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Possibility of using nutmeg flesh (M*yristica fragrans* houtt) extract in broiler diet to improve intestinal morphology, bacterial population, blood profile and antioxidant status of broilers under high-density condition

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Abstract. This study investigated the impact of nutmeg flesh extract on intestinal morphology, bacterial population, blood profile and antioxidant status of broiler chickens stocked at high density pens. After 15 days of rearing, 370 Lohmann broiler chicks (unsexed) were assigned to five treatment groups based on a completely randomized design, including T0 (chicks were raised at a density of 10 birds m^{-2} and received no nutmeg flesh extract), T1 (chicks were raised at a density of 16 birds m^{-2} and received no nutmeg flesh extract), T2 (chicks were raised at a density of 16 birds m^{-2} and received 0.5 mL kg^{-1} nutmeg flesh extract), T3 (chicks were raised at a density of 16 birds m^{-2} and received 1.0 mL kg^{-1} nutmeg flesh extract), and T4 (chicks were raised at a density of 16 birds m^{-2} and received 1.5 mL kg^{-1} nutmeg flesh extract). Sampling was conducted on day 35 of age. The results showed that the administration of nutmeg flesh extract (regardless of its levels) to broilers diets at high density (16 birds m^{-2}) significantly ($p \leq 0.05$) increased the performance, villi height in the duodenum, jejunum and ileum, decreased pH in the ileum and cecum, increased lactic acid bacteria and decreased coliform bacteria in the ileum and cecum, decreased malondialdehyde (MDA) and increased superoxide dismutase (SOD) and had no significant effect ($p > 0.05$) on the blood profile of broiler chickens. The findings demonstrated that nutmeg flesh extract improved performance, intestinal bacterial population and morphology and antioxidative status of broilers raised under high density condition.

Key words: antioxidant, broilers, intestinal bacterial population, intestinal morphology, nutmeg flesh extract.

INTRODUCTION

Raising of broilers at high densities causes broilers to experience pressure and stress during their growth period, although rearing at high densities is a strategy to gain profits by increasing meat production per square meter. Apart from the production efficiency, increasing the density of the cage has a negative influence on the intestinal microbial

balance, reduces beneficial bacteria, and increases pathogenic bacteria, resulting in decreased growth performance of broilers (Astaneh et al., 2018). Stress causes adverse effects on the physiology, immunology, and microbiology of broiler chickens which in turn can impair chicken performance (Sugiharto et al., 2017a). In particular, stress due to high density decreases body weight gain and feed consumption, decreases the quality of poultry products, and in severe cases increases mortality (Silas et al., 2014; Agusetyaningsih et al., 2022; Sugiharto & Turrini 2022). Stress due to high density has been reported to impair intestinal function associated with impaired nutrient absorption, causing an increase in the heterophile to lymphocyte ratio (H/L ratio) as well as involution of lymphoid organs (Astaneh et al., 2018). Furthermore, Sugiharto & Turrini (2022) reported that stress due to high density causes a decrease in antioxidant enzymes activities.

Molecular changes due to stress are reported to increase the production of free radicals or reactive oxygen species (ROS) and trigger oxidative stress (Sugiharto et al., 2019). To reduce the negative impact of oxidative stress, feed supplementation using synthetic antioxidants is common in broiler rearing practices. However, the use of synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) in the long term and in excess doses can leave residues in the meat that endanger the health of broiler consumers. Owing to these facts, natural sources of antioxidants are needed so that the use of synthetic antioxidants in broiler chickens can be reduced. Recent knowledge shows that plant extracts contain many phenolic compounds that can function as antioxidants. Herbal extracts have been reported to improve intestinal morphology (Liu et al., 2021), increasing the growth of beneficial bacteria in the digestive tract (Martínez et al., 2021), and improve broiler immunity (Agusetyaningsih et al., 2022). The application of herbal extracts in poultry feed has been reported to increase body weight gain, improve the rate of nutrient metabolism, and improve meat quality by lowering cholesterol levels and inhibiting lipid peroxidation (Oloruntola et al., 2020).

Nutmeg (*Myristica fragrans* Houtt) is a native Indonesian plant that is widely used as a spice. Nutmeg is an aromatic tropical plant (with a distinctive aroma) and has bioactive substances and can function as antioxidant, antimicrobial, painkiller, antiobesity, and hepatoprotective (Periasamy et al., 2016; Vangoori et al., 2019). Nutmeg consists of flesh, seed, and mace. Seed and mace are the main product of nutmeg which is used as spice, while nutmeg flesh is a waste that has no economic value. So far, studies regarding the use of nutmeg flesh extract on broilers to reduce the negative impact of stress due to high density (16 birds m^{-2}) have never been reported. Taken the antioxidative and antimicrobial properties of nutmeg flesh into consideration, administration of nutmeg flesh extract was expected to improve intestinal morphology, bacterial population, blood profile and antioxidant status of broilers reared under high density condition. Currently nutmeg flesh has not been used, and as waste nutmeg flesh can have a negative impact on environmental health if not treated properly. Therefore, the application of nutmeg flesh in broiler chicken production not only has a positive impact on chicken health and productivity, but also has a beneficial impact on the environment. Overall, the present study aimed to investigate the impact of nutmeg flesh extract on intestinal morphology, bacterial population, blood profile and antioxidant status of broilers reared under high density conditions. It was hypothesized that nutmeg

flesh extract improved intestinal morphology and bacterial population, blood profile and antioxidant status of broilers stocked in high density pens.

MATERIALS AND METHODS

Preparation of nutmeg flesh extract

Nutmeg flesh discarded by farmers after harvesting was obtained from the nutmeg plantation in Ternate City, North Maluku Province, Indonesia. Before use, the nutmeg flesh was peeled and thinly sliced, then air-dried and ground into flour before use. A 1 kg of nutmeg flesh flour was extracted based on a maceration technique by soaking in 4 litre of 96% ethanol solution for 3×24 hours. During the maceration process, stirring was carried out twice, i.e., in the morning and evening. The results of maceration in the form of filtrate were then filtered and evaporated using a rotary evaporator to produce a solution of nutmeg flesh extract (Sapsuha et al., 2021).

Antioxidant activity and phytochemical composition of nutmeg flesh extract

The AOAC method (AOAC, 2007) was used to determine the proximate content of nutmeg flesh extract. Total phenol content was determined using the Folin-Ciocalteu method (Sahreen et al., 2010), and total flavonoid content was determined using the spectrophotometric method (Mayur et al., 2010). The 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) assay was used to determine antioxidant activity (Sochor et al., 2010). The disc diffusion method (Kirby-bauer) was used to conduct the antibacterial inhibition test against *Escherichia coli*.

***In vivo* Experiment**

The Animal Ethical Committee of the Faculty of Animal and Agricultural Sciences, Universitas Diponegoro, Semarang approved the *in vivo* study with the approval number 57-01/A-7/KEP-FPP. A total of 370 Lohmann broiler chickens (unsexed) were reared together from 0 to 14 days of age. From day 15 onward, the chickens (average body weight of 420 ± 2.75 g) were allocated into five treatment groups (based on a completely randomized arrangement), including T0, T1, T2, T3, and T4, with five replicate pens in each group. The birds in T0 were reared at a density of 10 birds m^{-2} (i.e., normal stocking density), while the birds in T1, T2, T3, and T4 were reared at a density of 16 birds m^{-2} (i.e., high stocking density). Overall, the treatment group T0 consisted of 50 birds and for T1, T2, T3 and T4 each consisted of 80 birds. Chicks in groups T0 and T1 were not provided with nutmeg flesh extract. Chicks in groups T2, T3, and T4 were fed with diets containing nutmeg flesh extract at doses of 0.5, 1.0, and 1.5 g kg^{-1}, respectively. Because research using nutmeg flesh extract as an additive for broiler feed is still rare, the determination of the level of use of nutmeg flesh extract in feed was based on the fact that in general the use of feed additives for broiler chickens ranges from 0.05 to 0.2% of the total feed. The nutmeg flesh extract was added to the feed ('on top') after all the ingredients for the broiler ration were mixed. The chicks were distributed into treatment groups starting on the 15^{th} day because on that day the body weight of the chickens had reached more than 400 g $bird^{-1}$, allowing stress due to high density starting to occur in the chickens. In addition, the digestive organs in broilers have developed optimally in broilers aged 15 days so that the utilization of nutmeg flesh extract can be optimal. The

feed given was in the form of mash and formulated (Table 1) as starter feed (days 1–21) and finisher feed (days 22–35). The entire formulated feed did not contain antioxidants, enzymes, antibacterial, antifungal, and antiprotozoa. Feed and drinking water were provided *ad libitum* using manual feeder and drinker until day 35. Feeding was done little by little periodically to prevent spilled feed. On day 4, all chicks were vaccinated with Newcastle disease (ND)-infectious bronchitis disease (IBD) vaccines (Caprivac ND-R®, PT. Caprifarmindo Laboratories, Indonesia) through eye drop and ND-avian influenza (AI) vaccines (Caprivac ND-AI K®, PT, Caprifarmindo Laboratories) through subcutaneous injection at a dose of 0.15 mL bird^{-1}. Gumboro vaccine (Cevac Transmune IBD®, Ceva Animal Health, Indonesia) was also given on day 14 through the drinking water. During the rearing period, the chicks were raised in an open-sided broiler house with beds made of rice husks (thickness about 12 cm). A continuous lighting schedule was applied throughout the study period. During the study there was no blower fan provided in the broiler house, so air circulation occurred naturally. No mortality was observed during the *in vivo* experiment.

Table 1. Feed compositions of broilers as starter (days 1–21) and finisher (days 22–35)

Feed ingredients:	Starter (%)	Finisher (%)
Yellow corn	56.10	63.55
Soybean meal	36.64	29.83
Palm oil	2.40	2.40
DL-methionine	0.30	0.30
Bentonite	1.10	0.46
Limestone	1.42	1.42
Monocalcium phosphate	1.45	1.45
Premix[1]	0.20	0.20
Chlorine chlorite	0.08	0.08
NaCl	0.31	0.31
Nutrient contents:		
Metabolizable energy (kcal kg^{-1})[2]	2,924	3,040
Crude protein (%)	21.75	19.25
Crude fiber (%)	3.31	3.21
Crude fat (%)	4.43	4.82

[1]Premix contained (per kg of diet) of vitamin A 7,750 IU, vitamiin D3 1,550 IU, vitamin E 1.88 mg, vitamin B1 1.25 mg, vitamin B2 3.13 mg, vitamiin B6 1.88 mg, vitamiin B12 0.01 mg, vitamin C 25 mg, folic acid 1.50 mg, Ca-d-pantothenate 7.5 mg, niacin 1.88 mg, biotin 0.13 mg, Co 0.20 mg, Cu 4.35 mg, Fe 54 mg, I 0.45 mg, Mn 130 mg, Zn 86.5 mg, Se 0.25 mg, L-lysine 80 mg, Choline chloride 500 mg, DL-methionine 900 mg, CaCO3 641.5 mg, Dicalcium phosphate 1,500 mg.

Chicken body weight, feed consumption, and feed efficiency were measured at days 21 and 35. Daily weight gain, daily feed consumption and feed efficiency was then determined as described by Agusetyaningsih et al. (2022) as follows:

$$\text{Daily weight gain (g/bird/day)} = \frac{\text{Final body weight} - \text{initial body weight}}{\text{Days of rearing}}$$

$$\text{Daily feed consumption (g/bird/day)} = \frac{\text{Total feed consumption during rearing}}{\text{Days of rearing}}$$

$$\text{Feed efficiency (\%)} = \frac{\text{Daily weight gain}}{\text{Daily feed consumption}} \times 100\%.$$

On day 35, one male chick with a body weight close to the average body weight of each pen was taken and blood was taken from the wing veins and then put into a tube with anticoagulant (ethylenediaminetetraacetic acid/EDTA) for complete blood profile assessment. The remaining blood was stored in another tube (without coagulant) to produce blood serum after coagulation at room temperature for about 2 hours. Male

chicks were selected for blood sampling to avoid physiological errors due to sex variations. For practical reasons, the chicken from which the blood sample was previously taken was then slaughtered, and the internal organs were removed and weighed (empty condition). To measure the population of intestinal bacteria, digesta was taken from the ileum and cecum and put into a sterile sample container. Digesta was also collected from the duodenum, jejunum, ileum, and cecum for measurement of pH values (using an electronic pH meter, Thermo Fisher Scientific Inc.). For the assessment of the small intestine morphology, the segment of intestine was taken approximately 2 cm from each part of the small intestine and put into a sample tube containing 10% neutral formalin buffer solution. Samples were taken from the mid-point of the duodenum, from the mid-point between the point of entry of the bile duct and Meckel's diverticulum (for jejunum) and from the mid-point of the ileum.

A complete blood profile was determined using the Prima Fully-Auto Hematology Analyzer (PT. Prima Alkesindo Nusantara, Jakarta, Indonesia) following the manufacturer's protocol. To perform histological analysis, duodenal, jejunal, or ileal slices (5 μm each) were stained with hematoxylin and eosin. The villous height and crypt depth were measured using an optical microscope with a camera. Total coliform bacteria were counted on MacConkey agar (Merck KGaA, Darmstadt, Germany) medium and incubated under aerobic conditions for 24 hours at 38 °C. Coliform bacteria that grow on agar media turn red, and bacterial colonies were counted. Total lactic acid bacteria (LAB) were counted on de Man, Rogosa, and Sharpe agar (MRS; Merck KGaA) medium, then incubated anaerobically at 38 °C for 48 hours (Sugiharto et al., 2017b).

The thiobarbituric acid reactive substance (TBARs) method as described by Yuanita et al. (2019) was followed to determine malondialdehyde (MDA) levels in serum. The method was based on the ability to form a pink complex between MDA and thiobarbituric acid (TBA). A mixture of 0.5 mL of serum and 4.5 mL of saline phosphate buffer (PBS) was mixed and centrifuged for 15 minutes before removing 4 mL of the supernatant. The supernatant was combined with 1 mL of 15% trichloroacetic acid (TCA) and 1 mL of TBA and heated in an 80 °C water bath for 15 minutes before being cooled at room temperature for 60 minutes. After centrifugation for 15 minutes, the absorbance was measured with a spectrophotometer at a wavelength of 532 nm. The MDA concentration (nmol mL^{-1}) was calculated using the 1,1,3,3-tetramethoxypropane standard curve.

The measurement of superoxide dismutase (SOD) activity in the sample was carried out according to the method described by Peters (Peters et al., 2014). A total of 0.06 mL of the supernatant was reacted with a mixture of 2.70 mL of 50 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10), 0.06 mL of xanthine 10 mM, 0.03 mL of 0.5% bovine serum albumin (BSA) and 0.03 mL of 2.5 mM NBT. The xanthine oxidase (0.04) unit was then added. After 30 minutes, the absorbance was measured at a wavelength of 560 nm. The PBS containing 11.5 g L^{-1} KCl was used as a control solution. Enzyme activity was measured in units per milliliter of sample (U mL^{-1}).

Statistical analysis

The study was conducted based on a completely randomized design, and the data obtained were treated using analysis of variance according to the statistical model below:

$$Yij = \mu + \tau i + \varepsilon ij \quad (1)$$

where Yij is the observation value, μ is the average value of treatment, τi is the effect of treatment and εij is the treatment error.

RESULTS AND DISCUSSION

Antioxidant and antibacterial activity of nutmeg flesh extract

The chemical and functional compositions (antioxidant activity) as well as antibacterial activity of nutmeg flesh extract are shown in Table 2. The nutmeg flesh extract showed a good potential for antioxidant source as indicated by its high DPPH radical scavenging activity (3.45 μg mL^{-1}, based on IC_{50}) and the content of total phenolics (1.62 g per 100 g) and flavonoids (1.45 g per 100 g). The nutmeg flesh extract also exhibited antibacterial activity against *E. coli* (diameter of inhibition zone 13.7 mm).

Table 2. Chemical and functional compositions and antibacterial activity of nutmeg flesh extract

Items	Values
Moisture (%)	51.66
Ash (%)	0.15
Fat (%)	13.79
Proteins (%)	0.99
Carbohydrates (%)	33.42
Total phenolics (g per 100 g)	1.62
Flavonoids (g per 100 g)	1.45
DPPH radical scavenging activity (IC_{50} [μg mL^{-1}])	3.45
Antibacterial activity against *E. coli* (mm)	13.7

Phytobiotics (herbs and spices) are important sources of phytochemicals with strong antioxidant activity against free radicals, and they are commonly used in poultry and other livestock feeds to obtain various biological activities such as antibacterial, antifungal, antiviral, antiparasitic, and antioxidants (Sugiharto, 2020). The results of this study indicated that the nutmeg flesh extract showed strong antioxidant activity (based on the value of IC_{50} of DPPH radical scavenging activity) and contained the substantial amounts of phenols and flavonoids. These findings were consistent with previous studies showing the antimicrobial and antioxidant activities of the aromatic nutmeg plant (*Myristica fragrans* Houtt) (Gupta et al., 2013; Adu et al., 2020). The antibacterial activity test of nutmeg flesh extract against *E. coli* using the disc diffusion (Kirby-bauer) method were 13.7 mm, indicating that the nutmeg flesh extract had moderate inhibition against *E. coli* growth (Morales et al., 2003). Our present finding was similar to that of reported by Atmaja (2017) showing that nutmeg flesh extract had an inhibitory zone of 11.37 mm against the growth of *E. coli* at the concentration of 10×10^5 ppm.

Productive performance of broiler chickens

The data on performance of broilers are presented in Table 3. It was shown that for the entire study period (days 15–35) daily weight gain, daily feed consumption and feed efficiency were higher ($p < 0.01$) in T0 than that in T1 chicks. Moreover, the T4 group showed higher ($p < 0.01$) daily weigh gain as compared to T0, T1 and T2, but did not differ from that of T3 group. The T4 had the highest ($p < 0.05$) daily feed consumption

as compared to other treatment groups. Throughout the study period, feed efficiency was better (p = 0.02) in T0, T3 and T4 as compared to T1 group. In general, the treatment effects on daily weight gain, daily feed intake and feed efficiency were more pronounced during days 22–35 than days 15–21. It was most likely that the chicks weigh more during the finisher period, causing them to experience more stress from the high stocking density than they did during the days 15–21. With regard particularly to the performance of chicks, the results of this study showed that the daily weight gain of broiler chickens age 15–35 days ranged from 56.04–64.07 g/bird/day, which indicates a normal body weight gain range. Indeed, Dei & Bumbie (2011) showed that the daily weight gain of broiler chickens at 35 days ranged from 58.0 to 64.3 g/bird/day.

Table 3. Performance of broilers chickens

Items	Treatment groups					*SE*	*p* value
	T0	T1	T2	T3	T4		
Daily weight gain (g/bird/day)							
15–21 day	23.28	23.78	24.21	25,32	25.77	3.27	0.32
22–35 day	38.71^{b}	32.26^{a}	37.48^{b}	38.31^{b}	38.92^{b}	2.45	< 0.01
15–35 day	61.98^{b}	56.04^{a}	61.70^{b}	63.62^{cb}	64.67^{c}	1.28	< 0.01
Daily feed intake (g/bird/day)							
15–21 day	30.21	30.65	31.11	32.08	32.80	3.65	0.13
22–35 day	69.71^{b}	67.24^{a}	72.08^{c}	72,98^{c}	73,82^{d}	1.89	< 0.01
15–35 day	100.94^{b}	97.07^{a}	103.19^{c}	105.42^{d}	107.60^{e}	1.09	< 0.01
Feed efficiency (%)							
15–21 day	77.06	77.51	77.82	78.93	78.57	5.21	0.62
22–35 day	55.53^{b}	47.98^{a}	52.02^{b}	52.49^{b}	52.72^{b}	2.11	< 0.01
15–35 day	61.41^{b}	57.73^{a}	59.79^{ab}	60.35^{b}	60.10^{b}	1.73	0.02

^{a,b,c,d,e} On the same row, different superscripts indicated a significant variation ($p < 0.05$); T0: chicks raised at normal density of 10 chicks m^{-2}; T1: chicks raised at high density of 16 chicks m^{-2}; T2: chicks raised at high density and fed with 0.5 mL kg^{-1} nutmeg flesh extract; T3: chicks raised at high density and fed with 1.0 mL kg^{-1} nutmeg flesh extract; T4: chicks raised at high density and fed with 1.5 mL kg^{-1} nutmeg flesh extract; *SE*: standard error.

The data in this study clearly showed that rearing broilers in high density pens particularly during the finisher period had negative impacts on broiler production performance. Daily body weight gain, daily feed consumption and feed efficiency were lower in the chickens at high density pens, when compared to the chickens at normal density pens. In line with this, several studies have shown that high density condition is associated with impaired chicken growth and low feed efficiency (Kryeziu et al., 2018). The decrease in growth performance of broiler chickens at high density pens is often associated with several factors, including an increase in temperature and a decrease in air circulation in the pens, causing uncomfortable conditions (stress) for chickens, and limited space for movement and access to feed which can cause nutritional deficiencies in chickens (Yin et al., 2017; Ahmed et al., 2018).

The data in this study indicated that the extract of nutmeg flesh can reduce the negative impact of rearing broilers with high density (16 birds m^{-2}) on the growth performance of broilers. There are currently no studies in the literature that explain the effect of nutmeg flesh extract on increasing body weight gain in broiler chickens. The positive effect of nutmeg flesh extract could most likely be attributed to the synergistic

action of various phytochemicals present in the nutmeg flesh which in turn improve physiological conditions in chickens. The latter condition has an effect on increasing feed utilization and efficiency in order to improve broiler growth performance. Due to their ability to scavenge/neutralize free radicals and maintain intestinal mucosal integrity, phytochemicals such as flavonoids, phenols, and saponins have been shown to promote higher growth rates and better feed efficiency in broilers (Astaneh et al., 2018). In addition, previous studies have shown that nutmeg flesh has antibacterial, antiparasitic, antifungal, anti-coccidiotic, and hepatoprotective properties (Panggabean et al., 2019) which is able to stimulate the growth of beneficial bacteria, inactivate pathogenic bacteria, as well as facilitate the metabolism of nutrients and absorption in the digestive tract which in turn can improve the growth performance of broiler chickens.

In term of feed consumption, administration of nutmeg flesh extract resulted in higher feed consumption during the rearing period. Study showed that phytochemicals especially essential oil may improve feed taste and delicacy, thus increasing feed consumption and weight gain of animals (Sugiharto, 2016). Indeed, nutmeg flesh contains about 10% essential oil, which primarily consists of terpene hydrocarbons (sabinene and pinene), myrcene, phellandrene, camphene, limonene, terpinene, myrcene, p-cymene, and other terpene derivatives (Nagja et al., 2016). In this study, the increased feed efficiency was seen in broiler fed nutmeg flesh extract, indicating better feed utilization. In accordance with this, other study found that using plants as phytogenic agent in broiler feed can improve intestinal digestibility, which in turn increases broiler chicken growth (Amad et al., 2011). In addition, previous studies have shown that the introduction humic acids of natural origin in drinking water to the diet of broiler chickens has a positive effect on the body's natural resistance and feed digestibility (Korsakov et al., 2019).

Intestinal morphology of broiler chickens

Data on the intestinal morphology of broiler chickens are presented in Table 4. Villi height of duodenum and jejunum was lower ($p < 0.01$) in T1 than that in other treatment groups. Treatment with nutmeg flesh extract increased ($p < 0.01$) the villi height of duodenum, jejunum and ileum of broilers stocked at high density pens. The dietary treatment also increased villi height to crypt depth ratio of jejunum ($p = 0.04$) and ileum ($p = 0.02$) of broilers raised under high density pens. In this study, the treatment had no significant effect on crypt depth in the duodenum ($p = 0.31$), jejunum ($p = 0.99$) and ileum ($p = 0.51$) of broiler chickens. The current findings showed that the height of the villi of the duodenum, jejunum and ileum ranged from 933.55–1,250.03 µm, 984.58–1194.48 µm and 625.02–890.40 µm, where these respective values were within the normal conditions. According to Jazi et al. (2017), normally the villi height in the duodenum ranges from 1,058–1,318 µm, whereas Fard et al. (2014) mentioned that the normal height of the villi in the jejunum and ileum ranges from 1,010–1,239 µm and 508–635 µm.

Broilers raised at normal densities had higher villi in the duodenum, jejunum, and ileum than chickens raised at high density pens. Normal density-chickens also had a higher villi height to crypt depth ratio in the jejunum and ileum than the high density-broilers. These findings were consistent with the study of Tabeekh et al. (2017) who discovered that rearing chickens at normal density (12 birds m^{-2}) increased villi height

and crypt depth compared to high density (18 birds m^{-2}). Sugiharto et al. (2017a) reported that stress conditions due to high density have a negative impact on gut morphology.

Table 4. Intestinal morphology of broiler chickens

Items	Treatment groups					*SE*	*p* value
	T0	T1	T2	T3	T4		
Duodenum							
Villi height (µm)	998.18[b]	933.55[a]	1,097.74[c]	1,138.66[c]	1,250.03[d]	120.57	< 0.01
Crypt depth (µm)	98.93	94.17	94.10	102.92	110.50	13.84	0.31
VH/CD	10.14	9.99	9.52	11.38	11.53	1.55	0.21
Jejunum							
Villi height (µm)	1,145.53[b]	984.58[a]	1,155.84[b]	1,186.02[b]	1,194.48[b]	99.22	< 0.01
Crypt depth (µm)	72.46	71.24	71.77	72.47	72.68	4.85	0.99
VH/CD	15.95[b]	13.88[a]	16.15[b]	16.41[b]	16.44[b]	1.59	0.04
Ileum							
Villi height (µm)	682.36[a]	625.02[a]	749.94[a]	886.15[b]	890.40[b]	138.90	< 0.01
Crypt depth (µm)	67.57	65.72	68.38	70.24	70.47	4.71	0.51
VH/CD	10.19[a]	9.59[a]	11.00[ab]	12.62[b]	12.64[b]	1.94	0.02

[a,b,c,d] On the same row, different superscripts indicated a significant variation ($p < 0.05$); T0: chicks raised at normal density of 10 chicks m^{-2}; T1: chicks raised at high density of 16 chicks m^{-2}; T2: chicks raised at high density and fed with 0.5 mL kg^{-1} nutmeg flesh extract; T3: chicks raised at high density and fed with 1.0 mL kg^{-1} nutmeg flesh extract; T4: chicks raised at high density and fed with 1.5 mL kg^{-1} nutmeg flesh extract, VH/CD: villi height to crypt depth ratio; *SE*: standard error.

Moreover, the corticosterone hormone seemed to be responsible for intestinal mucosa damage because it can delay the proliferation of intestinal epithelial cells, resulting in a decrease in the height of the intestinal villi and the depth of the crypts in high temperature-stressed birds (Sugiharto et al., 2017a). With regard to the broilers raised at high density pens, administration of nutmeg flesh extract was able to increase the hight of the villi in the duodenum, jejunum, and ileum, as well as the villi hight to crypt depth ratio in the jejunum and ileum. The findings of this study were consistent with the findings of Saragih et al. (2019), who discovered that administration of the *Spirogyra jaoensis* plant increased villi hight and crypt depth in the duodenum, jejunum, and ileum. Pathogenic bacteria and stress, according to Sugiharto (2016), have a negative impact on intestinal microflora or intestinal epithelium, resulting in changes in cell permeability as the body's natural resistance, making it easier for harmful components and pathogenic bacteria to penetrate small intestinal cells, interfering with metabolism, digestion, and nutrient absorption. These conditions can cause chronic inflammation of the intestinal mucosa, which ultimately leads to decreased villi height, impaired digestion and absorption in chickens. The positive effect of nutmeg flesh extract on intestinal morphology has been attributed to its bioactive compounds that can stimulate the proliferation and growth of cells in the digestive tract, resulting in higher villi hight to crypt depth ratio. The mechanism by which nutmeg flesh extract provides benefits in improving intestinal morphology could be that the herbal plants can protect intestinal tissue from the microbial attack (Sugiharto, 2016).

pH values and selected bacteria population in intestine of broiler chickens

The pH values and numbers of selected bacteria in the intestine of broilers are presented in Table 5. While had no effect on the pH values of duodenum ($p = 0.59$), jejunum ($p = 0.71$) and ileum ($p = 0.02$), stocking the chicks at high density pens increased the pH values of cecum ($p < 0.01$). The nutmeg flesh extract considerably decreased pH in the ileum ($p = 0.02$) and cecum ($p < 0.01$) of broilers reared at high density, but had no effect on the pH of the duodenum ($p = 0.59$) and jejunum ($p = 0.71$). In this study, the pH values of the duodenum, jejunum, ileum and cecum of broiler chickens ranged from 6.23–6.56, 5.50–5.90, 4.73–6.10, and 6.28–7.73, respectively. These values were still within the normal ranges of pH of the digestive tract of broiler chickens. This was in accordance with Mabelebele et al. (2014) reporting that the normal pH values of the digestive tract of broiler chickens was between 6.62 and 6.43.

Table 5. pH and selected bacteria population in the intestine of broiler chickens

Items	Treatment groups					*SE*	*p* value
	T0	T1	T2	T3	T4		
pH							
Duodenum	6.35	6.56	6.42	6.23	6.26	0.34	0.59
Jejunum	5.89	5.90	5.87	5.64	5.50	0.53	0.71
Ileum	6.03^{b}	6.10^{b}	5.52^{ab}	5.24^{ab}	4.73^{a}	0.79	0.02
Cecum	7.10^{b}	7.73^{c}	6.58^{ab}	6.37^{a}	6.28^{a}	0.68	< 0.01
Lactic Acid Bacteria (log cfu g^{-1})							
Ileum	10.30^{b}	9.48^{a}	11.11^{bc}	11.00^{bc}	11.22^{c}	0.87	$p < 0.01$
Cecum	10.76^{b}	9.74^{a}	11.43^{c}	11.45^{c}	11.68^{c}	0.79	$p < 0.01$
Coliform (log cfu g^{-1})							
Ileum	6.20^{a}	7.95^{b}	5.85^{a}	5.76^{a}	5.61^{a}	1.37	< 0.01
Cecum	7.97^{b}	8.69^{b}	7.52^{ab}	7.52^{ab}	6.24^{a}	1.33	0.04

^{a,b,c} On the same row, different superscripts indicated a significant variation ($p < 0.05$); T0: chicks raised at normal density of 10 chicks m^{-2}; T1: chicks raised at high density of 16 chicks m^{-2}; T2: chicks raised at high density and fed with 0.5 mL kg^{-1} nutmeg flesh extract; T3: chicks raised at high density and fed with 1.0 mL kg^{-1} nutmeg flesh extract; T4: chicks raised at high density and fed with 1.5 mL kg^{-1} nutmeg flesh extract, VH/CD: villi height to crypt depth ratio; *SE*: standard error.

Our current study showed that the pH of cecum increased with raising broilers at high density condition. Similarly, Tsiouris et al. (2015) reported an increase in pH of the cecum of broilers reared at high density. The rise in cecum pH was most likely caused by a decrease in litter quality caused by an increase in humidity and temperature in the broiler house, which could affect the microbiota activity of intestinal commensal bacteria. The main factors that determine pH in the intestine are gastrointestinal secretions and volatile fatty acids produced by the gut microbiota. Giving nutmeg flesh extract to high-density broilers could lower pH in the ileum and cecum in the current experiment. This study supports the findings of Sunu et al. (2021) who discovered that supplementing plant extracts as phytobiotics can lower pH in the ileum and cecum. Carbohydrate content, particularly oligosaccharides found in almost all plants, can be a good substrate for fermentation, which promotes the growth of beneficial microbes. Lactic acid bacteria-fermentation produces a high concentration of lactic acid, which affects pH decrease and reduces the growth of harmful bacteria (Nkukwana et al., 2015). The decrease in the intestinal pH caused by the administration of nutmeg flesh extract in

this study was similar to the findings of Ferdous et al. (2016), who found that the administration of plant extracts in feed significantly reduced the intestinal pH of broiler chickens. The decrease in pH in the ileum and cecum due to the administration of nutmeg flesh extract was associated with an increase in the population of lactic acid bacteria in the ileum and cecum. Lactic acid is a metabolite of lactic acid bacteria and one of the organic acid components that contribute to pH reduction. The decrease in pH in the ileum and cecum caused by nutmeg flesh extract administration reduced the number of pathogenic bacteria in the intestines while increasing nutrient digestibility. Note that high acidity inhibits the growth of pathogenic bacteria (Song et al., 2014), resulting in healthier intestines.

Rearing broiler at high density pens increased ($p < 0.01$) the number of coliforms, while decreasing ($p < 0.01$) lactic acid bacteria numbers (Table 5). Moreover, the administration of nutmeg flesh extract significantly ($p < 0.01$) increased lactic acid bacteria and decreased ($p < 0.01$) coliform bacteria in the ileum and cecum of broilers raised at high density pens. Khosravifar et al. (2014) stated that the number of coliform bacteria in the intestine broilers of the finisher period was 6.04 log cfu g^{-1}. Previous study by Yeh et al. (2018) reported that the normal range of lactic acid bacteria in the digestive tract of broiler chickens ranged from 8.09 to 8.11 log cfu g^{-1}. It was very likely that differences in environmental conditions, hygiene and nutritional content of feeds led to variations in the population of lactic acid bacteria in the digestive tract between this study and other studies.

The data from this study showed that raising broilers at high density could reduce the population of lactic acid bacteria and increase coliform bacteria in the ileum and cecum of broilers. The effect of raising broilers at high density can cause stress, which is characterized by behavioural, biochemical, and physiological changes subjected to re-establishing homeostasis, which can alter the composition of gut microbes (Sugiharto et al., 2017b). Wang et al. (2021) reported the results of their study showing the abundance of lactic acid bacteria in broilers reared at normal densities compared to high densities. Our further research data confirmed that administration of nutmeg flesh extract increased the population of lactic acid bacteria and decreased coliform bacteria in the ileum and cecum of broiler chickens. These findings are consistent with the findings of Ferdous et al. (2016), who found that supplementing plant extracts as phytobiotics increased the population of lactic acid bacteria while decreasing *E. coli* and total coliform in the intestine. Acidic conditions in the digestive tract, particularly the ileum and cecum, aid in the balance of the digestive tract microflora, increasing the population of lactic acid bacteria while decreasing the population of pathogenic bacteria (Yadav & Jha, 2019). Most pathogenic bacteria grow in pH close to 7 or tend to be neutral. On the other hand, beneficial microorganisms live in acidic pH (5.8–6.2) and compete with pathogenic bacteria, which allows competitive exclusion (Rahmani et al., 2005). Lactic acid bacteria produce lactic acid which is able to maintain the pH of the digestive tract of broiler chickens to be acidic. Furthermore, acidic conditions in the digestive tract can improve the efficiency of the nutrient absorption by the digestive tract, ensuring that nutritional needs are satisfied (Mabelebele et al., 2014; Sugiharto, 2016).

Blood profiles of broiler chickens

The data on blood profile of broilers reared at high density are presented in Table 6. In general, blood profile of broilers was not affected ($p > 0.05$) by the treatments applied during the study. Broilers reared at high densities had blood profiles that are not substantially different from those reared at normal densities. Although the specific reasons for this condition were not known with certainty, broilers reared at high density pens from days 15 to 35 were very likely to be able to adapt to high density conditions so that the chickens can compensate and adjust physiological processes in their bodies to stress. Apart from the treatment effect, the levels of erythrocytes and leukocytes of broilers in this study were within normal haematological range. Scanes & Christensen (2014) reported that five-week-old broilers had erythrocyte values of $2.56–3.2\times10^{12}$ L^{-1}. In line with this, Ning et al. (2014) pointed out that the normal values of broiler red blood cells are around $2.0–3.2\times10^{12}$ L^{-1}. Likewise, the normal leukocyte counts in broiler chickens are in the range of $12–30\times10^{9}$ L^{-1} (Ullah et al., 2018)

Table 6. Blood profile of broiler chickens

Items	Treatment groups					*SE*	*p* value
	T0	T1	T2	T3	T4		
Erythrocytes (10^{12} L^{-1})	2.96	3.38	3.17	2.69	2.92	0.48	0.19
Leukocytes (10^{9} L^{-})	14.38	20.48	18.30	17.58	15.84	4.24	0.19
Haemoglobin (g dL^{-1})	10.30	12.20	10.40	9.70	10.20	1.71	0.18
Haematocrits (%)	36.40	41.20	35.90	32.60	35.20	5.80	0.22
Thrombocytes (10^{9} L^{-1})	13.80	23.20	28.80	16.20	22.80	13.84	0.47
Lymphocytes (10^{9} L^{-1})	137.00	198.10	176.40	169.10	151.50	41.43	0.17
Heterophils (10^{9} L^{-1})	7.40	6.70	13.20	4.99	6.90	7.20	0.46
MCV (10^{-15} L)	123.50	123.40	121.50	120.72	121.30	2.34	0.2
MCH (10^{-12} g)	34.74	36.00	35.06	35.52	34.96	1.44	0.69
MCHC (g L^{-1})	28.26	29.36	29.08	29.60	28.96	1.33	0.62
RDW-SD (10^{-15} L)	45.72	47.92	46.08	46.82	45.34	3.46	0.81
RDW-CV (%)	9.76	9.62	10.02	10.26	9.90	0.90	0.85
MPV (10^{-15} L)	9.20	7.90	7.36	8.72	8.54	1.41	0.74

T0: chicks raised at normal density of 10 chicks m^{-2}; T1: chicks raised at high density of 16 chicks m^{-2}; T2: chicks raised at high density and fed with 0.5 mL kg^{-1} nutmeg flesh extract; T3: chicks raised at high density and fed with 1.0 mL kg^{-1} nutmeg flesh extract; T4: chicks raised at high density and fed with 1.5 mL kg^{-1} nutmeg flesh extract; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; RDW-SD: red blood cell distribution width-standard deviation; RDW-CV: red blood cell distribution width-coefficient variation; MPV: mean platelet volume, *SE*: standard error.

Antioxidant activity of broiler chickens

Table 7 shows the MDA and SOD levels in the serum of broiler. MDA levels were lower ($p < 0.05$) in the T4 group compared to the T0, T1, T2, and T3 groups. There was no significant difference ($p > 0.05$) in MDA levels between the T0 and T1 groups. The SOD levels were lower ($p < 0.01$) in the T1 group than in the T0, T2, T3, and T4 treatments. Broiler chickens in the T4 treatment had the highest ($p < 0.01$) serum SOD level when compared to other treatments. The results of this study showed that the values of MDA and SOD ranged from 0.85 to 1.16 nanomol mL^{-1} and 37.58 to 51.84 U mL^{-1}, respectively. According to Sunu et al. (2019), SOD levels of chicken ranged from 44.79 to 66.49 U mL^{-1}, whereas according to Fathi et al. (2016) MDA levels of chicken range

from 2.30 to 3.17 nanomol mL^{-1}. The inconsistent levels of SOD and MDA in broilers across studies appeared to be associated with the various environmental factors and stress levels in chickens.

Table 7. Serum levels of superoxide dismutase and malondialdehyde of broiler chickens

Items	Treatment groups					*SE*	*p* value
	T0	T1	T2	T3	T4		
MDA (nanomol mL^{-1})	1.09^{bc}	1.16^{c}	1.04^{bc}	0.99^{b}	0.85^{a}	0.14	< 0.01
SOD (U mL^{-1})	45.69^{b}	37.58^{a}	50.80^{bc}	49.44^{bc}	51.84^{c}	6.34	< 0.01

[a,b,c] On the same row, different superscripts indicated a significant variation ($p < 0.05$); T0: chicks raised at normal density of 10 chicks m^{-2}; T1: chicks raised at high density of 16 chicks m^{-2}; T2: chicks raised at high density and fed with 0.5 mL kg^{-1} nutmeg flesh extract; T3: chicks raised at high density and fed with 1.0 mL kg^{-1} nutmeg flesh extract; T4: chicks raised at high density and fed with 1.5 mL kg^{-1} nutmeg flesh extract; *SE*: standard error.

Under most conditions, stress is associated with increased MDA and decreased SOD levels, which is a protective response of broilers against excessive free radical production (Akbarian et al., 2016). A recent study found that raising broilers at high densities increased MDA levels while decreasing SOD levels in serum (Nasr et al., 2021). A high MDA concentration indicates an oxidation process in the cell membrane. Aside from the effect of pen density, the use of nutmeg flesh extract in this study may have reduced MDA due to the antioxidant role of nutmeg flesh, which can inhibit lipid peroxidation. According to Sugiharto & Turrini (2022), MDA in the body is formed as a result of oxidative stress conditions, specifically an imbalance between the formation of reactive oxygen species (ROS) and the presence of antioxidants, where free radicals outnumber antioxidants. According to Zhao et al. (2014), a decrease in blood MDA levels indicates free radical inhibition by antioxidants, and thus a high antioxidant status is usually accompanied by a decrease in plasma MDA levels. In this study, provision of nutmeg flesh extract through the ration increased the concentration of SOD and decreased MDA in the blood serum of broiler chickens. It is well known that the flesh of nutmeg contains bioactive substances such as phenolics and flavonoids, both of which are powerful antioxidants. These findings were consistent with previous study reporting that plant extracts such as *Echinacea purpurea* containing flavonoids and other bioactive substances, can increase SOD levels in broiler blood (Lee et al., 2012). The increase in SOD activity caused by nutmeg flesh extract administration indicates that broiler chickens have a strong free radical scavenging capacity, as evidenced by lower MDA concentrations. In this regard, Lee et al. (2012) and Sugiharto & Turrini (2022) confirmed that SOD can prevent the formation of free radicals that can cause cell damage.

CONCLUSIONS

Treatment with nutmeg flesh extract up to 1.5 mL kg^{-1} of feed to high density reared broiler chickens increased the hight of intestinal villi, total lactic acid bacteria and decreased total coliforms, while having no effects on the blood profile. Aside from the effect of high stocking density, administration of nutmeg flesh extract decreased MDA levels and increase daily weight gain of broilers.

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