Effect of synbiotic from nutmeg flesh extract and *Lactobacillus plantarum* on small intestinal morphology, stress, and bacterial population of broiler chickens under high stocking density conditions

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Abstract  This study aims to examine the effect of synbiotic nutmeg flesh extract and *L. plantarum* on intestinal morphology, stress indicators and bacterial populations in broiler chickens reared at high densities. Broiler chickens (body weight 142 ± 4.71 g) were randomly divided into five treatments, and each treatment was repeated five times, namely, T0 (negative control with a normal density of 10 birds/m²), T1 (positive control with a high density of 18 birds/m²), T2, T3, and T4 with a high density of 18 birds/m². The synbiotic nutmeg flesh extract and *L. plantarum* were added to the feed from the eighth day at 0.5, 1.0, and 1.5 ml/kg for T2, T3, and T4, respectively. The results of the study showed that administering synbiotic nutmeg flesh extract and *L. plantarum* to broiler chickens reared at high density significantly (p<0.05) increased growth performance and villous length in the duodenum, jejunum, and ileum. In addition, this synbiotic also lowers the pH in the ileum and cecum, increases lactic acid bacteria and reduces coliform bacteria in the ileum and cecum, reduces the H/L and malondialdehyde (MDA) ratio and increases superoxide dismutase (SOD). Providing synbiotic nutmeg flesh extract and *L. plantarum* is also able to improve the performance and stress of broiler chickens raised at high densities.

Keywords: synbiotic, broilers, intestinal bacterial population, intestinal morphology, stress

1. Introduction

The maintenance of broiler chickens in the modern poultry industry has always experienced harsh and stressful conditions during their growth. This is especially the case when maintenance is carried out at high densities for efficiency reasons. Generally, 8 to 12 birds/m², 12 to 16 birds/m², and > 16 birds/m² are considered low, medium, and high densities, respectively (Yin et al 2017; Sapsuha, et al 2021; Sugiharto and Yudiarl 2022). A number of studies show that rearing at high densities has a negative effect on broiler chickens, especially related to physiological responses such as increased MDA levels, increased lipid peroxidation, and the production of free radicals as well as increased immunosuppression (Simitzis et al 2012; Ismail et al 2014; Sugiharto, 2016). This is a response to stress that can affect bowel function or gut health. Therefore, rearing broilers at high densities has a negative impact on broiler performance. The use of an antibiotic growth promoter (AGP) has become a promising strategy to improve the performance of broiler chickens under stress conditions (Peng et al 2016; Wu et al 2018). The routine use of antibiotic growth promoters (AGPs) can cause serious problems for consumers because they can cause residues in the products produced that have a negative impact on human health (Biagi et al 2017; Tang et al 2017). Thus, an alternative to AGP is needed.

One effort to replace the role of antibiotics is to use synbiotics. Synbiotics are defined as combinations of probiotics and prebiotics used together. Probiotics are live microorganisms that have a beneficial effect on the health of the host when administered in sufficient quantities. Probiotic bacteria have been used to improve animal performance by maintaining the normal microflora of the host animal. The main action of probiotics is strengthening the intestinal mucosal barrier against infectious agents (Anjum et al 2005; Jadhav et al 2015). Probiotic bacteria also stimulate antigen-specific and nonspecific immune responses. Prebiotics are defined as nondigestible feed ingredients that beneficially affect the host by selectively stimulating the growth or activity or both of a number of bacteria in the large intestine, thereby improving the health of the host (Blajman et al 2016). The use of synbiotics as natural additives in poultry feed has increased in recent years.
years. A number of studies have reported that the use of synbiotics in broiler feed can improve production performance. Synbiotics have been shown to have beneficial effects on the gut of broilers. Previous research results show that synbiotics can increase the survival and persistence of health-promoting organisms in the broiler intestine because specific substrates are available for fermentation (Hassanpour et al 2013, Sarangi et al 2016).

Nutmeg flesh extract can optimize the function of the digestive tract and metabolism so that feed use becomes more efficient. Apart from containing bioactive substances, nutmeg flesh also contains oligo and polysaccharides, so it can be used as a prebiotic (Sapsuha et al 2022). The contents of oligos and polysaccharides are carbohydrate compounds that can be used as prebiotics and can be a source of probiotic nutrients. L. plantarum is a microbrial culture of the lactic acid bacteria (LAB) class that is able to suppress the development of pathogenic microbes and lower the pH, thereby activating digestive enzymes. This LAB action will prevent infection and increase the digestibility of feed in the digestive tract. LAB can increase short-chain fatty acids and lactic acid in the intestine, lower pH, and activate digestive enzymes so that the digestive tract becomes healthier and increases protein digestibility (Kim et al 2015).

Recent research results show that nutmeg flesh extract can maintain the survivability of L. plantarum so that it can be used as a synbiotic in broiler chickens (Sapsuha et al 2023a). It was further found that giving it to broiler chickens can improve performance and intestinal morphology and improve intestinal microbes (Sapsuha et al 2023b), but its effect on broiler chickens reared at high densities has never been studied. Therefore, this study was conducted to evaluate the effect of using synbiotic nutmeg flesh extract and L. plantarum on intestinal morphology, stress indicators, and bacterial populations in broiler chickens reared at high density.

2. Materials and Methods

2.1. Synbiotic Production

Synbiotic production was carried out according to the method of Sapsuha et al (2023b). In short, this was done by mixing nutmeg flesh extract, with the extract method according to Sapsuha et al (2021), and 20 ml/100 ml of distilled water with 10 ml of Lactobacillus plantarum (bacterial concentration 1 x 10⁹ cfu/ml). The mixture was then incubated for 24 hours at 37 °C. The culture was then grown on MRSA by the pouring method and incubated at 37 °C for 48 hours. Synbiotics were stored in the refrigerator until use.

2.2. In vivo experiment

The Animal Research Ethics Committee of the Faculty of Agriculture, Universitas Khairun, approved the in vivo research with approval number 07/KEPH/PH/2023. A total of 450-day-old Lohmann broiler chicks (unsexed) were used for this research. The research was conducted at the Poultry Cage, Animal Husbandry Program, Universitas Khairun, for five weeks. Chickens were randomly divided into five treatments with a total of 50 chickens in treatment T0 (positive control), while in treatments T1 (negative control), T2, T3, and T4 had 90 birds per treatment. Chickens in Treatment T0 were further divided into five replicates with 10 birds per repetition, whereas those in Treatments T1, T2, T3, and T4 were further divided into 18 birds per repetition. Chickens were kept in ventilated broiler coops with rice husks as bedding and equipped with feeding and drinking containers. The feed given is mash and is formulated (Table 1) as starter feed (days 1-21) and finisher feed (days 22-35).

Chickens were fed the T0 and T1 treatments without the synbiotic nutmeg flesh extract and L. plantarum, while the T2, T3, and T4 treatments were supplemented with the synbiotic nutmeg flesh extract and L. plantarum, as much as 0.5 ml/kg feed, 1.0 ml/kg feed, and 1.5 ml/kg feed, respectively.

2.3. Growth Performance

At the end of rearing, chicken body weight, feed consumption, and feed efficiency were measured. Daily weight gain, daily feed consumption, and feed efficiency were determined as described by Agusteyaningsih et al (2022) as follows:

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

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

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

2.4. Intestinal Morphology

For the morphological assessment of the small intestine, approximately 2 cm of intestinal segments were taken from each part of the small intestine and put into a sample tube containing 10% neutral buffered formalin. Samples were taken from the midpoint of the duodenum, from the midpoint between the entry point of the bile duct and Meckel’s diverticulum (for the jejunum), and from the midpoint of the ileum. To perform histological analysis, duodenal, jejunal, or ileum slices (5 μm each) were stained with haematoxylin and eosin. Villous height and crypt depth were measured using an optical microscope with a camera.

2.5. Gut Microbial Populations

To measure the intestinal bacterial population, the 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.). Total coliform bacteria were counted on MacConkey agar (Merck KGaA, Darmstadt, Germany) and incubated under aerobic conditions for 24 h at 38 °C. Coliform bacteria growing on the agar media turned red, and bacterial colonies were counted. Total lactic acid
bacteria (LAB) were counted on de Man, Rogosa, and Sharpe agar (MRS; Merck KGaA) media and then incubated anaerobically at 38 °C for 48 hours (Sugiharto et al. 2017).

<table>
<thead>
<tr>
<th>Items (% except that otherwise mentioned)</th>
<th>Starter (1-21)</th>
<th>Finisher (22-35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow corn</td>
<td>54.25</td>
<td>65.25</td>
</tr>
<tr>
<td>Fine bran</td>
<td>20.38</td>
<td>13.11</td>
</tr>
<tr>
<td>Fish flour</td>
<td>17.85</td>
<td>14.12</td>
</tr>
<tr>
<td>Palm oil</td>
<td>2.45</td>
<td>2.45</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>Bentonite</td>
<td>1.15</td>
<td>1.15</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>MCP</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Premix</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Chlorine chlorite</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Nutrient content based on laboratory analysis:
- ME (kcal/kg): 2,998 for Starter (1-21), 3,103 for Finisher (22-35)
- Dry matter: 84.76 for Starter (1-21), 85.68 for Finisher (22-35)
- Crude protein: 21.32 for Starter (1-21), 19.75 for Finisher (22-35)
- Extract ether: 4.52 for Starter (1-21), 4.69 for Finisher (22-35)
- Crude fiber: 3.22 for Starter (1-21), 3.12 for Finisher (22-35)
- Ash: 8.29 for Starter (1-21), 8.86 for Finisher (22-35)

2.6. MDA, SOD, and Heterophil to Lymphocyte Ratio

On the 35th day, one rooster with a body weight close to the average body weight of the cage was taken, and blood was taken from its wing veins. The blood was then put into a tube with anticoagulant (ethylenediaminetetraacetic acid/EDTA) to determine the ratio of heterophils and lymphocytes. The remaining blood was stored in another tube (without coagulant) to produce blood serum after freezing at room temperature for approximately 2 hours to determine the SOD and MDA levels.

Determination of heterophile and lymphocyte content was carried out using a Prima Fully Auto Hematology Analyzer (PT. Prima Alkesindo Nusantara, Jakarta, Indonesia) following the manufacturer’s protocol. Determination of malondialdehyde (MDA) was performed according to Sapsuha et al. (2022) using the thiobarbituric acid reactive substances (TBARS) method. The principle of this method is based on the ability to form a pink complex between MDA and thiobarbituric acid (TBA). Blood serum (0.5 mL) and 4.5 mL of phosphate-buffered saline (PBS) were mixed and centrifuged for 15 minutes, and then 4 mL of the supernatant was taken. The supernatant was mixed with 1 mL of 15% trichloroacetic acid (TCA) and 1 mL of TBA, heated in a water bath at 80 °C for 15 minutes, and cooled to room temperature for 60 minutes. Then, the results were centrifuged for 15 minutes, and the absorbance was measured with a spectrophotometer at a wavelength of 532 nm. The MDA concentration (nmol/mL) was determined based on the 1,1,3,3-tetramethoxypropane standard curve. Measurement of superoxide dismutase (SOD) activity in samples was carried out according to the method of Yuanita et al. (2019). A total of 0.06 mL of supernatant was reacted with a mixture consisting of 2.70 mL of 50 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10), 0.06 mL of 10 mM Xanthine, 0.03 mL of bovine serum albumin (BSA) 0.5%, and 0.03 mL of NBT 2.5 mM. Next, xanthine oxidase (0.04) units were added. The absorbance produced after 30 minutes was measured at a wavelength of 560 nm. As the control solution, PBS containing 11.5 g/L KCl was used, which was a blood preparation solution. The units per milliliter (U/mL) of sample are used to express enzyme activity.

2.7. Data Analysis

The data were statistically analyzed with analysis of variance (SPSS version 25.0). Duncan’s test was performed if there was a significant effect (p<0.05) of the treatment on the measured parameters and if there was a significant effect of the treatment.

3. Results

3.1. Broiler chicken performance

Broiler chicken performance data are presented in Table 2. During the study period, daily weight gain, daily feed consumption and feed efficiency were higher (p<0.01) at T0 than at T1. In addition, the T4 group showed higher daily weight gain (p<0.01) than the T0, T1, and T2 groups but did not differ from the T3 group. T4 had the highest daily feed consumption (p<0.05) compared to the other treatment groups. During the study period, feed efficiency was better (p = 0.02) in groups T0, T3, and T4 than in group T1.

3.2. Intestinal morphology of broiler chickens

Data on the intestinal morphology of broiler chickens reared at high densities are presented in Table 3. The results showed that the symbiotic treatment of nutmeg flesh extract and L. plantarum significantly (p<0.01) increased the length of the villi in the duodenum, jejunum, and ileum and the
ratio of villi length: crypt depth in the jejunum and ileum but had no significant effect (p><0.05) on crypt depth in any segment of the small intestine or the ratio of villi length: crypt depth in the duodenum of broiler chickens reared at high density.

3.3. pH Value and Total Broiler Gut Bacteria

The average pH and gut microbes of broiler chickens reared at high densities are presented in Table 4. The results showed that symbiotic treatment with nutmeg flesh extract and L. plantarum in broilers reared at high densities significantly (p<0.05) reduced the pH of the ileum and cecum broiler chickens and had no significant effect (p>0.05) on the pH of the duodenum and jejunum.

The data presented in Table 4 indicated that the administration of nutmeg flesh extract and L. plantarum to broiler poultry raised at high densities had a significant effect (p<0.05). This resulted in a significant increase in lactic acid bacteria and a significant decrease (p<0.05) in coliform bacteria in the ileum and cecum.

3.4. Antioxidant status of Broiler chickens

Data on the H/L ratio as well as MDA and SOD levels in broiler serum are presented in Table 5. The results showed that the administration of symbiotic nutmeg flesh extract and L. plantarum significantly reduced the H/L ratio (p<0.05) in broiler chickens reared at high densities. MDA levels were lower (p<0.05) in the T4 treatment than in the T0, T1, T2, and T3 treatments. No significant difference (p>0.05) in MDA levels was observed between the T0 and T1 groups. SOD levels were lower (p<0.05) in the T1 group than in the T0, T2, T3, and T4 treatments. Compared with other treatments, broiler chickens in treatment T4 showed the highest serum SOD levels (p<0.05).

Table 2 Performance of broiler chickens (days 14 - 35).

<table>
<thead>
<tr>
<th>Items</th>
<th>Treatment groups</th>
<th>SE</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily weight gain (g/bird/day)</td>
<td>T0</td>
<td>T1</td>
<td>T2</td>
</tr>
<tr>
<td>57.14b</td>
<td>51.95a</td>
<td>56.49b</td>
<td>58.07c</td>
</tr>
<tr>
<td>Daily feed intake (g/bird/day)</td>
<td>T0</td>
<td>T1</td>
<td>T2</td>
</tr>
<tr>
<td>95.42b</td>
<td>90.63a</td>
<td>96.26b</td>
<td>96.87c</td>
</tr>
<tr>
<td>Feed efficiency (%)</td>
<td>T0</td>
<td>T1</td>
<td>T2</td>
</tr>
<tr>
<td>60.47b</td>
<td>57.33a</td>
<td>58.69b</td>
<td>59.95c</td>
</tr>
</tbody>
</table>

**In the same row, different superscripts indicate a significant variation (p<0.05). T0: chicks raised at a normal density of 10 chicks/m², T1: chicks raised at a high density of 18 chicks/m², T2: chicks raised at high density and fed with 0.5 mL/kg symbiotic nutmeg flesh extract and L. plantarum, T3: chicks raised at high density and fed with 1.0 mL/kg symbiotic nutmeg flesh extract and L. plantarum, T4: chicks raised at high density and fed with 1.5 mL/kg symbiotic nutmeg flesh extract and L. plantarum, SE: standard error.**

Table 3 Intestinal morphology of broiler chickens.

<table>
<thead>
<tr>
<th>Items</th>
<th>Treatment groups</th>
<th>SE</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>T0</td>
<td>T1</td>
<td>T2</td>
</tr>
<tr>
<td>Villi height (µm)</td>
<td>1687.46b</td>
<td>1554.26a</td>
<td>1697.65b</td>
</tr>
<tr>
<td>Crypt depth (µm)</td>
<td>238.65</td>
<td>228.54</td>
<td>237.84</td>
</tr>
<tr>
<td>VH/CDF</td>
<td>7.07</td>
<td>6.80</td>
<td>7.14</td>
</tr>
<tr>
<td>Jejunum</td>
<td>T0</td>
<td>T1</td>
<td>T2</td>
</tr>
<tr>
<td>Villi height (µm)</td>
<td>1278.06b</td>
<td>1011.72a</td>
<td>1289.14b</td>
</tr>
<tr>
<td>Crypt depth (µm)</td>
<td>176.42</td>
<td>173.21</td>
<td>176.89</td>
</tr>
<tr>
<td>VH/CDF</td>
<td>7.42b</td>
<td>5.84a</td>
<td>7.29b</td>
</tr>
<tr>
<td>Ileum</td>
<td>T0</td>
<td>T1</td>
<td>T2</td>
</tr>
<tr>
<td>Villi height (µm)</td>
<td>888.23b</td>
<td>665.34a</td>
<td>717.05a</td>
</tr>
<tr>
<td>Crypt depth (µm)</td>
<td>138.24</td>
<td>135.62</td>
<td>140.04</td>
</tr>
<tr>
<td>VH/CDF</td>
<td>6.42b</td>
<td>4.91a</td>
<td>5.12a</td>
</tr>
</tbody>
</table>

**In the same row, different superscripts indicate a significant variation (p<0.05). T0: chicks raised at a normal density of 10 chicks/m², T1: chicks raised at a high density of 18 chicks/m², T2: chicks raised at high density and fed with 0.5 mL/kg symbiotic nutmeg flesh extract and L. plantarum, T3: chicks raised at high density and fed with 1.0 mL/kg symbiotic nutmeg flesh extract and L. plantarum, T4: chicks raised at high density and fed with 1.5 mL/kg symbiotic nutmeg flesh extract and L. plantarum, VH/CDF: villi height to crypt depth ratio, SE: standard error.**

4. Discussion

Raising broiler chickens at high densities interferes with chicken growth. FCR is also significantly exacerbated by high-density rearing systems, resulting in inefficient use of feed. Raising broiler chickens at high densities causes several negative factors that affect growth performance, such as heat stress, limited movement, and reduced access to feed and drink areas (Cengiz et al 2015; Selvam et al 2017). Cengiz et al (2015) reported that when cage density was increased from 10 to 20 birds/m², final body weight and cumulative feed consumption were reduced by 15.51% and 12.56%, respectively. Therefore, in this study, the daily weight gain of broiler chickens at high density (18 birds/m²) was significantly worse (10.03%), and feed consumption decreased (5.01%) compared to normal density (10 birds/m²). The rearing of broilers at high densities results in the movement of chickens to a limited area of the coop. Broiler chickens kept at normal density may have easier access to food and water than those kept at high density where the birds cannot move freely in the coop. Thus, feed intake may decrease at high rearing densities, resulting in reduced body weight and FCR.
In this study, the administration of synbiotic nutmeg flesh extract and *L. plantarum* improved the performance of broiler chickens raised at high densities. The synbiotic effectiveness of nutmeg flesh extract and *L. plantarum* can most likely be attributed to the synergistic action of various phytochemicals present in nutmeg pulp as well as the probiotic role of *L. plantarum*, which can maintain microbial balance and improve digestive function and intestinal absorption in broiler chickens (Blajman et al. 2014; Sapsuha et al. 2022). Altf et al. (2019) found the same result: Synbiotics could improve the performance of broiler chickens raised at high densities. The beneficial effects of synbiotics, which can stimulate the activity of one or more bacteria in the large intestine and thereby enhance health and body weight (Cengiz et al. 2015), may be responsible for weight gain caused by the administration of synbiotics. Other research reports that giving synbiotics to broiler chickens causes the secretion of different enzymes (amylolytic, proteolytic, and lipolytic) in the chicken intestine by probiotic microbes that help digestion nutrients (Abdel-Raheem et al. 2012; Hassanpour, 2013; Sarangi et al. 2016; Mohammed et al. 2018). Consequently, there is an increase in body weight gain and feed intake, which leads to better chicken performance. Kridtayopas et al. (2019) also reported that providing synbiotics to broiler chickens can facilitate better growth performance of broiler chickens.

### Table 4 pH and selected bacterial populations in the intestine of broiler chickens.

<table>
<thead>
<tr>
<th>Items</th>
<th>Treatment groups</th>
<th>SE</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>T0</td>
<td>6.13</td>
<td></td>
</tr>
<tr>
<td>Jejunum</td>
<td>T0</td>
<td>5.38</td>
<td></td>
</tr>
<tr>
<td>ileum</td>
<td>T0</td>
<td>6.14</td>
<td></td>
</tr>
<tr>
<td>Cecum</td>
<td>T0</td>
<td>7.32</td>
<td></td>
</tr>
<tr>
<td>Lactic Acid Bacteria (log cfu/g)</td>
<td>T0</td>
<td>9.20</td>
<td></td>
</tr>
<tr>
<td>ileum</td>
<td>T0</td>
<td>9.66</td>
<td></td>
</tr>
<tr>
<td>Cecum</td>
<td>T0</td>
<td>7.00</td>
<td></td>
</tr>
<tr>
<td>Coliform (log cfu/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ileum</td>
<td>T0</td>
<td>8.95</td>
<td></td>
</tr>
<tr>
<td>Cecum</td>
<td>T0</td>
<td>8.51</td>
<td></td>
</tr>
</tbody>
</table>

a,b,cIn the same row, different superscripts indicate a significant variation (p<0.05). T0: chicks raised at a normal density of 10 chicks/m², T1: chicks raised at a high density of 18 chicks/m², T2: chicks raised at high density and fed with 0.5 mL/kg synbiotic nutmeg flesh extract and *L. plantarum*, T3: chicks raised at high density and fed with 1.0 mL/kg synbiotic nutmeg flesh extract and *L. plantarum*, T4: chicks raised at high density and fed with 1.5 mL/kg synbiotic nutmeg flesh extract and *L. plantarum*, SE: standard error.

### Table 5 Serum levels of superoxide dismutase and malondialdehyde in broiler chickens.

<table>
<thead>
<tr>
<th>Items</th>
<th>Treatment groups</th>
<th>SE</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio H/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA (nanomol/mL)</td>
<td>T0</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>SOD (U/mL)</td>
<td>T0</td>
<td>47.70</td>
<td></td>
</tr>
</tbody>
</table>

a,bIn the same row, different superscripts indicate a significant variation (p<0.05). T0: chicks raised at a normal density of 10 chicks/m², T1: chicks raised at a high density of 18 chicks/m², T2: chicks raised at high density and fed with 0.5 mL/kg synbiotic nutmeg flesh extract and *L. plantarum*, T3: chicks raised at high density and fed with 1.0 mL/kg synbiotic nutmeg flesh extract and *L. plantarum*, T4: chicks raised at high density and fed with 1.5 mL/kg synbiotic nutmeg flesh extract and *L. plantarum*, SE: standard error.

The height of the villi in the duodenum, jejunum, and ileum in chickens reared at high density was lower than that in chickens reared at normal density. Shakeri et al. (2014) reported that chickens reared at high densities experienced a significant reduction in villi height in the duodenum, jejunum, and ileum compared to chickens reared at normal densities. The results showed that the addition of synbiotic nutmeg flesh extract and *L. plantarum* to feed for broiler chickens reared at high density increased villi height in the duodenum, jejunum, and ileum when compared to chickens reared at high density without the addition of synbiotics. Stress due to maintenance at high density can cause dysfunction in the mucosa (Song et al. 2014) and the formation of lipid peroxidation (Altan et al. 2003). In addition, microbiota dysbiosis under stress conditions can negatively impact gut structure and epithelial development (Sugiharto and Yudiarti, 2022).

In terms of the ratio of villus height: crypt depth in the jejunum and ileum, the addition of the synbiotic nutmeg flesh extract and *L. plantarum* to broiler chickens reared at high density significantly increased this ratio compared to that without synbiotic administration. Dizaji et al. (2012) found that supplementation with synbiotics (MOS mixed with *B. subtilis*) in broiler diets significantly increased the villi height and the ratio of villus: crypt depth when compared to broilers fed diets supplemented with probiotics or prebiotics alone. Under stress conditions, this indicated that synbiotic supplementation of nutmeg flesh extract and *L. plantarum* obviously improved intestinal morphology and consequently increased nutrient utilization.
There is an increase in the pH of the cecum when broiler chickens are kept at high densities. This is consistent with the results of Tsbouris et al. (2015), who reported that there was an increase in cecum pH in broiler chickens reared at high densities. The increase in cecal pH in broiler chickens reared at high densities is most likely due to a decrease in litter quality caused by increased humidity and temperature in the cage, which can affect the activity of the intestinal microorganisms. The main factors determining pH in the intestine are gastrointestinal secretions and volatile fatty acids produced by the intestinal microbiota. Giving symbiotic nutmeg flesh extract and L. plantarum to broiler chickens reared at high densities can reduce the pH in the ileum and cecum. Sunu et al. (2021) reported similar findings, stating that symbiotic treatment of onion extract and L. acidophilus might lower pH in the ileum and cecum. The decrease in intestinal pH due to administration of the symbiotic nutmeg flesh extract and L. plantarum in this study was similar to the findings of Ferdous et al. (2016), who found that giving 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 symbiotic nutmeg flesh extract and L. plantarum 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 plays a role in lowering pH. The decrease in pH in the ileum and cecum due to the administration of this symbiotic causes a decrease in the number of pathogenic bacteria in the intestine while increasing the digestibility of nutrients.

Symbiotics play an important role in the digestive tract, including increasing the number of beneficial bacteria (Kritdayopas et al. 2019), producing nonstarch polysaccharide enzymes and short-chain fatty acids (Mohammed et al. 2018), and improving the structure of the small intestine (Sunu et al. 2021). Rearing broiler chickens at a high density can alter the composition of bacteria in the intestine due to changes in intestinal microflora, specifically a decrease in the number of lactic acid bacteria in the ileum and cecum and an increase in the number of E. coli in the ileum compared to normal density maintenance. This clearly shows that rearing broiler chickens at high densities can disrupt the intestinal microbial population. Zhang et al. (2016) reported that stress causes overgrowth of pathogenic bacteria and suppresses the population of beneficial bacteria.

Administration of symbiotic nutmeg flesh extract and L. plantarum in broiler feed reared at high densities resulted in an increase in beneficial bacteria in the digestive tract, which could contribute to microbial balance and symbiosis. On the other hand, administration of symbiotics reduced the number of E. coli in the intestines of broiler chickens raised at high density. Pourabedin et al. (2014) showed that the administration of MOS and Bacillus sp. in broiler chickens, feed can increase the number of Lactobacillus sp., Bacillus sp., and Clostridium sp., as well as decrease the number of E. coli and Salmonella spp. in the digestive tract. The results of other studies show that the administration of a symbiotic consisting of a mixture of isomalto-oligosaccharides with 11 Lactobacillus strains can increase the number of Lactobacillus sp. in the cecum and decreased the number of E. coli in the cecum (Mookiah et al. 2014). This demonstrates the beneficial function of symbiotics on the microbial ecological balance in the gastrointestinal tract of broilers.

This increase in the H/L ratio can increase stress and indicate an increase in infections detected by the immune system. In high stress conditions, hepatophils will increase and lymphocytes will decrease as a result of tissue damage in the Bursa Fabricius, which causes the H/L ratio to increase (Akhhavan-Salamat and Ghasemi, 2015). A number of researchers have reported an increase in the H/L ratio with increasing cage density (Cengiz et al. 2015; Thaxton et al. 2006). Administration of symbiotic nutmeg flesh extract and L. plantarum to broiler chickens reared at high densities can reduce the H/L ratio. Similar results were reported by Sunu et al. (2021), who found that symbiotic administration of garlic extract and L. acidophilus bacteria can reduce the H/L ratio.

Stress is usually associated with a decrease in SOD values and an increase in MDA, which is the reaction of broiler chickens to excess free radicals (Sugiharto and Yudiarti., 2022). Li et al. (2019) found that stress caused by high stocking densities reduced broiler SOD levels. The increase in MDA levels in broiler chickens housed in high-density cages appears to be a response of broiler chickens to excessive free radical production. MDA is an indicator of lipid peroxidation in the body that is often used and is associated with oxidative stress (Binder et al. 2016). A high MDA concentration indicates an oxidation process in the cell membrane. Magnuson et al. (2020) also revealed that rearing broiler chickens at high densities increases the concentration of glutathione (a powerful nonenzymatic antioxidant) in chicken plasma. In addition to the effect of high density, administration of the symbiotic nutmeg flesh extract and L. plantarum increased SOD levels in broiler chickens. This finding is consistent with Sunu et al. (2021), who confirmed that administration of symbiotics increases SOD levels and reduces MDA levels in broiler chickens. This shows that symbiotics act as antioxidants that inhibit lipid peroxidation. According to Zhao et al. (2014), a decrease in plasma MDA levels indicates inhibition by antioxidants, and high antioxidant status is usually accompanied by a decrease in plasma MDA levels.

5. Conclusions

Providing symbiotic nutmeg flesh extract and L. plantarum can improve the performance and stress of broiler chickens reared at high densities.

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Ethical considerations

The Animal Research Ethics Committee of the Faculty of Agriculture, Universitas Khairun, approved the in vivo research with approval number 07/KEPH/PH/2023.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Sapsuha et al. (2023)


