in Indonesia.

Keywords: Eggs; feed additive; physical and chemical quality; Spirulina; Tegal-laying duck

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INTRODUCTION

The population of Indonesian is increasing each year. This condition leads to several issues, including food insecurity. In 2020, according to the Ministry of Agriculture's Food Security Agency, 15.8% of 416 regions, or 4% of 98 cities in Indonesia, had low food security index, with 22.5% of prbeing vulnerable. The prevalence of malnutrition in the region is 30.22%, which is classified by WHO as unfavorable (30-40 %) (BKPKP, 2020). According to the RISKESDAS (2020), the prevalence of children under five was 17.9%, and 4.9% were malnourished. The approach research of inexpensive, high-quality food is a priority. Agriculture has provide food security issues. The food supply pillar is a subsector comprising livestock and fisheries, including poultry and aquaculture.

The increasing demand for protein-rich foods is contributed by poultry products. Currently, feed ingredients of poultry as well as antibiotics, growth factors, or chemicals can be substituted by natural ingredients. However, the cost of feed ingredients continue to increase throughout the year. This condition leads to the increases of production costs and reduces the productivity (Kumar & Sibi, 2020). Microalgae is considered as a worthy substance in the search for alternative protein sources. Microalgae is a natural feed with a high nutrient content that promised as a poultry feed additive. Spirulina contains vitamin B, calcium, iron, magnesium, manganese, potassium, and zinc, among other vitamins and minerals. Additionally, microalgae are also a source of essential fatty acids, γ-linolenic acid, pigments including chlorophyll, phycocyanin, carotene, and xanthophyll (El-Hady & El-Ghalid, 2018).

The deterioration of physical quality of eggs is a current issue for breeders of laying ducks during cultivation process. To assess the overall quality of duck eggs and their marketability, local duck breeders in Indonesia conduct an exhaustive evaluation of varieties that leading into physical indicators, as well as egg weight, haugh unit (HU), egg white weight, egg yolk weight, egg yolk index (EYI), and shell thickness (Sunarno *et al*., 2023).

The availability of eggs as a healthy protein source is a consumers demand as many food products pose health risk. Recently, the interest of natural, safe, and environmentally friendly functional food products is increased (Borowitzka, 2013). Developing aquaculture-livestock technology and products is a solution to the increasing poultry and aquaculture production and conserving tropical biological resources. Spirulina and poultry are commodities that need to be designed to meet the needs of food production and quality, especially eggs. The development of aquaculture-poultry food still faces obstacles in production capacity, quality, and relatively high prices. Based on this, research on microalgae as an organic product will be carried out. Products from these microalgae will be developed into feed additives to produce quality eggs.

In this study, we observed and analyzed the effect of spirulina flour as a feed additive in the late growth and early egg-laying periods on the physical and chemical quality of eggs in Tegal-laying ducks.

METHODS

Preparation of Spirulina Flour

Spirulina sample was taken from cultivation area in Semarang City, central Java, Indonesia. At the growth phase, Spirulina was harvested as filaments or spiral threads with more giant cells than other types of phytoplankton. The obtained spirulina biomass was rinsed three times with fresh water to remove the remaining culture medium. The paste or gel is first weighed and then spread thinly on a baking sheet covered with mica plastic for the drying process. The maximum thickness of Spirulina biomass each plastic is 0.5 centimeters or 100 grams. The Spirulina biomass was dried in an incubator at 60°C for 12 hours until the dry weight was constant. The dried Spirulina was ground into flour using a blender and then sifted to produce finer flour grains. The refined Spirulina flour is then stored in aluminum foil and a vacuum sealer so that it is airtight, has a longer shelf life, is mold-resistant, and is ready to be processed as a feed additive.

Proximate Analysis of Spirulina Flour

Proximate analysis of water and crude ash, protein, fat, and Spirulina biomass carbohydrates content were determined by the AOAC method (1995), Kjeldahl (SNI 01-2354.4-2006, phenolic acid sulfate (SNI 01-2891-1992) and method Soxhlet (SNI 01-2891-1992), respectively.

Spirulina Flour Extraction

The Spirulina flour was extracted using ethanol as a solvent with a biomass-to-ethanol ratio of 1:10, macerated for one day, and centrifuged at a speed of 2455 G and temperature of 10 (C for ten minutes. The dark-colored supernatant was collected and concentrated using a Buchi R-205 and Buchi 215 vacuum rotary evaporator until no solvent remained. The extract that has dried in a rotary evaporator flask is then placed in dark bottles and dried with nitrogen gas. This extraction procedure was performed thrice.

Phytochemical Test of Spirulina Flour Extract

Preparation of test solutions for phytochemical screening (alkaloids, saponins, tannins, steroids, triterpenoids, and glycosides) by dissolving *Spirulina* sp. extract flour in ethanol pro analysis at 1:10 ratio. The alkaloid content was determined by reacting the test solution with Dragendorff reagent and the test solution with Mayer's reagent.

The presence of alkaloids is indicated by an orange precipitate upon reaction with the reagent Dragendorff and a yellow precipitate upon reaction with Meyer's reagent. Shaking was used to examine saponin by observing the formation of foam. The indicator presence of saponins is the stability of a 1-10 cm-high foam for at least 10 minutes after adding a single drop of 2N HCl and the absence of foam loss. The tannins were evaluated by combining 3 ml of the test solution with five drops of 1% NaCl and three drops of gelatin solution. If the precipitate is formed, an extract solution is positive and contains tannins.

The Liebermann-Burchard reaction was used to examine steroid and triterpenoid compounds. At the surface of the solution, brown or violet rings indicated the presence of triterpenoids, whereas a greenish-blue ring indicates the presence of steroids. Liebermann-Burchard was performed to determine the glycoside content, which is blue or green. The flavonoid content of ethanol extract of Spirulina flour was determined using the Taubeck test, characterized by a solution that fluoresces yellow under UV light at 366 nm.

Determination of Pigment Content in Spirulina Flour

Phycocyanin was analyzed by spectrophotometer at 615 and 650 nm to detect phycobiliprotein. Extracted phycocyanin was diluted according to each solvent and measured using a spectrophotometer at 615 nm and 625 nm. Aquadest was used as a neutral control. The phycocyanin concentration (PC) was calculated by Bennett equation and Bogorard.

Xanthophyll was macerated using ethanol, while carotene was extracted using acetone/hexane at 2:1 ($^v/_v$). Two grams of Spirulina flour was dissolved with 50 mL of ethanol, shaken for 30 min, then filtered; the treatment was repeated twice. The filtrate was adjusted to a volume of up to 100 mL and then measured at a wavelength of 464 nm. The residue obtained was dehydrated and stored for β-carotene extraction.

The dry residue obtained from xanthophyll extraction was extracted with a mixture of acetone-hexane 2:1 (v/v) using the maceration method with two times the addition of 50 ml of solvent and shaking for 30 min. The extracts obtained were combined and made up to 100 ml in volume, and then the β-carotene was detected at a wavelength of 493 nm.

Formulation of Duck Feed from Spirulina Flour

Duck feed from Spirulina flour was formulated using standard duck feed for the grower and layer periods. Standard feed (grower phase) was treated for ducks aged 3-20 weeks. Layer standard feed was treated for ducks aged more than 20 weeks. Standard grower feed contains a minimum 16% of crude protein and 2750 kcal/kg metabolic energy. Layer standard feed includes a minimum 18% of crude protein and 2650 kcal/kg metabolic energy. Feedstock was formulated once a week. The concentration of spirulina powder used in this study was 0%, 0.3%, 0.6%, 1.2%, and 2.4% (Table 1). Each standard feed is mixed separately until homogeneous with spirulina flour according to the concentration of each that has been determined. Formulated feed was treated to the ducks for four weeks.

Table 1. Formulation of duck feed from spirulina flour

Feed ingredient composition	Treatment feed additive levels (%)				
	0	0.3	0.6	1.2	2.4
Standard feed (%)	100	99.7	99.4	98.8	97.6
Spirulina flour (%)	0	0.3	0.6	1.2	2.4
Total (%)	100	100	100	100	100

Experimental Animals

This research protocol was approved by the Ethics Committee of the Faculty of Medicine, Diponegoro University (Approval Letter No. 29/EC/H/FK-UNDIP/III/2022). Twenty-five female Tegal-laying ducks, 18 weeks old, were purchased from breeders in Delanggu, Klaten, Central Java, Indonesia.

Each duck was maintained in an individual battery cage sized 40×35×60 cm^3 at 23°C ± 2°C, 12 : 12 h light/dark cycle. Food and drink are provided ad libitum, feeds twice in a day, i.e., every morning (07.00) and evening (15.00). Vaccinations against Newcastle Disease (ND), ND1, and ND2 were given when the ducks were 7 and 14 days old, respectively. Sanitation of the duck coop and measurements of temperature and humidity were carried out every day.

Tegal-laying ducks were randomly divided into five treatment groups, comprising five ducks per group. The first group was the control (P0), fed without spirulina flour. Groups 2 to 4 (P1, P2, P3, and P4) were treated as standard feed with added feed additives at 0.30%, 0.6%, 1.2%, and 2.4%. All groups were acclimatized for seven days before the treatment. The feed requirements for laying ducks during acclimation and treatment are shown in Table 2, meanwhile Table 3 explains the nutritional and energy needs of laying ducks at each growth phase and laying period.

Table 2. The feed requirements for laying ducks during acclimation and treatment

Growth Phase	Age (week)	Treatment	Feed (g/day)
Late grower period	18	Acclimation	150
Late grower period	19-20	Feed additive treatment	160
Layer period	21-22	Feed additive treatment	180

Table 3. The nutritional needs of laying ducks in each growth phase

Nutritional type	Grower (18 weeks)	Grower (19-20 weeks)	Layer (21-22 weeks)
Water content maximum (%)	14	14	14
Ash content maximum (%)	11	14	14
Protein content (%)	15	17	17
Fat content (%)	3	3	3
Carbohydrate content (%)	9	10	10
Metabolic energy (kcal/kg)	2750	2750	2650

After 28 days, the number of eggs produced, egg weight, and physical and chemical quality indicators of Tegal duck's eggs were measured. The measured variable of egg's physical characteristics were egg shape index, yolk diameter, yolk height, yolk weight, yolk index, egg white height, egg white weight, eggshell thickness, and the haugh unit value. The chemical indicators of the measured egg quality included protein content and the egg quality.

Measurement of the Physical Quality of Eggs

The physical quality variable of duck eggs observed and measured included the haugh unit (HU), egg yolk index, egg yolk weight, albumen weight, egg weight, shell thickness, and egg shape index. Haugh Unit was calculated by the method of as follows:

$$\text{Haugh unit (HU)} = 100 \log (H + 7.57 - 1.7\, W^{0.37})$$

where H = albumen height and W = weight of egg. Egg yolk index were calculated by using (yolk height/yolk diameter) of egg. Egg yolk weight, albumen weight, and egg weight was measured by analytical weight, and thickness of eggshell was measured using micrometer. Shell thickness = (pointed end + equator + blunt end). Egg shape index (shape index) were calculated by using (breadth/length) of egg (Anggita *et al*., 2023).

Chemical Quality Determination of Eggs

The Kjeldahl method measured the egg protein levels (Mori *et al*., 2020). The protein content was obtained by breaking duck eggs and then separating the egg yolks and whites using an egg separator. In the first stage, 0.3 g of egg white sample was destroyed, and 0.3 g of selenium was added (1:1). Then 10 ml of H_2SO_4 was added to the digestion flask and digested for ± 45 minutes (until the color turned clear). The second stage of distillation was pouring the results from the digestion flask into a 1000 ml Erlenmeyer, adding 40 ml of 45% NaOH and 90 ml of distilled water, preparing 250 ml of Erlenmeyer with 20 ml of H_3BO_3, then adding two drops of MR (manual reset) and MB (manual button) indicators, distillation was carried out until the flask turned into green. The third stage of the titration is the result of 250 ml Erlenmeyer, added 0.1 N HCl, and observed until it turns pink. A blank solution is made by replacing the sample with distilled water carrying out digestion, distillation, and titration as in the sample.

Analysis of cholesterol levels was carried out using the Liebermann-Burchard method. Cholesterol levels were obtained by breaking duck eggs and then separating the egg yolks and whites using an egg separator. Egg yolks are dried in an oven at 60^0C. The egg yolk sample weighed 0.02 g, then put in a test tube and added 3 ml of alcohol: ether (3:1), then shaken and left for 30 minutes. The solution was centrifuged at 5000 rpm for 3 minutes. The supernatant (clear part) formed is put in a test tube and then evaporated into a water bath to dry as a paste. The paste or extraction product developed was diluted with 5 ml of chloroform. Each standard solution was prepared with 5 ml of various concentrations and 5 ml of chloroform blank. Each tube was added with 2 ml of acetic anhydride and 0.1 concentrated sulfuric acid and then shaken. The tube is stored in a dark room for 15 minutes. Absorbance was measured at a wavelength of 420 nm (Ukachukwu, *et al*., 2017).

Analysis of egg yolk beta carotene levels refers to the method by Stutz *et al*. (2015). Determining beta carotene levels in egg yolk begins with creating a standard curve. Beta carotene standard of 10 mg was weighed on an analytical balance and then put into a volumetric flask until the volume reached 10 ml. Petroleum ether was added to the volumetric flask until the tera mark (1000 ppm). Using a standard pipette, a 1000 ppm solution was taken and diluted to 50, 100, 250, 500, and 750 ppm. An absorbance test is conducted against one of the standards at a wavelength between 400-700 nm to determine the maximum wavelength. The following procedure measures the absorbance of the sample. A sample of 0.1 g was weighed using a digital scale and put into a measuring flask. 3 ml of petroleum ether was added to the volumetric flask, and the solution was then incubated for 30 minutes. The next stage is conducting an absorbance test at the maximum wavelength (450nm) and calculating the β-carotene content using a linear regression curve from the β-carotene standard.

Statistical Analysis

The data were analyzed to determine the distribution pattern and homogeneity utilizing the Shapiro-Wilk test for normality. The results of the data analysis revealed as a normal and homogeneous distribution, followed by an Analysis of Variance (ANOVA) with a 5 percent significance level. The Duncan Multi Range Test at a significance level of 5% using the SPSS (Statistical Product and Solution) application for Windows version 26.0 was used to determine the differences between treatment groups after the ANOVA revealed a significant difference.

RESULTS AND DISCUSSION

A proximate analysis of spirulina flour was conducted to analyze the chemical composition of the feed. The spirulina flour in this study contained low moisture and high metabolic energy (Table 4). Low water content means the presence of high dry matter content. The water content in spirulina flour dramatically affects the quality and storage capacity of the material. With a low water content, spirulina flour's shelf life increases or lasts longer (Pandey *et al.*, 2023).

Table 4. Proximate analysis results of Spirulina flour

Nutritional type	Content (%)
Water content maximum (%)	8.1032
Ash content maximum (%)	10.5192
Crude protein content (%)	19.2182
Crude fat content (%)	8.3261
Nitrogen-free extracts content (%)	10.3817
Metabolic energy (kcal/kg)	2527.28

The ash content of a feed ingredient indicates these materials' mineral content. According to Yusriadi *et al.* (2020), ash comprises dissolved minerals, detergents, and minerals, including the spirulina source, cultivation techniques, and flour processing methods. Spirulina is typically cultivated in controlled environments as well as ponds or tanks, where it absorbs minerals from the water and stores them within its cells. Higher ash levels may indicate a more natural and nutrient-dense source.

Spirulina flour's organic matter consists of crude protein, crude fat, and nitrogen-free extracts. High digestibility and solubility of monosaccharides, disaccharides, and polysaccharides in acidic and basic solutions characterize nitrogen-free extracts. The natural protein content is more significant than oil fats and nitrogen-free extracts. To some extent, the proteins in Spirulina flour stimulate the growth and reproduction of livestock, such as laying ducks. Proteins contribute to the productivity of livestock. A sufficient amount of fat in spirulina flour promotes the growth and reproduction of livestock. In addition, crude fat contains fat-soluble nutrients, such as vitamins A, D, E, and K, and fat-extracted pigments like chlorophyll and xanthophyll. A phytochemical compound was screened to determine the active secondary metabolite in spirulina flour (Thamilmaraiselvi & Steffi, 2018). Flavanoid, alkaloids, saponins, tannins, steroids, triterpenoids, and glycosides were qualitatively detected in the ethanolic extract of Spirulina flour, which is presented in Table 5.

Table 5. Groups of phytochemical compounds in spirulina flour

Group of phytochemical compounds	Test results	Description
Flavonoids	+	Intensive yellow fluorescence
Alkaloids	+	Orange precipitation
Saponins	+	Permanent foam
Tannins	+	Formation of a precipitation
Steroids	+	Greeny ring
Triterpenoids	-	No brown ring appears
Glycosides	+	Blue or green color

According to Rosihan & Savitri (2013), flavonoids, alkaloids, saponins, tannins, steroids, triterpenoids, and glycosides showed an immunomodulatory effect. Behl *et al*. (2021) reported that the immunomodulator activity of flavonoids is via lymphocyte proliferation and macrophage activation. Macrophages phagocytize bacteria, and T lymphocytes differentiate into $CD4^+$ and $CD8^+$. $CD4^+$ cells will produce γ-interferon stockings and stimulate natural killer (NK) cells. These cytokines play a role in macrophage activation, which leads to the formation of several compounds, one of which is nitric oxide. Nitric oxide plays a role in killing bacteria by oxidizing lipids in the bacterial cell membrane. Alkaloids help support the functions of animal organs by modulating the immune system via lymphocytes (Poluan *et al*., 2023). Saponins are bioactive compounds that function as antioxidants (Mustafa *et al*., 2022) and immune enhancers to boost the immune system. Stated further stated that saponins can modulate the cellular immune system and increase antibody production.

Aftab *et al*. (2017) reported that tannins as antioxidants stabilize free radicals by capturing free electrons and play a role in inhibiting the chain reaction of free radical formation. Metabolites secondary steroid groups, including β-sitosterol, fucosterol, and isofucosterol (Corral-Rosales *et al*., 2019), which can reduce cholesterol levels in the blood, and fucosterol and isofucosterol have the potential as antioxidants (Hanan *et al*., 2020). Terpenoid compounds such as monoterpenes and sesquiterpenes have antioxidant potential in the form of essential oils. Soltys *et al*. (2022) added that triterpenoids, as derivatives of terpene compounds, function as hydrogen donors so that they have antioxidant properties. Antioxidant activity can be in the form of inhibition at the chain reaction initiation stage, blocking at the propagation stage by damaging or binding free radicals, and stabilizing hydrogen peroxide (Poluan *et al*., 2023).

Glycoside is a secondary metabolite compound that binds with sugars by glycosidic bonds. The sugar moiety of a glycoside is bonded to the anomeric C atom to form a glycosidic bond. Glycosides can be bound by O-(O-glycoside), N-(aminoglycosides), S-(thioglycosides), C-(C-glycosides). The sugar part of a glycoside is called glycones, and the non-sugar portion is called aglycones or genin. Glycosides play an important role in the living system of an organism. Flavonoid-type glycosides showed antioxidant activity and improved the circulatory system. Glycones can consist of a single sugar (monosaccharide) or several sugar units (oligosaccharides). The results of the analysis of phycocyanin, xanthophyll, and β-carotene pigments in spirulina flour are depicted in Table 6.

Table 6. The content of phycocyanin, xanthophyll, and beta-carotene pigments in spirulina flour

Pigment type	Content
Phycocyanin (mg/mL)	1.962
Xanthophyll (%)	0.005
β-carotene (%)	0.146

The physical quality of the Tegal laying duck after treatment is shown in Table 7. The ANOVA results showed significant differences in the values of all variables between the treatments and the controls. This indicates that spirulina flour as a feed additive significantly affects the physical quality of eggs in Tegal-laying ducks.

Table 7. The egg physical quality of Tegal-laying duck after 28 days treated with spirulina flour, the feed additive

Variable	Spirulina flour feed additive levels (%)				
	0	0,3	0.6	1,2	2.4
Haugh unit eggs	74.1^a±1.10	76.7^b±1.03	79.8^c±1.06	81.9^d±1.14	83.7^e±1.05
Yolk index (YI)	0.42^a±0.02	0.44^a±0.01	0.46^b±0.01	0.49^c±0.02	0.52^d±0.02
Yolk weight (g)	21.8^a±0.11	22.3^{ab}±1.08	23.2^b±0.12	24.6^c±0.38	26.2^d±0.14
Albumen weight (g)	32.0^a±1.02	33.2^b±1.03	34.4^c±0.32	35.7^d±0.20	38.6^e±0.16
Egg weight (g)	60.2^a±0.25	63.2^b±1.11	64.2^c±0.26	66.7^d±1.12	72.6^e±1.09
Eggshell thickness (mm)	0.33^a±0.02	0.33^a±0.02	0.35^a±0.02	0.43^b±0.05	0.48^c±0.02
Egg shape index (%)	72.2^a±1,20	73.4^{ab}±1.23	75.6^b±2.07	79.4^c±2.12	84.4^d±1.16

Note: The data displayed is the average ± standard deviation. Different superscripts on the same line show significant differences (p<0.05). P0 (Ducks fed basal diet without additive spirulina feed/control). P1, P2, P3, and P4 were ducks fed basal feed with spirulina flour as a feed additive of 0.3, 0.6, 5, 1.2, and 2.4%.

The Haugh unit egg (HU) value is within the normal range. Anggita *et al.* (2023) reported that good egg quality has a HU value greater than 72. Haugh Value Egg units are divided into several qualities. AA quality chicken eggs have a HU value of 72 to 100, A quality chicken eggs have a HU value of 60 to 71, B quality chicken eggs have a HU value of 30 to 59, and C quality chicken eggs have a HU value below 29 (Manazes *et al*., 2012).This condition indicates that spirulina flour's additive treatment effectively increases eggs' HU value. Feed additive treatment of spirulina flour with a content of 2.4% gave the highest egg HU value of 83.7. Protein content affects the HU value of eggs. Spirulina powder feed additive treatment can increase the HU value of eggs. This result is in line with the study of El-Shall *et al.* (2023), which reported that when spirulina flour is included as a feed additive in poultry diets, it can positively impact the HU value of eggs. This is primarily attributed to the nutritional composition of spirulina, which is rich in essential nutrients such as protein, vitamins, and minerals. When hens consume feed containing spirulina flour, they receive these nutrients, which can lead to improved egg quality. The protein in spirulina, for example, may enhance the structural integrity of the egg white (albumen), resulting in a higher HU value. The bioactive compounds in spirulina flour (phycocyanin, xanthophyll, ß-carotene) act as activators of proteases, which produce reduction products from proteins, such as smaller polypeptides, oligopeptides, peptides, or amino acids, which are smaller in size. It will be absorbed by the cells that make up the intestinal villi as material for egg formation.

The Haugh Unit value was influenced by egg weight and albumen height. The ambient temperature affects the HU value because of the rate of CO_2 loss which caused the albumen height to decrease, so that the more CO_2 loss, the lower HU value. The HU value was influenced by the value of egg weight and albumen thickness. Lower the egg weight causes the decreasing of albumen thickness and the HU value (Anggita *et al*., 2023).

Spirulina flour feed additive treatment had a significant effect on the average yolk index (YI) (p<0.05). The value of this variable belongs to the normal range. Fresh eggs have a YI value of 0.33-0.52. The YI value of the results of this study is good because it has a value above 0.40. Based on the data in Table 7, it shows that the additive treatment of spirulina flour is effective in increasing the YI value. Spirulina flour feed additive treatment with 2.4% content yielded the highest YI value of 0.52. YI is a value to determine the quality of the egg yolk. The YI value was influenced by the availability of protein in the feed. Treatment with spirulina flour feed additives increases the YI value because the content of active compounds in the treatment material can mediate cross-linking between the amine groups and hydroxyl groups in proteases so that they will increase protease activity. The end product of protein digestion process is amino acids, which will be used to form egg yolk material or lipoprotein synthesis in the liver and form the egg yolk vitelline membrane (Kasiyati *et al*., 2016). Biyatmoko (2016) stated that protein availability in feed strongly influences the YI value. Protein is the main component in forming the egg yolk vitelline membrane to wrap the yolk and prevent water from the egg white into the yolk so that the yolk is not soggy. According to Rostini *et al*. (2021), protein content affects the quality of egg yolks in forming egg yolk material (vitellogenin). This material is the result of lipoprotein synthesis in the liver and will then accumulate in the follicles of the ovaries carried by the bloodstream. The mature follicle, ready to be ovulated, enters the infundibulum.

Spirulina flour feed additive significantly affected the average weight of egg yolk (p<0.05). The value of this variable belongs to the normal range. Ismoyowati & Sumarmono (2019) stated that the percentage of egg yolk ranged from 30-35% of egg weight. The weight of duck egg yolks generally ranges from 20-26 g. The average value of the egg yolk weight variable in various treatments shows that the effectiveness of spirulina flour feed is 0.6, 1.2, and 2.4%, effectively increasing the egg yolk weight in tegal ducks. Feed additive treatment with a content of 2.4% affected the heaviest egg yolk weight in Tegal ducks, which was 26.2 g. The increase in egg yolk weight is thought to occur due to increased protein content. Sunarno *et al*. (2023) stated that feed additives significantly affect the yolk weight of laying ducks. Laying ducks that are given food with nutritional content according to their body's needs will increase egg production and egg yolk weight. Egg yolk weight increases because the amino acids resulting from protein digestion can be absorbed efficiently by enterocyte cells in the small intestine. Increased protein levels and absorption of amino acids in the small intestine contribute to an increase in egg yolk volume, which causes a higher egg yolk weight. Otherwise, a lack of protein and low absorption of amino acids in the small intestine can cause the opposite effect. The bioactive compounds in spirulina flour play a role in increasing protease activity. The product of protein degradation in the form of amino acids will then be absorbed by the intestinal villi for lipoprotein synthesis (egg yolk material) in the liver. The bioactive compounds in spirulina flour (phycocyanin, xanthophyll, ß-carotene) act as protease activators by forming cross-links between the amine groups and the hydroxyl groups in proteases. This condition can increase protein degradation in the form of amino acids needed to form egg yolks. Ismoyowati & Sumarmono (2019) reported that protein, water, and minerals are the components of egg yolks.

Spirulina flour feed additives significantly affected the average albumen weight (p<0.05). The value of this variable belongs to the normal range. Ismoyowati & Sumarmono (2019) stated that the percentage of egg white is around 52.6% of egg weight. Egg white weight generally ranges from 31-38 g. The data in Table 7 shows that spirulina flour feed additive at 0.3%, 0.6%, 1.2%, and 2.4% can effectively increase egg white weight. Feed additive treatment with a content of 2.4% affected the weight of the heaviest egg white, which was 38.5 g. The viscosity of the egg white influences the high egg white weight. Ismoyowati & Sumarmono (2019) stated that high egg white weight has thick egg white, which is controlled by high protein content in the feed. Quan & Benjakul (2019) noted that the main proteins in duck eggs consist of ovalbumin, ovomucin, ovomucoid, and lysozyme. Xie *et al*. (2011) stated that the content of ovomucin and lysozyme in albumin causes albumin to thicken by binding to water and forming an albumin gel. Ramadhani *et al*. (2018) stated that egg white consists of protein (8.9%) and water (88.1%). Sunarno *et al*. (2023) added that albumen weight can be affected by egg protein content because as the level of albumen viscosity increases, it will be followed by egg weight continuing to increase. Spirulina flour feed additive treatment was able to increase egg white weight. This was related to increased protein degradation due to increased protease catalytic activity by bioactive compounds in spirulina flour (phycocyanin, xanthophyll, ß-carotene) by forming cross-links between nano chitosan and protease. More protein reduction products in the form of amino acids are produced and absorbed by the intestine to form egg whites in the magnum. Ismoyowati & Sumarmono (2019) stated that the amine groups of bioactive compounds form cross-links with the hydroxyl groups of proteases, which can increase the stability and catalytic activity of proteases in degrading proteins into amino acids which the intestinal villi will absorb, then distribute to the magnum for egg white formation.

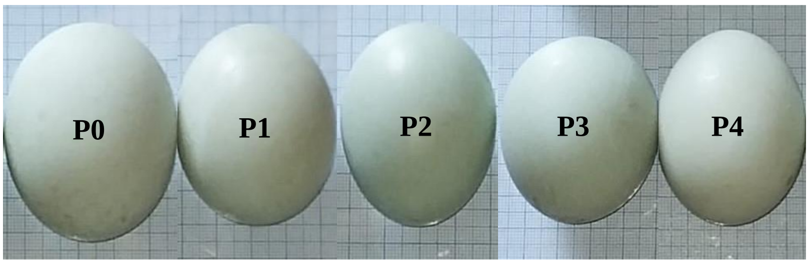

Figure 1. Size of duck egg between control and Spirulina flour feed additive treatment

Spirulina flour feed additives significantly affected the average egg weight (p<0.05). Based on Rostini *et al*. (2021), the best quality eggs weigh over 60 g. The treatment data in Table 7 shows that feed additives at 0.3, 0.6, 1.2, and 2.4% can effectively increase egg weight. Feed additive treatment with a content of 2.4% gave effect to the heaviest egg weight, 72.6 g. Egg weight in all treatment groups significantly differed from the control (Figure 1). The picture shows that the egg with the heaviest size has the largest size, namely treatment P4, and the lightest one has the smallest size. Egg weight is influenced by yolk weight and egg white weight. Kasiyati *et al*. (2016) stated that increasing egg weight is related to increasing serum estradiol secretion, ovary weight, weight and follicle diameter. This increase in egg weight is immediate in proportion to the weight of the larger and heavier yolk, and consequently has Impact on egg weight.

The size of yolk and the thickness of the egg white will add to the total egg weight. Sunarno *et al*. (2023) stated that the measurement of egg weight encompasses the combined weights of various egg components, including the eggshell, egg yolk, and egg white. The development of these three essential elements is intricately influenced by specific nutrients in the feed, including protein, lipids, carbohydrates, calcium, and phosphorus. Ismoyowati & Sumarmono (2019) reported that eggs consist of 53% egg white (albumen), 30-35% egg yolk (yolk), and 9-12% eggshell of the total egg weight. Sunarno *et al.* (2023) stated that the size of the yolk and the thickness of the egg white affect the total egg weight.

The increase in egg yolk weight and egg white weight after administration of spirulina flour feed additives will be followed by an increase in egg weight. The increase in egg weight is influenced by protein intake in the feed. Ipek & Sozcu (2017) reported that the formation of egg yolks and egg whites is greatly influenced by the effectiveness absorption of nutrients from feed, especially protein. Moran (2016) reported that protein would be converted into amino acids by proteases in the duodenum, then absorbed in the intestinal villi and distributed to the liver via the portal vein for lipoprotein synthesis, which is the formation of egg yolk material. Some are carried to the magnum for the formation of white eggs. Increased protein absorption in the intestine optimizes the formation of egg yolks and egg whites, so egg weight also increases.

In this research, feed additive treatment with spirulina flour has also been shown to influence the size of duck eggs (Figure 1). Curabay *et al.* (2021) research studies have indicated that ducks receiving spirulina flour as a feed additive tend to produce eggs with slightly different size characteristics than the control group. The size of duck eggs is typically assessed based on parameters such as egg weight, eggshell thickness, egg shape index, and circumference. Research studies have indicated that ducks receiving spirulina flour as a feed additive tend to produce eggs with slightly different size characteristics than those in the control group. For instance, in a study conducted by Sverguzova *et al*. (2021), it was found that ducks fed with a diet containing 2% spirulina flour produced eggs with an average weight that slightly higher than the the control group that received a standard diet without spirulina added.

Spirulina flour feed additives significantly affected the average eggshell thickness ($P<0.05$). Hargitai *et al*. (2021) stated that regular duck eggs have a shell thickness of around 0.35-0.56 mm. Data in Table 7 shows that the 1.2 and 2.4% of feed additives can effectively increase the eggshell thickness. Feed additive treatment of spirulina flour with a content of 2.4% had the highest effect on eggshell thickness, which was 0.48 mm. Eggshell formation is strongly influenced by the availability of calcium in the feed. Jiang *et al*. (2013) stated that high calcium in feed can improve eggshell quality. Treatment of spirulina feed additives can increase eggshell thickness. This is due to the role of bioactive compounds (phycocyanin, xanthophyll, ß-carotene) in increasing protease catalytic activity and protein digestibility so that absorption and transport of calcium are also high for eggshell formation. Nys & Roy (2018) stated that eggshell biomineralization is closely related to calcium homeostasis, involving both the transfer of calcium in the intestine and uterus, along with the resorption and mobilization of medullary bone. High protein digestibility can accelerate the absorption and transport of calcium in the intestine. High protein digestibility promotes the increased of calcium absorption due to Ca-binding protein (CaBP), or calcium-binding protein, which plays a role in transporting calcium into the intestinal mucosal cells. Kerschnitzki *et al*. (2014) stated that protein intake affects calcium mass in the eggshell because it plays a role in transporting calcium, namely the presence of calcium-binding protein (CaBP) or calbindin bonds, which play a role in transporting calcium into the intestinal mucosal cells in the duodenum. Calcium is absorbed in the intestinal mucosa, enters the capillaries, and is carried into the uterus for eggshell formation. Through the mobilization of calcium in the medulla of the bone, calcium stores in this section can provide 40 to 60% of the calcium requirements for egg shell formation. Zhang *et al.* (2022) stated that eggshell formation starts with forming the inner and outer shell membranes at the isthmus. Eggs wrapped by membranes then enter the uterus, and calcium deposition occurs in the eggshell, which takes about 17-20 hours. The shell ultrastructure is established from the sequential precipitation of mineral carbonate and organic matrix during the mineralization stages, during which large quantities of ions (Ca^{2+} and HCO^{3-}) are transferred across the uterus to supply the mineral precursors of the eggshell CaCO3 (Nys and Roy, 2018).

Spirulina flour feed additive significantly affected the average egg shape index ($P<0.05$). Duman *et al*. (2016) stated that the duck egg shape index was between (<70%) categorized as sharp, (72-76%) classified as normal or standart, and (>76%) ranked as round. The ideal egg shape index value ranged from 72-76%. The data in Table 7 shows that the spirulina flour feed additive at 0.6, 1.2, and 2.4% can effectively increase the egg shape index value. Feed additive treatment of spirulina flour with a content of 2.4% gave the highest egg shape index, namely 84.4%.

Egg size and egg weight affect the egg shape index value. A high egg shape index produces round eggs, while a low egg shape index value produces oval eggs. Treatment with spirulina flour feed additives can increase the value of the egg shape index. This is due to the positive effect of the bioactive compounds in spirulina flour (phycocyanin, xanthophyll, ß-carotene), which optimize digestive activity, significantly increasing the catalytic activity of proteases in degrading protein into amino acids needed for egg formation. It has been reported that carotenoids have a significant effect on egg quality. Carotenoids have a great effect to the color of egg yolk and egg quality (Hien *et al*., 2013). Emtyazjoo *et al.* (2022) indicated that spirulina could result a slightly altered egg shape index compared to eggs from birds on a standard diet. Eggs from spirulina-treated birds have been observed to exhibit a slightly higher egg shape index, indicating a relatively rounder or more symmetrical shape when compared to eggs from the control group. The value of the egg shape index can also be affected by differences in the size of the reproductive organs of ducks, such as differences in the size of the isthmus and uterus in each duck. The inner and outer membranes of the egg are formed in the isthmus organ; then, the egg enters the uterus to deposit calcium carbonate to form the eggshell. Rostini *et al*. (2021) stated that the isthmus and uterus with a wide diameter tend to produce round eggs, while the isthmus and uterus with a narrow diameter have oval eggs. King'ori (2012) states that the anatomical structure of the reproductive organs of ducks, such as the structure of the oviduct, distribution of the reproductive organs, and differences in the lumen diameter of the reproductive organs, influence egg size.

The ANOVA results showed a significant difference in egg protein, cholesterol, and β-carotene levels in Tegal ducks between treatment and control (P<0.05). Spirulina flour feed additives influence the quality of Tegal duck eggs, as indicated by an increase in albumen protein content, a decrease in cholesterol levels, and an increase in beta-carotene in egg yolk (Table 8). The analysis of the ANOVA test showed a significant difference in the average albumen egg protein content between treatments (P<0.05). Duncan's test at a 5% significance level showed that spirulina flour feed additive affected egg white protein content starting at P2 with a concentration of 0.6%. This indicates that adding feed additives causes an increase in egg white protein content in Tegal duck eggs. The highest protein content occurred at P4, with a concentration of 2.4%. This study's average value of Tegal duck egg white protein levels was average. Ganesan *et al*. (2014) stated that normal duck egg white protein levels ranged from 8.60-10.50%. Normal protein levels indicate that feed nutrient absorption is optimal for egg production, so the protein in egg albumen is high.

Table 8. The analysis results of the average chemical quality of Tegal duck eggs after the feed additive treatment with spirulina flour for 28 days.

Variable	Spirulina flour feed additive levels (%)				
	0	0.3	0.6	1.2	2.4
Albumen protein levels (%)	7.52^{a}±0.11	8.40^{a}±0.12	8.82^{b}±0.12	9.09^{c}±0.11	9.52^{c}±0.12
Yolk cholesterol levels (mg/100g)	432.65^{c}±9.19	402.46^{b}±8.02	408.25^{b}±7.05	367.21^{a}±3.61	357.54^{a}±13.15
Yolk beta carotene (mg/100g)	472.30^{a}±20.09	561.13^{b}±23.46	891.34^{c}±26.57	1006.95^{d}±8.93	1013.71^{d}±10.20

Note: The data displayed is the average ± standard deviation. Different superscripts on the same line show significant differences (p<0.05). P0 (Ducks fed basal diet without additive spirulina feed (control)). P1, P2, P3, and P4 were ducks fed basal feed with spirulina flour as a feed additive of 0.3, 0.6, 5, 1.2, and 2.4%.

Table 8 shows that spirulina flour feed additives treatment can increased the protein content in egg whites as the concentration of feed additives increased. Feed additive treatment with spirulina flour has been studied for its potential effects on albumen protein levels in eggs. Albumen, commonly known as egg white, is a essential component of eggs, contributing to the structure and nutritional value. Spirulina, a nutrient-rich microalgae, is often supplemented for poultry diets. The results of El-Sabrout *et al.* (2022) research have shown that when spirulina flour is added to the diet of laying hens or duck, the production of albumen protein levels in the eggs is increased. Spirulina is notably rich in high-quality protein, containing all essential amino acids, and this nutrient profile can positively impact egg composition. Feed added with spirulina flour feed additives can increase protease activity in the intestine. Proteases are enzymes that play a role in hydrolyzing proteins into simpler ones, namely peptides and amino acids. Sunarno *et al*. (2023) stated that protein content dramatically influences the process of egg formation. Amino acids absorbed by enterocyte cells in the intestinal villi will be circulated to the liver and then accumulate in the follicles in the ovaries, which are carried by the bloodstream. The mature ovum is ready to enter the infundibulum and then enter the magnum as a place to secrete albumen.

ANOVA results on the Tegal duck egg yolk cholesterol level variable showed a significant difference between treatments *(p*<0.05). Duncan's test with a significance of 5% indicated that spirulina flour feed additives affected egg yolk cholesterol levels starting at P1 with a concentration of 0.3%. This shows that the feed additives given caused a decrease in egg cholesterol levels, lower than the control. Feed additive concentrations of 0.3, 0.6, 1.2, and 2.4% can effectively reduce cholesterol levels in Tegal duck eggs. The lowest cholesterol levels occurred at P3 and P4 with 1.2 and 2.4% feed additive concentrations. Spirulina flour feed additives have various kinds of bioactive compounds such as flavonoids, alkaloids, saponins, tannins, steroids, triterpenoids, glycosides, phycocyanins, xanthophylls, and beta carotene. Some bioactive compounds have a positive charge or are cationic and can reduce egg cholesterol levels by binding mechanisms. Zhou *et al*. (2022) stated that research has shown that when spirulina flour is included as a dietary component for laying hens or ducks, it can reduce yolk cholesterol levels. This effect is attributed to the unique nutritional composition of spirulina, which contains various bioactive compounds, including phytosterols and specific peptides, known for their cholesterol-lowering properties. These bioactive compounds may interfere with the absorption of dietary cholesterol in the bird's digestive system, resulting in lower cholesterol incorporation into the developing egg yolk. The effect of spirulina in lowering the cholesterol levels is inhibits cholesterol absorption. Cholesterol will come out along with spirulina bioactive compounds in the form of feces. Spirulina bioactive compounds can bind bile acids and mixed micelle components such as monoacylglycerol, free fatty acids, or free cholesterol. This causes reduced micelle dissolution of the lipid portion and reduced cholesterol absorption in the small intestine. Decreased absorption of cholesterol in the intestine can cause a decrease in the level of cholesterol formed in eggs. Tomaszewska *et al*. (2021) stated that the formation of cholesterol in eggs begins when the feed contained in the intestine occurs through the absorption of feed nutrients. The absorbed nutrients are circulated through the hepatic portal vein to the liver, channeled through the blood vessels in the form of lipoproteins, and channeled into the ovarian follicles. The mechanism of spirulina bioactive compounds in reducing cholesterol levels can also occur through inhibiting cholesterol metabolism. The bioactive spirulina compound can inhibit the activity of the HMG-CoA reductase enzyme, which is an enzyme that has a role in converting HMG-CoA substrates to mevalonate in cholesterol biosynthesis, which results in the flow of egg cholesterol formation being blocked so that the egg cholesterol formed was low.

ANOVA results on the variable beta carotene levels in Tegal duck egg yolks showed significant differences between treatments (P<0.05). Duncan's test with a significance of 5% indicated that the spirulina flour feed additive affected beta carotene levels in egg yolk starting from treatment P1, namely giving Spirulina flour at a level of 0.3%. This shows that the feed additives caused a more significant increase in beta carotene levels in egg yolk than in the control. Feed additives at higher levels will increase beta-carotene levels in egg yolk, as shown in P2, P3, and P4. The highest levels of beta carotene were found in treatments P3 and P4, higher than the control and other treatments (Table 8). Beta carotene levels in egg yolk are closely related to orange color. High levels of beta carotene will contribute to a more orange egg yolk. Figure 2 shows that treatments P1, P2, P3, and P4 affect the egg yolk color, it has a more intense orange than the control. The more Spirulina flour is added to the feed, the more intense orange color of egg yolk will be. Duck egg yolks with an intense orange color indicate that the beta carotene content in the egg yolk is higher. Khan *et al*. (2023) research showed that spirulina supplementation can lead to a notable increase in beta-carotene levels in the yolks of Tegal duck eggs. Spirulina is naturally rich in beta-carotene, transferred from the duck's diet to the developing egg yolk. As a result, the yolks of eggs produced by ducks on a spirulina-supplemented diet take on a vibrant orange-yellow color, visually indicative of higher beta-carotene levels.

Figure 2. Egg yolk color between control and Spirulina flour feed additive treatment

Duck feed with the addition of Spirulina flour additives containing beta carotene causes this pigment, after being absorbed by the cells of the small intestine, to be deposited into vitellogenin. This material forms egg yolk in the liver. Vitellogenin, which contains beta-carotene, will then be deposited into the duck egg yolk, giving it an orange color. The more beta carotene content will make the orange color stronger and brighter. Calislar (2019) stated that the beta carotene pigment content in feed will influence the orange color of egg yolks. Bovskova *et al*. (2014) noted that beta-carotene is the compound that causes yolk to turn yellow. This type of pigment will give a bright yellow, orange, or orange solid color to the egg yolk, and this pigment is fat-soluble. Beta carotene pigment has a significant influence in giving egg yolk color, and this pigment is known to not contain oxygen atoms in its chemical structure, either in the form of hydroxyl, carbonyl, or aldehyde. Beta carotene is absorbed by enterocyte cells in the duodenum of the small intestine, and its absorption will be faster by lipids in the intestinal tract. Beta carotene can be converted into vitamin A through an oxidative process, especially on the outer membrane surface of enterocyte cells, in organs such as the liver and kidneys, and lungs. Beta carotene in egg yolks will be transported to and stored in the immune organs and similar network (Calislar, 2019)

The absorption of beta carotene in the duck's body occurs through the consumption of feed containing this pigment and through the formation of a complex between fat micelles and beta carotene. Beta carotene pigment is not absorbed alone but together with fat. The absorption of the fat-beta carotene complex is carried out by enterocyte cells in the small intestine's villi, then transported by lipoproteins in the blood plasma and deposited into the target tissue, namely yolk in ducks during the production period. Increasing Spirulina flour levels gives duck egg yolks an increasingly orange color. This condition provides evidence that the color of the yolk is influenced by the level and rate of deposition of the beta-carotene pigment contained in the feed consumed by the ducks. The higher level of beta carotene in Spirulina flour caused higher the deposition rate of beta carotene in the egg yolk, so that the egg yolk will have an intense orange color. The evidence from this research is strengthened by the results of research conducted by Gao *et al*. (2021), which states that adding 500-700 mg/l of beta carotene can significantly increase the color of yolk in laying hens. Apart from being an indicator of egg quality, the beta carotene pigment in egg yolk also has an essential function as a precursor for forming vitamin A and antioxidants in the duck's body. Duck eggs with a high beta carotene content are very beneficial for health because they can improve the function of the body's defense system, increase the production of red blood cells, improve reproductive performance and other body organs.

CONCLUSIONS

Spirulina flour-based feed additive of 0.3-2.4% concentration can improve the physical and chemical quality of Tegal-laying ducks' egg.

Further research is needed to examine the dominant phytochemical compounds, purification of beta carotene and phycocyanin pigments in Spirulina flour and their potential in improving the physical and chemical quality of eggs in Tegal laying ducks.

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