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Intestinal Health in Broiler Chickens Treated with Nanoencapsulation of *Terminalia catappa* Leaf Extract as an Antibacterial Agent

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ABSTRACT

This study aimed to observe the characteristics of nanoencapsulation of *Terminalia catappa* leaf extract (NETLE) in drinking water as an antibacterial agent and its effects on growth traits, intestinal microflora population, and intestinal micromorphology in broiler chickens. In this study, as many as 192 male broiler chickens were kept in a semi-closed house and distributed into six treatments with four replications, each replication consisting of eight birds, with the experimental treatments consisting of water without any additive (T0; negative control), water added with 50 ppm antibiotics tetracycline (T1; positive control), water added with 15 mL/L *T. catappa* leaf extract (T2), water added with 30 mL/L *T. catappa* leaf extract (T3), water added with 15 mL/L NETLE (T4), water added with 30 mL/L NETLE (T5). The diet consisted of yellow corn and soybean meal with 22.09% crude protein, 3155.05 kcal/kg metabolizable energy, 1.10% calcium, and 0.67% available phosphorus. The study showed that the size of NETLE was 77.2 nm with a Polydispersity Index of 0.417 and a zeta potential value of +44.8 mV. It proved that NETLE had antibacterial activity against *Escherichia coli*, *Salmonella typhimurium*, and *Lactobacillus acidophilus*. The administration of NETLE in drinking water did not affect growth performance, villus width, and crypt depth, yet reduced *Salmonella* sp. population ($p<0.01$) and increased lactic acid bacteria population ($p<0.01$), villus height ($p<0.01$), and the ratio of villus height to crypt depth ($p<0.05$). The findings showed the beneficial function of NETLE additions in drinking water to improve histomorphology and reduce pathogens in the intestinal of broiler chickens.

***Keywords:** broiler chickens; intestinal histomorphology; intestinal microbes; nanoencapsulation technology; Terminalia catappa leaves*

INTRODUCTION

Intestinal health is a crucial matter to improve the welfare and performance of poultry, where problems with the gut can lead to financial loss for the producers or result in food safety issues for consumers (Robert *et al.*, 2015). Meanwhile, there are beneficial microbiota living in the GI tract of the poultry and are essential in maintaining gut health through several abilities. They can modulate the physiological functions of the host, necessarily in maintaining intestinal homeostasis, preventing pathogens colonization, reducing adverse competitive and pathogenic microorganisms, and optimizing energy expenditure used to boost the immune system. Therefore, when gut health is maintained, it will indicate energy savings and lead to a better condition to improve poultry growth performance and productivity (Carrasco *et al.*, 2019).

In the past, antibiotic growth promoters (AGPs) were widely used to improve livestock productivity. Antibiotics as antimicrobials were given to broiler chickens to control disease, promote growth, and improve feed efficiency (Costa *et al.*, 2017). However, the administration of in-feed antibiotics is no longer allowed for usage as feed additives for poultry as the public was afraid of the potential growth and development of various pathogenic microbes that are resilient to antibiotics, as well as of the presence of residues of this additive in the products of livestock.

To overcome such problems, the use of phytogenic feed additives (PFAs) as botanical products has recently gained attention with their prospectives among such alternatives to replace the use of AGPs (Ahsan *et al.*, 2018). The administration of PFAs was reported effective in stabilizing the intestinal microflora. It also has been reported to reduce the toxic metabolites of gut microbes due to their antimicrobial properties in various pathogenic bacteria (Gadde *et al.*, 2017). One alternative of plants that are used as a phytobiotic is *Terminalia catappa*. Extract of *T. catappa* leaves was known to have

the ability to inhibit the growth and colonization of *Staphylococcus aureus*, *Escherichia coli*, *Salmonella thypii*, *Bacillus cereus*, *Zymomonas mobilis*, and *Serratia marcescens* (Rajesh *et al.*, 2015). *T. catappa* leaf also contains phytochemicals, such as flavonoids (kaempferol or quercetin), some tannins (punicalin and punicalagin, or copied), saponins, phytosterols, β-carotene, glycosides, cyanogenic glycosides, alkaloids, phenols, and steroids (Offor *et al.*, 2015).

However, bioactive compounds are sensitive to some factors, including feed processing, distribution, storage processes, and all processes in the digestive tract (pH, enzymes, the presence of other nutrients) resulting in the limitation in absorption and utilization in the body of animals (Munin & Edward-levy, 2011). One possible alternative solution that are applicable is nanotechnology. Nanotechnology was reported to have the ability to increase the bioavailability of drugs that commonly have low solubility in systemic circulation. Therefore, they can provide pharmacological effects in smaller doses. One method of making nanoparticles is ionic gelation with the principle of ionic interactions. Ionic gelation can be made from chitosan with a positive charge and polyanion tripolyphosphate (Martien *et al.*, 2012). As encapsulation materials, the interaction between chitosan and sodium tripolyphosphate (STPP) forms a biocompatible cross-link that efficiently delivers bioactive phytobiotics in the intestines of broiler chickens. The application of nanoencapsulation technology to protect the bioactive of *T. catappa* leaf is expected to increase its utilization as a feed additive. However, the nanoencapsulation of *T. catappa* leaf extract (NETLE) used as an antibacterial agent that supports intestinal health in broiler chickens has never been studied. Therefore, the current study aimed to investigate the characteristics of NETLE and the effects of NETLE addition in drinking water on the intestinal microbial population and intestinal cell wall histomorphology of broiler chickens.

MATERIALS AND METHODS

This study was conducted in a semi-closed house poultry site at the Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia. The experimental methods used in the present research were ratified by the Research Ethics Committee, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia (Approval No. 0087/EC-FKH/Eks./2020). A total number of 192 male day-old New Lohmann MB 202 broiler chicks were distributed into six treatments and four replications, with eight birds in each replicate pen. Each pen was 1x1.5 m with rice husks as litter. The basal diet was based on yellow corn and soybean meal with 22.09% crude protein (CP), 3155.05 kcal/kg metabolizable energy (ME), 1.10% calcium (Ca), and 0.67% available phosphorus (Pav). The formulation and chemical composition of the basal diet are shown in Table 1. For the 35 consecutive days, each bird in the study was given one of the following treatments: drinking water without any additive (T0; negative control), drinking water + 50 ppm antibiotic tetracycline (T1; positive control), drinking water + 15 mL/L *T. catappa* leaf extract (T2), drinking water + 30 mL/L *T. catappa* leaf extract (T3), drinking water + 15 mL/L NETLE (T4), or drinking water + 30 mL/L NETLE (T5).

Nanoencapsulation Preparation

The formulation of NETLE applied the ionic gelation method (Sundari *et al.*, 2014) with the ratio of *T. catappa* leaf extract (1%): chitosan (0.1%): STPP (0.1%) was 1:5:1/60. *T. catappa* leaves were extracted using the maceration method (Ningsih *et al.*, 2019) using 70% ethanol (1:5 w/v) for three days. The filtrate was then filtered using filter paper and evaporated using a waterbath at 55 °C to remove ethanol. One gram of *T. catappa* leaf extract was dissolved in 5 mL of ethanol and 1 mL of tween 80. Then, 94 mL of distilled water was put into the mixture to obtain a 1% *T. catappa* leaf extract solution. Nanoencapsulation was obtained by mixing 1% *T. catappa* leaf extract solution with 0.1% chitosan that had been dissolved with the acetic acid solution. The extract and chitosan were mixed using a magnetic stirrer (C-MAG HS 7, IKA, Selangor, Malaysia) for 30 minutes, then was added with 0.1% STPP solution and mixed for 60 minutes.

Table 1. Ingredients and calculated chemical composition of basal diet

Ingredients	Proportion (%)	CP (%)	ME (kcal/kg)	Ca (%)	Pav (%)	Lys (%)	Met (%)	Thr (%)
Yellow corn	55.00	4.95	1815.00	0.01	0.14	0.16	0.10	0.20
Soybean meal	30.30	13.94	712.05	0.08	0.17	0.78	0.15	0.49
Meat bone meal	6.00	3.00	144.00	0.57	0.33	0.16	0.04	0.09
Rice brand	2.00	0.20	42.00	-	0.03	0.01	-	0.01
Palm oil	5.00	-	442.00	0.01	0.01	-	0.01	-
Vitamin premix	0.25	-	-	0.09	-	-	-	-
L-Lysine HCl	0.10	-	-	-	-	0.08	-	-
DL-Methionine	0.15	-	-	-	-	-	0.15	-
$CaCO_3$	1.00	-	-	0.34	-	-	-	-
NaCl	0.20	-	-	-	-	-	-	-
Total	100.00	22.09	3155.05	1.10	0.67	1.18	0.45	0.79

Note: CP= Crude Protein; ME= Metabolizable Energy; Ca= Calcium; Pav= available Phosphorus; Met= Methionine; Lys= Lysine; Thr= Threonine. Vitamin premix (Masamix-Bro) composition was Vit. A: 12.500.000 IU; Vit. D3: 2.500.000 IU; Vit. E: 10.000 mg; Vit. K3: 2.000 mg; Vit. B1: 2.000 mg; Vit. B2: 4.000 mg; Vit. B6: 1.000 mg; Vit. B12: 12.000 mg; Vit. C: 40.000 mg; Niacin: 40.000 mg; Biotin: 200 mg.

Nanoencapsulation Characteristics Determination

Particle size and zeta potential. Analysis of the particle size and zeta potential were determined by the Dynamic Light Scattering (DLS) method. The DLS measurements were carried out using a commercial laser light scattering instrument or particle size analyzer (SZ – 100, HORIBA, Fukuoka, Japan) at a temperature of 25 °C and with a scattering angle of 90° (Liang *et al.,* 2017).

Nano morphology. The morphology (shape) of the nanoencapsulation was analyzed using Transmission Electron Microscopy (JEOL JEM 1400 Pluss, Jeol, Peabody, USA). The nanoencapsulation sample was dropped on a copper grid (Chopper grid) and then coated with carbon with an auto carbon coating tool for 2-3 minutes. Samples with copper grids were immersed in phosphotungstic acid for 2-3 minutes. Then, the samples were observed using TEM, following the method used by Liang *et al*. (2017).

Antibacterial Activity Analysis

Antibacterial activity was analyzed using the well diffusion method (Masjid *et al.,* 2019). The pure cultures of bacteria were subcultured on Mueller Hinton Agar (MHA). The nanoencapsulation of *T. catappa* leaf extract was tested against three strains of bacteria (*E. coli*, *Salmonella typhimurium*, and *Lactobacillus acidophilus*). Each was swabbed uniformly on a plate using a sterile cotton swab and wells (6mm) were made using puncture. The samples that contained aquadest, antibiotic tetracycline 50 ppm, 0.1% chitosan, 0.1% STPP, 1% *T. catappa* leaf extract and nanoencapsulation of *T. catappa* leaf extract were poured into wells using a micropipette. The mixtures were stored in 35 °C room incubator (Memmert Incubator Oven INB 200, Memmert, Schwabach, Germany) for one day. Determination of bacterial inhibition was measured by measuring the diameter of the clear zone using a ruler with millimeter gradations.

Growth Performance Analysis

Growth performance parameters, including feed intake (FI), water intake (WI), and mortality, were recorded daily, whereas feed conversion ratio (FCR) and body weight gain (BWG) was calculated weekly. One chicken was selected from each subgroup at 35-day with masses close to average, where it included 24 samples (six treatments and four replications). Samples were slaughtered by cervical dislocation and eviscerated manually.

Intestinal Microbial Population Analysis

Determination of the number of intestinal microbes was carried out by calculating the Total Plate Count (TPC). Variables observed included the number of lactic acid bacteria and *Salmonella* sp. colonies. The TPC calculation media used were de-Man's, Rogosa, Sharpe Agar (MRSA) media for LAB, and Salmonella Shigella Agar (SSA) media for *Salmonella* sp. Samples were taken at the end of the 35-day maintenance periods where one bird per group with a weight close to the median among 24 samples was chosen (six treatments and four replications). The jejunum digesta sample was collected and put into a sterile bottle. One gram of jejunal digesta sample from each treatment was inserted into the first test tube containing 9 mL of distilled water. The mixture then was homogenized and obtained 10^{-1} dilution. One mL of the mixture from the first tube was then put into the second tube. The tubes were shaken until homogenous and gained a 10^{-2} dilution. Then, 1 mL from the second tube was then inserted into the third tube. The tubes were shaken, and a 10^{-3} dilution was obtained. The processes were repeated until a 10^{-10} dilution was obtained. The sterilized medium was then poured into a petri dish and waited for it to solidify. Afterward, 1 mL of the solution was removed from the dilution tube and poured into a petri dish containing the medium. Petri dishes were incubated in reverse position at 37 °C for 48 hours for lactic acid bacteria and 24 hours for *Salmonella* sp. (Halimatunnisroh *et al.,* 2017).

Intestinal Histomorphology Analysis

Samples of jejunal sections were taken from healthy birds from each replicate pen. The jejunum was removed from the endpoint of the duodenal loop up to approximately 1 cm proximal to the Meckel's diverticulum. For histomorphology examination, segments of about 6 cm were taken from the middle of the jejunum. The digesta was removed by flushing them with physiological sodium chloride (0.89% NaCl in sterile water) and cut into three equal pieces. The tissues slices were then immediately stored in 10% formalin buffered solution, shaken slowly, and kept in a proper place, according to the method by Dono (2012).

For glass slides preparation, the samples were removed from the stored solution, dehydrated in acetone concentrations (35%, 50%, 70%, and 95%), and embedded in paraffin. Villi tissues were then cut into 4 μm slices using a microtome and placed on slides to be stained by the hematoxylin-eosin. The observations of histomorphology of the jejunal villi, including villus height, villus width, crypt depth, and villus height : crypt depth (VH:CD) were conducted using a light microscope with 4X magnification equipped with an Optilab digital camera (Optilab Advance, Miconos, Yogyakarta, Indonesia) according to the method used by Choiri *et al.* (2018).

Statistical Analysis

All data except nanoencapsulation characteristic parameters were statistically analyzed using a completely randomized design in a one-way ANOVA based on less than 5% probability value. Data with significant differences were further tested using the Duncan test (antibacterial activity) and the orthogonal contrast test (microbial populations and intestinal histomorphology). Statistical analysis calculations were performed using SPSS (version 20; International Business Machines Corporation, Armonk, NY).

RESULTS

Nanoencapsulation Characteristics

NETLE was obtained using the ionic gelation method by utilizing the positive charge of chitosan and the negative charge of STPP. The results obtained were the particle size of the NETLE of 77.2 nm with a poly-dispersity index value of 0.471 and zeta potential value of +44.8 mV. The morphology of NETLE is shown in Figure 1.

Antibacterial Activity

The *in vitro* study in Table 2 showed that NETLE was efficacious to limit the growth and population of *E. coli*, *S. typhimurium*, and *L. acidophilus* ($p<0.01$).

Growth Performance

The nanoencapsulation of *T. catappa* leaf extract did not significantly affect feed intake, water intake, body weight gain, feed conversion ratio, and liveability of broiler chickens. Data are presented in Table 3.

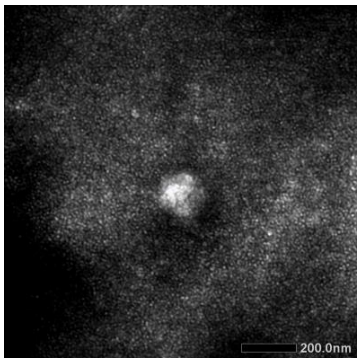

Figure 1. Morphology of nanoencapsulation of *Terminalia catappa* leaf extract

Intestinal Microbial Populations

Results in Table 4 revealed that drinking water with 15-30 mL/L *T. catappa* leaf extract administration, whether nano-capsulated or not, were effective to stop the growth and population of *Salmonella* sp. ($p<0.01$) and stimulating the growth of lactic acid bacteria ($p<0.01$) in the jejunum of broiler chickens. The result was similar to the use of 50 ppm antibiotic tetracycline. Orthogonal contrast tests indicated that the use of tetracycline, *T. catappa* leaf extract, and NETLE increased the population of LAB ($p<0.01$) and decreased the population of *Salmonella* sp. ($p<0.01$) in the jejunum. If compared to those of non-nanoencapsulated, the application of nanoencapsulation technology to *T. catappa* leaf extract maximized the growth and population of lactic acid bacteria ($p<0.01$).

Intestinal Histomorphology

Results in Table 5 show that drinking water with the addition of tetracycline or *T. cattapa* leaf extract stimulated the proliferation of the villus cells ($p<0.01$) and tended to stimulate the growth of crypt cells ($p=0.09$) in the jejunal wall of broiler chickens ($p<0.01$). Orthogonal contrast test shows that administration of 15 or 30 mL/L *T. catappa* leaf extract, capsulated or not, resulted in higher villus when compared to that of tetracycline addition ($p<0.01$). There was no differential effect between nanocapsulated and non-nanocapsulated extracts on the stimulation of villus cell growth. Results also revealed that the addition of *T. cattapa* leaf extract increased the villus height to crypt depth ratio ($p<0.05$). The orthogonal contrast test also showed that additions of 15 or 30 mL/L *T. catappa* leaf extract, capsulated or not, resulted

Table 2. Antibacterial activity of nanoencapsulated extract (*Terminalia catappa* leaf extract: chitosan: STPP)

Isolate	Inhibition zone diameter (mm)						SEM	p-value
	T0	T1	T2	T3	T4	T5		
Eschericia coli	0^{a}	18.92^{e}	9.25^{b}	0^{a}	14.92^{d}	13.25^{c}	0.22	<0.01
Salmonella typhimurium	0^{a}	0^{a}	8.03^{b}	0^{a}	7.83^{b}	7.83^{b}	0.24	<0.01
Lactobacillus acidophilus	0^{a}	0^{a}	0^{a}	0^{a}	6.33^{c}	1.75^{b}	0.07	<0.01

Note: Means in the same row with different superscripts differ significantly ($p<0.01$). T0= Aquadest (negative control); T1= antibiotic tetracycline 50 ppm (positive control); T2= 0.1% chitosan; T3= 0.1% STPP; T4= 1% *T. catppa* leaf extract; T5= NETLE. NETLE= nanoencapsulation of *Terminalia catappa* leaf extract.

Table 3. Performance of broiler chickens given drinking water with nanoencapsulation of *Terminalia catappa* leaf extract for 35 days

Variables	Treatments						SEM	p-value
	T0	T1	T2	T3	T4	T5		
FI (g/bird)	3127.60	3280.38	3119.19	3063.07	3058.22	3120.97	30.63	0.35
WI (l/bird)	6.23	6.60	6.24	6.24	6.16	6.49	0.05	0.06
BWG (g/bird)	1950.88	2109.13	1996.35	1988.76	1984.85	2035.64	19.36	0.24
FCR	1.60	1.56	1.56	1.54	1.54	1.53	0.01	0.59
Live ability (%)	100.00	100.00	100.00	100.00	100.00	96.88	0.52	0.45

Note: FI= feed intake; WI= water intake; BWG= body weight gain; FCR= feed convertion ratio. T0= water without feed additives (negative control); T1= water + 50 ppm antibiotic tetracycline (positive control); T2= water + 15 mL/L *T. catappa* leaf extract; T3= water + 30 mL/L *T. catappa* leaf extract; T4= water + 15 mL/L NETLE, T5= water + 30 mL/L NETLE. NETLE= nanoencapsulation of *Terminalia catappa* leaf extract.

Table 4. Microbial populations (log cfu/g) in the jejunum of broiler chickens given drinking water with nanoencapsulation of *Terminalia catappa* leaf extract for 35 days

Species	Treatments						SEM	p-value
	T0	T1	T2	T3	T4	T5		
Lactic acid bacteria	2.45[a]	3.49[b]	4.29[c]	4.23[c]	5.00[d]	5.19[d]	0.21	<0.01
Salmonella sp.	1.60[a]	0[b]	0[b]	0[b]	0[b]	0[b]	0.34	<0.01

Note: Means in the same row with different superscripts differ significantly (p<0.01). T0= water without feed additives (negative control); T1= water + 50 ppm antibiotic tetracycline (positive control); T2= water + 15 mL/L *T. catappa* leaf extract; T3= water + 30 mL/L *T. catappa* leaf extract; T4= water + 15 mL/L NETLE, T5= water + 30 mL/L NETLE. NETLE= nanoencapsulation of *Terminalia catappa* leaf extract.

Table 5. Jejunal histomorphology responses to administration of nanoencapsulation of *Terminalia catappa* leaf extract in broiler chickens for 35 days

Histomorphology variables (μm)	Treatments						SEM	p-value
	T0	T1	T2	T3	T4	T5		
VH	1307.89[a]	1407.15[b]	1723.00[c]	1577.89[c]	1521.01[c]	1650.20[c]	37.72	<0.01
VW	186.64	156.54	173.57	196.36	165.49	158.67	31.06	0.41
CD	237.45	246.34	233.59	154.95	185.09	192.17	11.11	0.09
VH:CD	5.68[a]	6.15[b]	7.48[c]	10.61[d]	8.34[cd]	9.20[cd]	0.50	0.02

Note: Means in the same row with different superscripts differ significantly (p<0.01). T0= water without feed additives (negative control); T1= water + 50 ppm antibiotic tetracycline (positive control); T2= water + 15 mL/L *T. catappa* leaf extract; T3= water + 30 mL/L *T. catappa* leaf extract; T4= water + 15 mL/L NETLE, T5= water + 30 mL/L NETLE. NETLE= nanoencapsulation of *Terminalia catappa* leaf extract; VH= Villus height; VW= Villus width; CD= Crypt depth.

in higher villus compared to that of tetracycline addition (p<0.05). There was no differential effect between nanocapsulated and non-nanocapsulated extracts on the VH:CD ratio.

DISCUSSION

Nanoencapsulation Characteristics

The particle size of NETLE was 77.2 nm with a polydispersity index (PI) value of 0.417. The size of nanoparticles is in agreement with Deng *et al*. (2020) that nanoparticles have a size range from 1 to 1000 nm. Katouzian & Jafari (2016) stated that the smaller the nanoparticle size, the higher the rate of release and distribution. In this case, the particle size might be affected by the extract ratio, chitosan, and STPP used, and the homogenization methods used to make encapsulation. Soo *et al.* (2016) reported that PI above 0.5 indicates heterogeneity in the system so that NETLE has a uniform particle size distribution and is single dispersed.

Based on the results, NETLE had a zeta potential value of +44.8 mV. These results indicated that nanoencapsulation of *T. catappa* leaf extract was stable because the value was more than +30 mV. The suitable zeta potential value indicated the stability of the nano size in delivering tiny particles. Nano particle with zeta potential above +-30 mV showed a stable suspension, as the surface charge prevents aggregation of the particles (Servat-Medina *et al*., 2015).

Figure 1 is the result of the morphological NETLE using TEM, observed on the scale of 100 nm and 200 nm. The image shows that the morphology of the NETLE was spherical. The spherical shape was essential since a less spherical particle shape will facilitate a stronger contact between particles, leading to aggregation (Martien *et al*., 2012).

Antibacterial Activity

Ionic gelation is a nanotechnology method through ionic cross-linking with polyanions via the ionic gelation technique. The complexation occurs spontaneously between the positively-charged chitosan and the negatively-charged polyanion resulting in physically cross-linked due to the electrostatic interaction of nanoparticles. The encapsulation of active compounds in chitosan nanoparticles, which are usually molecules of high crystallinity, results in their amorphization. This condition can reduce the crystallinity and thus enhance their bioavailability (Michailidou *et al*., 2020).

The *in-vitro* bacterial inhibition analysis in the present study shows that the NETLE had antibacterial activity, which was beneficial for the alternative for in-feed antibiotics. The antibacterial activity in NETLE was attributed to the bioactive compounds in the *T. catappa* leaf extract. It is in line with a previous study conducted by Rajesh *et al.* (2015), stating that bioactive compounds in *T. Catappa* leaf extract reduced the growth and population of pathogenic bacteria in both gram-positive (*Staphylococcus aureus* and *Bacillus cereus*) and gram-negative (*Salmonella typhii, Serratia marcescens,* and *Escherichia coli*). Antibacterial activity of plant extract was closely related to the presence of secondary metabolites, such as tannins, saponins, terpenoids, alkaloids, and flavonoids. The bioactive secondary metabolites from different classes of phytochemicals were reported to inhibit growth or even kill the pathogens via different mechanisms of action (Dono, 2012). Meanwhile, the antibacterial agents in the *T. catappa* extract can inhibit nucleic acid, protein, cell wall, and membrane phospholipids biosynthesis (Kankia, 2014).

Growth Performance

The result showed no significant difference in the growth performance parameters among all treatments. The lack of direct positive response in growth traits might be attributed to the conditions where dietary *T. catappa* supplementations did not change the nutrient and energy content of the treatment diets. On the other hand, the supplementary dosages were still considered to be small and were still accepted by the birds and did not affect the growth performance. Diaz-Sanchez *et al.* (2015) stated that plant selection and the optimal dosages are vital to improve growth performance, as improper dosages did not affect growth performance.

Microbial Populations

The increase in the lactic acid bacteria population and the reduction in *Salmonella* sp. population indicated the positive effects of supplying drinking water added with NETLE. The improved microbial environment in the current study might also be attributed to the essential bioactive substances in *T. catappa* leaves that have antibacterial properties. Saki *et al*. (2014) similarly reported that drinking water with phytobiotics addition reduced the number of pathogenic bacteria and increased the population of beneficial bacteria. The prospect of phytobiotic as a natural antibiotic is related to the ability of bioactive compounds to break down the cellular membrane and interfere with the cytoplasm ecosystem of the pathogens (Gheisar & Kim, 2017). Those abilities will result in bacteriostatic and bacteriocidal activities of phytobiotic. The decreasing population of pathogens should increase the growth and population of beneficial microbes in the intestine of the host animal.

In this case, the addition of NETLE resulted in a higher LAB population in the jejunal section of broiler chickens compared to the addition of non-capsulated *T. catappa* leaf extract. In this study, although *T. catappa* leaf extract inhibited the growth of *L. acidophilus* bacteria, it also contained bioactive that could impede the growth of pathogenic bacteria, proven by a higher inhibition zone for *S. typhimurium* and *E. coli* bacteria. Moreover, *T. catappa* leaves contain several organic acids (Oyeleye *et al.,* 2017), which increased the LAB population in other studies (Sugiharto, 2016). The mechanism of why organic acids in *T. catappa* inhibited the growth of *L. acidophilus* but stimulated the growth of LAB has still remained unknown.

Intestinal Histomorphology

The addition of NETLE to the drinking water did not affect villus width and crypt depth, yet it contributed to the increase in villus height and villus height to crypt depth ratio (VH:CD). The increase in villus height and VH:CD values indicated that the administration of NETLE had a better effect on the intestinal health of broiler chickens. It is in line with the results of Khan *et al*. (2017) study showing that the increase in villus height, along with the increased VH:CD, had a direct correlation with the increase in epithelial cell turnover and thus activated cell mitosis. Olukosi & Dono (2014) also reported that the longer villi support, the greater nutrient absorption due to the increased surface area. In contrast, deeper crypts show higher cell turnover in response to normal cell shedding or inflammatory responses. The higher ratio of villus height to crypt depth indicated healthy intestinal development. Nevertheless, the improvements in the micro-villus growth and the ratio between villus height and crypt depth in the current study were not enough to facilitate better growth performance of the host animal. These phenomena might be due to the other aspects of nutritional physiology rather than only a single causative improvement in the intestinal absorptive cells. Many factors that have also been reported to affect growth performance include genotype (strain), diets content, sex, design of pen, stocking density, and room temperature and ventilation (Baracho *et al.,* 2019; Ikusika *et al.,* 2020).

The higher villus and VH:CD in the NETLE treatment might be caused by the antibacterial activity of the bioactive compounds in *T. catappa* leaves. The antibacterial activity might cause a reduction in the growth and colonization of pathogenic microbes in the intestine. That reduction should positively reduce the adverse effects of the toxic metabolites produced by the pathogens (Podolsky, 1993) and hence reduce the intestinal mucus secretion by Goblet cells (Ferket, 2004). Excessive growth of pathogens in the gut can lead to harmful metabolites production that, in turn, damages the intestinal wall (Lovland & Kaldhusdal, 2001; Sugiharto, 2016). Therefore, the reduction of pathogenic bacteria can improve the intestinal structure of chickens. Hashemi *et al*. (2014) stated that dietary supplementation of phytobiotics decreased the production of toxic compounds, reduced intestinal damage, and reduced lumen reconstruction.

CONCLUSION

Nanocapsulation of *T. catappa* leaf extract might be a promising alternative for in-feed antibiotics because it has a stable antibacterial activity against *E. coli*, *S. thypimurium*, and *L. acidophilus*. It also increases the population of lactic acid bacteria and stimulates the proliferation of absorptive cells in the jejunum of broiler chickens.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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