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Effect of supplementing red pigment from *Monascus* sp. fermented native black rice (Maepayathong Dum Rice) on production performance and egg quality in laying hens

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Abstract The results indicated that the addition of red pigments led to a deeper-colored yolk compared to the control group, with the highest intensity observed at a 6% red pigments. Furthermore, the addition of red pigments is promoted the quality of chicken eggs and production performance. It indicated that Maepayathong dum rice could be used as a raw material for producing natural pigments to replace synthetic dyes in egg-laying hen feed.

Keywords: Red pigment, *Monascus* sp., Production performance, Egg quality, Egg yolk

Introduction

The poultry industry plays a crucial role in egg production to meet the global consumption demands, as eggs are a widely consumed source of protein. Egg production efficiency and quality are also important factors for the success of laying hen production. Eggs are a widely consumed source of protein. Egg production efficiency and quality are also important factors for the success of laying hen production. Producers strive to maximize egg production while maintaining high egg quality standards, such as shell strength, egg white quality, and yolk color. In addition, among the factors affecting consumer demand and market value, egg yolk color is a particularly important factor. Brightly colored and deep-yolk colored are often associated with higher nutritional quality,

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leading manufacturers to seek ways to increase the color of egg yolks. In general, synthetic colors are used to achieve this goal. If used in large quantities or for a long period of time, it will cause residues in the chicken and in the chicken eggs, affecting the safety of consumers. Therefore, there is a study on the use of herbs, natural antioxidants, plant or microbial colorants that have properties to promote the health of laying hens, increase production and egg quality, to replace the use of synthetic colors and antibiotics. Currently, microbial colorants are products that are gaining much attention for use as additives in laying hen feed (Naraini and Latif, 2012; Punyatong, 2012; Sun *et al.*, 2015).

Red pigment, also known as red rice (Angkak or red yeast rice), is a product derived from the fermentation of the fungus *Monascus* sp. on cereals such as rice, millet, and agricultural wastes. Typically, this fungus initially produces white hyphae, which later turns red as it matures due to the production of yellow-red pigments. In addition to these pigments, *Monascus* sp. also produces monacolin K, a compound that can help reduce cholesterol levels in blood (Naraini and Latif, 2012), and GABA (Gamma amino butyric acid), an amino acid with neurotransmitter properties that can reduce stress and lower blood pressure (Su *et al*., 2003). Because of these beneficial properties, pigments from *Monascus* sp. are widely used as ingredients in various products, including pickled bean curd, health drinks, cosmetic products, and animal feed. In addition, Wang and Pan (2003) reported that feeding egg-laying hens with red pigment from *M. purpureus* NTU803, cholesterol in eggs can be decreased than the control group (no red pigment). Wang *et al*. (2006) also found that adding red pigment from *Monacus* sp. to the diet of Arbor Acres broiler chickens, their meat products contain greater level of unsaturated fatty acids but triglyceride and choreterol concentration in serum were lower in experimental group when compared to control group.

Maepayathong dam rice is a native rice variety of the Chong people, the indigenous population of Chanthaburi Province, Thailand (Mongkontanawat *et al.*, 2018). The bran of this rice has a purplish-black color. According to Sangkitikomon *et al.* (2008), the anthocyanins from this black rice have higher antioxidant activity than those found in red rice and Riceberry rice. Moreover, it is nutritionally rich, containing higher amounts of dietary fiber, phytic acid, and various vitamins (E and B) compared to ordinary rice. However, this rice variety is currently cultivated in only one location and can only be grown once a year. Additionally, it is not widely consumed, which could lead to its extinction. The research investigation aimed to use Maepayathong dam rice as a raw material for pigment production by *Monascus* sp. The produced pigment was added to the diet of laying hen and the effect of pigment on the production performance and quality of chicken eggs were investigated.

Materials and methods

Microorganisms and raw material

In this study, the fungus *Monascus purpureus* TISTR3090 was obtained from Thailand Institute of Scientific and Technological Research (TISTR), Thailand and used for red pigment production. It was maintained on potato dextrose agar (PDA) (HiMedia, India) slants and transferred monthly.

Maepayatong dum rice (Native black rice) was purchased from a local farmer in Makam city, Kao Kitchakut district, Chanthaburi province, Thailand and used as substrate for producing red pigment.

Preparation of seed culture

The seed culture was prepared by growing *M. purpureus* on PDA plate at 30°C for 7 days. Then, the tip of the mycelium was cut using cork borer and placed in PDA slants. After, the slants were incubated at 30°C for 7 days, The spore suspension was performed by adding 10 mL of sterilized distilled water. Next, the spore suspension was performed by scrapping the spore under aseptic condition and used as the seed culture (1.0×10^6 spores/mL).

Production of red pigments by the fermentation of M. purpureus on Maepayathong dum rice

The production of red pigment was conducted by washing and soaking Maepayathong Dum rice in water at room temperature for overnight. Then, it was filtrated with a sieve and 50 grams of the soaked rice was added into 250-mL Erlenmeyer flasks, containing 10 mL of distilled water. Next, the flasks were autoclaved at 121°C for 15 minutes and cooled to room temperature. The 5% of seed culture was added to the sterilized rice and mixed together. After, 10 days of incubation at 30°C the red pigment was harvested by autoclaving and drying in a hot air oven at 50°C for 8 h. Finally, the dry red pigment was grinded to obtain a red powder for use as a feed additive for laying hens.

Proximate analysis of red pigment

The carbohydrate, protein, fat, ash, moisture, pulp and fiber content of red yeast rice from *M. purpureus* were analyzed according to the AOAC (2000) method.

Pigment analysis

The amount of the produced pigments was analyzed according to the method of Babitha *et al.* (2007) by a slightly modification. One grams of red pigment powder was added into a test tube containing 5 ml of 95% ethanol. The test tube was shaken at 200 rpm for an hour and then filtrated with Whatman No.1 filter paper (red pigment extract). The filtrated was measured the absorbance at 412 (yellow) and 500 (red) nm, using a spectrophotometer (Lambda 365, Perkin Elmer, USA). The amount of pigments produced by the culture was reported as the unit of absorbance (OD) per gram dry weight.

Analysis of total phenolic content

The total phenolic content (TPC) was examined using a modified method of Wong *et al.* (2005). The 50 µL of red pigment extract was added into a test tube. Then, the 2.0 mL of 10% Folin-Ciocalteu reagent (Sigma, Swizerland), 2.0 mL of 7% sodium carbonate solution and 2.0 mL of distilled water were transferred into the test tube as a sequence. The mixer was placed in the dark room for and hour. Next, the absorbance was measured at 765 nm using a spectrophotometer (Lambda 365, Perkin Elmer, USA) and compared with a standard curve of gallic acid solution. The TPC result was expressed as µg gallic acid equivalent per mililiter of sample (µg GAE/mL sample).

Antioxidant activity assay

For the antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay according to method of Shimada *et al.* (1992) by a slightly modification. The 100 µL of red pigment extract and 209 mL of 0.1 mM DPPH solution (Sigma, USA) was added into a test tube, respectively. Then, the mixer was placed in the dark room for 30 minutes. The absorbance of DPPH solution ($OD_{control}$) and red pigment sample with DPPH solution (OD_{sample}) was measured at 517 nm using a spectrophotometer (Lambda 365, Perkin Elmer, USA). The DPPH scavenging activity was calculated using the below equation:

$$\text{DPPH scavenging activity (\%)} = [(OD_{control} - OD_{sample})/OD_{control}] \times 100$$

Animal and experimental design

A total of 120 commercial laying hens (Isa Brown), aged 28 weeks, were used for this study over an 8-week period. The hens were kept in cages measuring

60 cm x 60 cm x 40 cm, with two hens per cage, in an open housing environment maintained at around 30 degrees Celsius. The experiment was employed in a Completely Randomized Design (CRD). The hens were randomly assigned to four treatments: control (0% red pigment), and supplemented with red pigment at levels of 2%, 4%, and 6%, with 5 replicates of 6 birds each. The hens were fed a diet containing 16% crude protein and 2,700 ME kcal/kg feed, with other nutrients provided to meet the needs of laying hens (complete feed). During the experiment, feed and clean drinking water were available ad libitum, and the diets were provided twice daily at 8:00 AM and 4:00 PM.

Production performance and egg quality

The number of eggs and their weight were recorded daily to determine the laying rate and average egg weight. Weekly feed consumption was measured and expressed as grams per day per hen. The feed conversion ratio was determined by dividing the total weekly feed intake by the total weekly egg weight.

Egg quality was assessed weekly. For each treatment group, three eggs from each replication were randomly sampled for analysis. The evaluation included eggshell thickness, eggshell weight, yolk color, yolk weight, albumen weight, albumen height, and Haugh Unit. Eggshell thickness was measured at three locations (air cell, equator, and sharp end) using a vernier caliper. Yolk color was determined with a yolk color fan (scale of 1-15, from light yellow to orange), and albumen height was measured using an egg quality analyzer.

Statistical analysis

All experimental treatments were done in three replications (n = 3). The data were analyzed with analysis of variance (ANOVA). Results are presented as means and standard deviations (SD). Differences between groups were assessed using Duncan's Multiple Range Test (DMRT) at a 95% confidence level ($p<0.05$). Except, comparison of pigment assay using water versus ethanol was statically analyzed by student’s *t*-test

Results

Production of red pigment from M. purpureus

Red pigment production through fermentation using *M. purpureus* on Maepayathong dam rice showed that the fungus initially forms white mycelium during the first 2-3 days. The mycelia are changed to a yellow-orange color, and after drying, the rice becomes a deep red color (Figure 1.). It was observed that 1 kilogram of Maepayathong dam rice could be produced 504 grams dry weight of red pigment powder (50.4% yield).

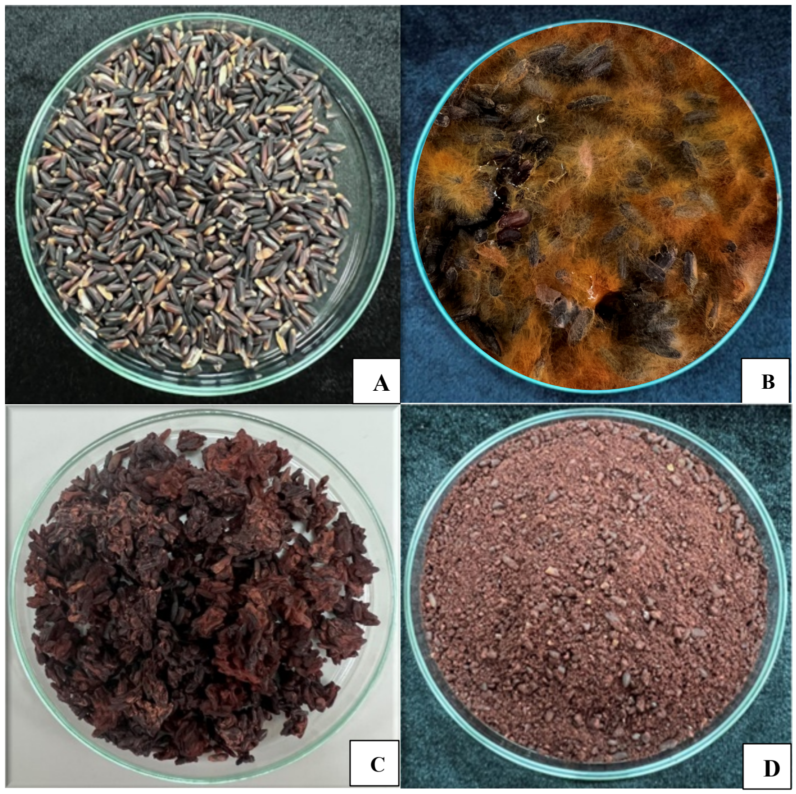

Figure 1. Characteristics of (A) Maepayathong dam rice (B) mycelium of *M. purpureus* growing on Maepayathong dam rice (C) red pigment obtained after drying and (D) red pigment powder

Pigment quantity and nutritional value of red pigments

The amount of red pigment produced when extracted with water ranged from 7.16±0.07 to 13.17±0.17 OD per gram of dry weight, with the highest yield of yellow pigment, followed by orange and red pigments in that order. In contrast, extraction with 95% ethanol yielded pigment amounts ranging from 8.32±0.35 to 22.10±0.98 OD per gram of dry weight, with the highest yield of yellow pigment, followed by red and orange pigments, respectively. The study showed that *M. purpureus* was grown on Maepayathong dam rice produced the highest quantity of yellow pigment, and extraction with 95% ethanol was more effective than extraction with water (Figure 2). The produced red pigment composed of the highest carbohydrate content, followed by crude fiber, moisture, crude protein, ash and the lowest fat content (Table 2).

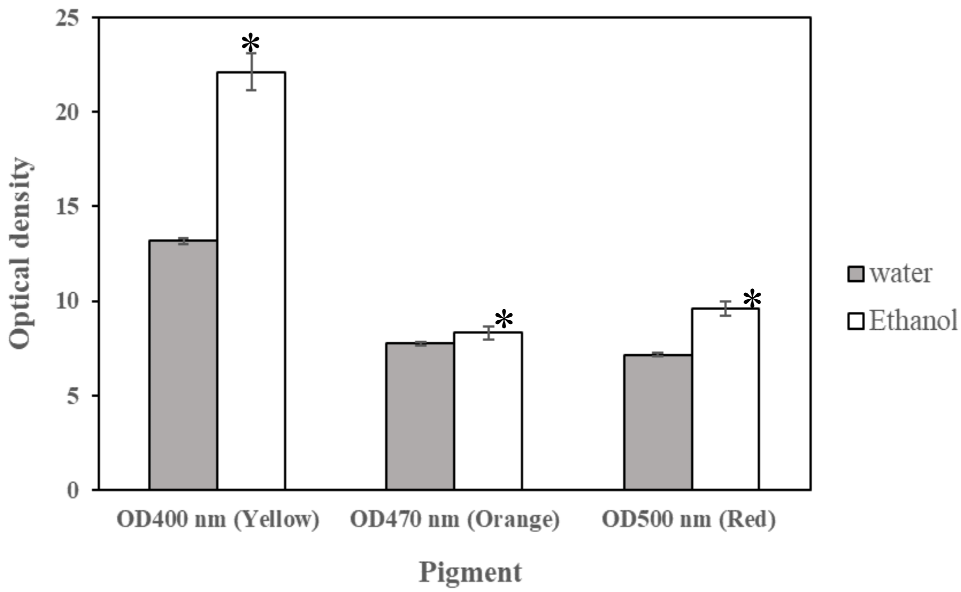

Figure 2. The amount of pigments obtained from extraction with different solvents: Results obtained are presented as means ± SD. Data were analyzed using student's t-test. * significantly (P<0.05)

Table 2. Nutritional composition of red pigment produced by fermentation of *M. purpureus*

Nutritional composition	(%)
Crude protein	2.39
Carbohydrate	68.27
Fat	1.20
Ash	1.23
Moisture	6.25
Crude fiber	20.66

Total phenolic content and antioxidant activity

The results of the total phenolic compounds and DPPH antioxidant activity of the red pigment produced by the fermentation process of the *M. purpureus* on Maepayathong dam rice showed that the total phenolic compound content was 29.14±9.74 mg GAE/mg, and the DPPH radical scavenging activity was 42.77±4.46%.

Production performance

Throughout the rearing period, the layer hens had a 100% survival rate. Egg production data showed that a 6% red pigment supplementation in the feed resulted in the highest egg production, followed by 2% red pigment supplementation (P<0.05). Laying hens supplemented with 4% red pigment had the lowest feed intake. The feed conversion ratio in the groups supplemented with 4% and 6% red pigment showed no significant difference and was lower than that of the control group and the group supplemented with 2% red pigment (Table 3).

Table 3. Effect of red pigment supplementation on laying hen production performance

Items	Red pigment in diet (%)			
	0	**2**	**4**	**6**
Egg production rate (%)	$84.29 \pm 2.74^{b/1}$	88.57 ± 2.16^{a}	85.71 ± 1.93^{b}	91.43 ± 1.87^{a}
Feed intake (g/hen/day)	123.43 ± 1.21^{ab}	125.14 ± 1.03^{b}	122.86 ± 1.25^{a}	124.71 ± 0.98^{b}
Feed conversion ratio	2.05 ± 0.31^{b}	2.01 ± 0.52^{b}	1.98 ± 0.80^{a}	1.92 ± 0.42^{a}

1/: Data are presented as means ± SD. Mean values within each row with different superscript differ significant (P<0.05)

Egg quality

Result showed that supplementation with red pigment was statistically increased the egg weight, yolk weight, and yolk color compared to the control group (P<0.05) as shown in Table 4. Supplementation at a 6% level led to the highest egg white weight, which likely contributed to the greater total egg weight in this group. Eggshell thickness was similar across all treatments, with no significant differences observed (P>0.05). In terms of Haugh unit values, the 6% red pigment supplementation showed no significant difference from the control group, but it was higher than the values recorded in the 2% and 4% supplementation groups. However, all groups maintained Haugh unit values within the AA quality grade, as per both the Thai standard for egg freshness (H.U. > 72) and the international standard (H.U. between 83-100).

Table 4. Effect of red pigment supplementation on egg quality in laying hens

Items	Red pigment in diet (%)			
	0	2	4	6
Egg weight (gram)	60.17±0.94$^{d/1}$	63.54±1.02^{c}	65.38±0.85^{b}	68.60±1.31^{a}
Eggshell thickness (mm.)	0.38 ± 2.11	0.38±.97	0.37±1.67	0.39±1.89
Eggshell weight (gram)	8.35±1.56^{c}	9.45±0.98^{b}	10.05±1.34^{b}	11.58±1.06^{a}
Yolk color (Roche 1-15)	9.00±1.59^{c}	10.00±1.24^{b}	12.00±1.13^{a}	12.75±1.65^{a}
Yolk weight (gram)	14.33±0.88^{b}	16.68±0.76^{a}	16.20±0.92^{a}	16.40±0.63^{a}
Albumen weight (gram)	37.83±0.59^{b}	36.23±0.83^{b}	35.93±0.77^{bc}	41.95±0.85^{a}
Albumen high (mm.)	12.36±0.67^{a}	10.57±0.64^{b}	10.65±0.72^{b}	12.36±0.81^{a}
Haugh Unit	98.57±0.73^{a}	91.56±0.85^{b}	91.96±0.79^{b}	97.28±0.83^{a}

1/: Data are presented as means ± SD. Mean values within each row with different superscript differ significant (P<0.05)

In addition, adding diets with red pigment is produced by *Monascus* sp. led to increase egg yolk color at all levels compared to the control group (P<0.05). The yolk color varied from yellow to orange, depending on the amount of red pigment added to the diets (Figure 2).

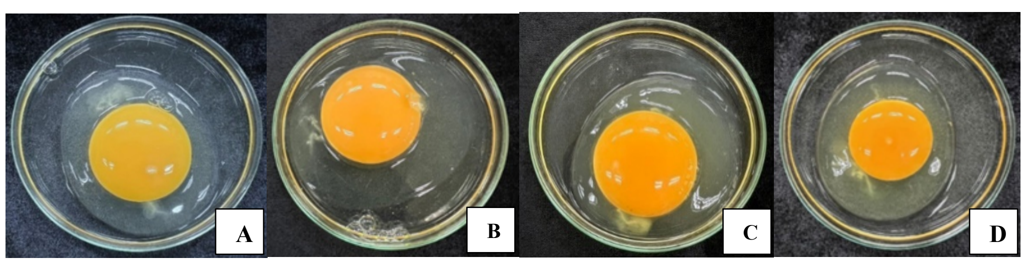

Figure 2. Eggs supplemented with red pigment at various levels: (A) 0%; (B) 2%; (C) 4% and (D) 6%

Discussion

Rice is the most popular raw material for producing pigments from *Monascus* sp. (Husakova *et al.,* 2021; Zhang *et al.,* 2024). In this study Maepayathong dam rice was used as the raw material in the fermentation process of *Monascus* sp. to produce pigments for use as a supplementary substance to the diet of laying hen. The *M. purpureus* was choosen as a pigment producing microorganism because it is a commercially important strains and is widely used in food, beverage and pigment production (Lagashetti *et al.,* 2019). This fungus can produce various bioactive compounds, including phenols, tannins, flavonoids, and polyketides (pigments) (Kaur *et al.,* 2023). The analysis of pigment content revealed that extraction with 95% ethanol yielded better results than extraction with distilled water. This may be due to that distilled water has a very high polarity and contains only hydroxyl (OH^-) groups in its molecules. In contrast, 95% ethanol composed of 5% water and 95% ethanol and it has a unique structure that allows it to react with both non-polar and polar substances. This is because it contains a hydroxyl group with high electronegativity of oxygen indicating polarity while the ethyl (C_2H_5) group indicates non-polarity. The hydrogen bonds in water are mixed with ethanol, resulting in a polarity similar to the pigment produced by *M. purpureus*, which leads to better pigment extraction. This finding is consistent with a previous study (Daud *et al.,* 2020). The amount of red pigment was analyzed and range from 7.16±0.07 to 9.59±0.40 OD/g dry weight, which differs from the report by Chairote *et al.* (2007). In their study the production of red pigment by *M. purpureus* isolated from commercial Chinese red yeast rice using various Thai glutinous rice varieties including Mali105, Kam black glutinous rice (Kam), Korkor 6 white glutinous rice (RD6) and Sanpatong 1 white glutinous rice (SPT1) as raw materials were conducted. It was found that after two weeks of fermentation, the highest production of red pigment was observed on RD6, followed by SPT1, Mali105 and Kam, with values of 30.11, 29.63, 4.51, and 3.63 AU/g, respectively. Mehri *et al.* (2021) report that the highest red pigment production using demineralized whey medium with 75 g/L of lactose, 25 g/L of monosodium glutamate, 2%(v/v) of inoculation ratio and fermentation medium pH of 7.0 by simultaneous hydrolysis and fermentation (SHF) process was received as 38.4 AU/g. The differences in the amount of red pigment could be attributed to raw materials, composition of culture medium and the conditions for culturing this fungus. The amount of red pigment produced in this study was relatively low. In order to increase the yield, it would be necessary to optimize the components of the medium, such as the carbon source, nitrogen source, and supplementary substamnces, as well as the conditions that promote growth and pigment production.

In the produced red pigment powder, the phenolic compounds were detected in the range of 29.14±9.74 mg GAE/g, and the DPPH radical scavenging activity was 42.77±4.46%. Chaudhary *et al.* (2024) produced pigment from *M. purpureus* MTCC-369 using broken rice as a solid substrate. They found that the 1-640 µg/mL fractions of the extracted red pigment in indicated increase in the total phenolic content from 3.25 µg GAE/g to 62.70 µg GAE/g (19.29-fold). Liu *et al.* (2022) reported that red yeast rice was produced by *M. purpureus* and extracted by ultrasonic-assisted extraction method. It showed the maximum scavengenging DPPH activity of 34.29%. The presence of bioactive compounds and antioxidant activity in the pigment produced by the *Monascus* sp. may be attributed to the production of certain metabolites, polyketide pigments, and dimerumic acid during the fermentation process, which have antioxidant and ion scavenging potential (Cheng *et al.,* 2016; Chaudhary *et al.,* 2022; Smith *et al.,* 2015). Additionally, Huynh *et al.* (2014) found that during the filamentous growth phase, enzymes such as β-glucosidase, cellulase, esterase, and xylanase are produced, which play a role in releasing phenolic compounds, tannins, and flavonoids. The varying amounts of phenolic compounds and DPPH antioxidant activity observed in each study are due to differences in cultivation conditions, varying quantification methods, and the strains of fungi used.

The addition of red pigment derived from the fermentation of *Monascus* sp. enhanced egg production and quality. It was found that the egg production rate, egg weight, yolk color, yolk weight, and albumen weight in the group supplemented with red pigment were higher than the control group, with the optimal results observed at a 6% supplementation level. This aligns with the research by Naraini and Latif (2012), who studied the use of pigment from *M. purpureus* fermented in Sago and Tofu wastes as a feed additive for Isa Brown laying hens. Their study found that the group supplemented with *M. purpureus* pigment showed improved feed intake, egg production, feed conversion, and yolk color compared to the control group (P<0.01), with the best results at a 30% supplementation level. Additionally, cholesterol levels in eggs were reduced by 31.49%, the highest observed reduction, while yolk color increased by 18.56%. Similarly, Sun *et al.* (2015) found that adding red yeast rice (pigment from *Monascus* sp.) to the feed of Lohmann Brown laying hens at 1 and 5 g/kg increased albumen height and Haugh units in proportion to the supplementation amount. They also observed a reduction in yolk cholesterol levels (P<0.05) at the 5 g/kg supplementation level after 56 days, and red rice supplementation reduced serum cholesterol and triglycerides, though egg production efficiency remained unaffected. Wang and Pan (2003) studied the effects of red pigment from fermentation of *M. purpureus* at levels of 0, 2, 5, and 8% on serum and egg yolk cholesterol in White Leghorn hens. They discovered that monacolin K in red pigment significantly reduced cholesterol in eggs (P<0.05). Triglycerides and low-density lipoprotein (LDL) levels also decreased with higher red pigment supplementation (P<0.05), but there was no effect on high-density lipoprotein (HDL). This is consistent with Wong *et al.* (2005), who found that red pigment supplementation at 2 and 4% in Hyline Brown layers reduced egg yolk cholesterol levels (P<0.05) without affecting egg production or feed conversion ratio. Similarly, Yong *et al.* (2021) reported that monacolin K reduced egg cholesterol by 11.16% (P<0.05) when red yeast rice was supplemented at 0.8%, though it had no effect on egg production efficiency. In a study on quails, Pengnoi *et al.* (2018) reported that supplementing red pigment at levels of 6, 12 and 24 mg/day/bird had no effect on body weight, egg weight, eggshell weight, egg white weight, or yolk weight. However, it decreased feed intake, feed conversion ratio, and yolk cholesterol levels compared to the control group. Egg production was higher in the red pigment-supplemented groups, and cholesterol levels were lower in the groups receiving 12 and 24 mg/day/bird compared to the group receiving 6 mg/day/bird.

The study results of Maepayathong dam rice could be used to produce pigments by the fermentation process of *Monascus* sp. The pigments contained bioactive compounds, such as phenolic compounds, and exhibit DPPH antioxidant activity. When supplemented in laying hen feed, it was found to enhance production performance and egg quality, especially improving the yolk color, which is found to be desirable for consumers and the bakery industry. The supplementation at a level of 6% yielded the best results.

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