

Nutrient Utilizations and Intestinal Morphology of Broilers Treated with *Lactobacillus plantarum* AKK30 – Oligosaccharides Synbiotic

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ABSTRACT

Lactobacillus plantarum AKK30 inoculum in *de Maan Rogosa Sharpe Broth* (MRSB) grown on media containing oligosaccharides (inulin or mannan oligosaccharides/MOS) was studied *in vitro* and *in vivo*. *In vitro* assay was conducted to characterize metabolite profiles and its effect on pathogenic bacteria, while *in vivo* assay was conducted to study the metabolic energy, nitrogen retention, and intestinal morphology of broilers. A total of 24 male broilers-40-day-old (average body weight= 1.725±0.05 kg) strains Cobb-500 were used, where 20 birds were added 1% inoculum *L. plantarum* AKK30 of daily intake and randomly distributed in five treatments and four replications consisted of: A) MRSB + *L. plantarum* AKK-30 (10^7 cfu g⁻¹); B) MRSB + inulin 0.5% (w v⁻¹); C) MRSB + MOS 0.5%(w v⁻¹); D) *L. plantarum* AKK-30 (10^7 cfu g⁻¹) + MRSB + inulin 0.5% (w v⁻¹); and E) *L. plantarum* AKK-30 (10^7 cfu g⁻¹) + MRSB + MOS 0.5% (w v⁻¹), while 4 birds were fasted as endogenous chicken. The results showed that the highest isoleucine and threonine were found in group E. The highest oleic acid and the lowest conjugated linoleic acid (CLA) were observed in all treatments and inoculum did not affect antibacterial activity. The metabolizable energy values and nitrogen retention in group D and E were higher than other treatments (p<0.05). The lowest height of villi was observed in group A (p<0.05). In conclusion, the addition of oligosaccharides inulin or mannan oligosaccharide (MOS) affects the metabolite profiles of *Lactobacillus plantarum* AKK30 and their synbiotic effects improve intestinal morphology, metabolizable energy, and nitrogen retention in broilers.

Keywords: *Lactobacillus plantarum* AKK30; metabolizable energy; nitrogen retention; oligosaccharides; synbiotic

INTRODUCTION

The gastrointestinal tract (GIT) is important in poultry for digestion and poultry-host defense; however, GIT is easily attacked by the pathogenic bacteria (Sethiya, 2016). Millions of bacteria are exposed in the chicken intestine, which affects the health of the chicken (Forkus *et al.*, 2017) and nutrients digestibility (Pourabedin & Zhao, 2015). More than 900 species of bacteria are found in chicken intestine (Apajalahti & Vienola, 2016). Currently, probiotics are being used as antibacterial agents, especially to suppress the population of pathogenic bacteria in the intestine (Palaniyandi *et al.*, 2017) and nutrients digestion in chicken (Chang *et al.*, 2019).

The use of probiotics like *Lactobacillus* bacteria in poultry diets has substantially increased in the last years. Furthermore, a meta-analysis of studies of probiotics across Brazil until 2005 showed that probiotics are feasible as feed additives for broilers (Filho *et al.*, 2006).

Lactobacillus bacteria have antibacterial activity (Cao *et al.*, 2019), improve intestinal health (Yan *et al.*, 2017), affect intestinal morphology, and the thickness of broiler intestine (Tsirtsikos *et al.*, 2012). Roberfroid *et al.* (2010) reveal that *Lactobacillus* genera secrete enzyme for fermenting the undigested carbohydrates that are further converted into monosaccharides.

Concentrations of fatty acids, lactic acid, and pH in ileum and caeca are not affected by *Lactobacillus johnsonii* (Olnood *et al.*, 2015). However, there are still many problems related to the ability of *Lactobacillus* bacteria to increase nutrient digestibility (Wealleans *et al.*, 2017), the growth of villi (Rodjan *et al.*, 2018), and improve intestinal morphology (Peng *et al.*, 2016).

Exploration of symbiosis between probiotics and oligosaccharides is needed and the expected results are the increase in protein digestibility and improve intestinal microflora in broiler. Roberfroid *et al.* (2010) reported that oligosaccharides were non-digestible compounds and providing beneficial effects on host physiology.

Although those studies provided valuable information about probiotics and oligosaccharides, the potencies of synbiotic *L. plantarum* AKK30 and inulin to improve nutrient digestibility in chickens has not been reported. Therefore, this experiment aimed to study the nutrient utilizations and intestinal morphology of broilers treated with *Lactobacillus plantarum* AKK30 – oligosaccharides synbiotic on metabolizable energy, nitrogen retention, fatty acids, amino acids, inhibitory activities of pathogenic bacteria, and villi performance in broiler chicken.

MATERIALS AND METHODS

The ethical clearance for this experiment was endorsed by the commission for Clinical Experiment the Integrated Laboratory of Research and Testing (LPPT) at Universitas Gadjah Mada (UGM), Yogyakarta-Indonesia. The commission issued the ethical clearance certificate no. 00097/04/LPPT/VIII/2018.

Experimental Design, Birds, and Diets

The study was conducted in the laboratory of Bio-Feed Additive Technology and poultry closed house at the Research Division for Natural Product Technology (BPTBA), Indonesian Institute of Sciences (LIPI), Gading, Gunungkidul, Yogyakarta.

This study was conducted in two stages of experiments. The first stage was carried out by *in vitro* assessment of *L. plantarum* AKK30, which was cultured on the *deMann Rogosa Sharpe*/MRS broth (Oxoid) medium in combination with oligosaccharides (Inulin from Orafit, Beneo, USA and MOS from Technomos, Biochem, Germany). A completely randomized design (CRD) with five treatments consisting of A) MRSB + *L. plantarum* AKK-30 (10^7 cfu g^{-1}); B) MRSB + inulin 0.5% (w v^{-1}); C) MRSB + MOS 0.5% (w v^{-1}); D) *L. plantarum* AKK-30 (10^7 cfu g^{-1}) + MRSB + inulin 0.5% (w v^{-1}); and E) *L. plantarum* AKK-30 (10^7 cfu g^{-1}) + MRSB + MOS 0.5% (w v^{-1}), and 3 replications in each treatment. The measured parameters were optical density, anti-pathogenic bacterial activity, and the profiles of fatty acid and amino acids. 1% *L. plantarum* AKK30 in 2 mL of MRSB was added to the microtube and was vortexed, then incubated 24 hours at 37°C. A sequence of falcon tubes filled with 10 mL of sterile MRSB containing inulin or MOS (0%, 0.5% w v^{-1}), then inoculated with 1% (v v^{-1}) starter *L. plantarum* AKK30 as previously reported by Sofyan *et al.* (2019).

The second experiment was carried out by *in vivo* assessment of synbiotic on nutrient utilization in broilers. The guideline for the treatment in this study was conducted according to McGlone (2010). The broilers diet was formulated according to the recommendations of the nutrient requirement for chicken (NRC, 1994). The ingredient content and composition of the broiler diet are shown in Table 1. Chickens were fed diets in crumble form during the experiment period. Drinking water was available *ad libitum*.

A total of 24 male broilers-40-day-old (average body weight= 1.725±0.05 kg) strains Cobb-500 were

used, where 20 birds were added 1% inoculum *L. plantarum* AKK30 of daily intake and randomly distributed in five treatments and four replications consisted of: A) MRSB + *L. plantarum* AKK-30 (107 cfu g^{-1}); B) MRSB + inulin 0.5% (w v^{-1}); C) MRSB + MOS 0.5% (w v^{-1}); D) *L. plantarum* AKK-30 (107 cfu g^{-1}) + MRSB + inulin 0.5% (w v^{-1}); and E) *L. plantarum* AKK-30 (107 cfu g^{-1}) + MRSB + MOS 0.5% (w v^{-1}), while 4 birds were fasted as endogenous chicken. The treatments were arranged in a completely randomized design. All birds were individually reared in the metabolic cage (width 30 cm× length 40 cm× height 40 cm), and the room temperature was set at 23°C in a closed house system and illumination was provided for 24 h during the experiment. The observed parameters were apparent metabolize energy (AME) without nitrogen correction and AME with nitrogen correction (AMEn), true metabolize energy (TME) without nitrogen correction and TME with nitrogen correction (TMEn), nitrogen retention (NR), and intestinal morphology.

Chemical Analysis

Fatty acids consisted of myristic acid, palmitic acid, oleic acid, and conjugated linoleic acid (CLA) were determined by the gas chromatography-mass spectrometry (GCMS) type QP2010S (Shimadzu, Japan) equipped with column: Rtx 5 MS length: 30 meter ID: 0.25 mm Film: 0.25 μ m vehicle gas: Helium for ionization: EI 70 Ev. The n-hexane solution was used for the preparation of sample solutions, sterilizing, and heating for 2 hours. N-hexane was evaporated; the remainder of evaporation was a sample solution. Furthermore, the sample solution was esterified by adding KOH methanolate (1: 2) and sonicated 1.5 hours at 60-70°C. The sonication solution was extracted as well with hexane, which was evaporated using a hairdryer, and as much as \pm 0.5 mL of extractant were analyzed by GCMS.

The determination of metabolites amino acid was conducted by using the ultra-performance liquid chromatography (UPLC) with column AccQ.Tag Ultra C18 1.7 μ m (Waters Corp., Milford, MA, USA) and HPLC with column Lichospher 25 cm x 4.0 mm, 5 μ m from Merck. The value of the essential amino acid index (EAAI) of metabolites was estimated according to Istiqomah *et al.* (2009), by comparing the essential amino acid content from the standard (whole egg) as recommended by USDA (2017).

Turbidimetric measurements with a microplate reader (MultiSkan GO, ThermoScientific) at a wavelength of 590 nm were carried out for analyzing the growth activity of *Lactobacillus plantarum* AKK30. The absorbance value of the inoculum was recorded every 2 hours for 24 hours. Previously, a wheel tube containing 150 μ L inoculum (1% 0.1 mL/10 mL) was inserted in a plate reader. The pH of the sample was measured by pH meter and before being used, the pH meter was calibrated with phosphate buffer (pH 6.86) and acetate buffer (pH 4.00).

Antibacterial-activity assay of *L. plantarum* AKK 30 used twenty-five microlites of cell-free supernatant from inoculum (1% 0.1 mL/10 mL) which was inserted into a

Table 1. Ingredient composition (%) and nutrients content of broiler diet

Ingredients	Composition (%)	Nutrients contents	
Corn	55.00	Metabolize energy (kcal kg ⁻¹)	2,775.20
Rice bran	6.00	Crude protein (%)	19.14
Soybean meal	29.50	Ether extract (%)	6.90
Meat bone meal	3.00	Crude fiber (%)	6.91
Crude palm oil	2.50	Ash (%)	6.73
Premix*	0.50	Calcium (%)	1.76
DCP	0.80	Total Phosphorus (%)	1.17
Salt	0.10	Available Phosphorus (%)**	0.49
Limestone	1.70	Dry matter (%)	89.18
L-Lysine	0.60	Amino acids (%)	
DL-Methionine	0.30	Lysine	0.96
		Methionine	0.25
Total	100.00	Methionine + Cysteine	0.71

Note: *Premix/kg containing vitamins A: 12.500.000 IU, D3: 2.500.000 IU, E: 10.000 mg, K3: 2.000 mg, B2: 4.000 mg, B6: 1.000 mg, Niacin: 40.000 mg, Ca-d-Panthotenate: 4.000 mg, Choline: 20.000 mg, Fe: 30.000 mg, Cu: 5.000 mg, Mn: 80.000 mg, Co: 2.000 mg, I: 200 mg, and Zn: 70.000 mg.
**It was calculated according the cumulative content in feed formula.

blank paper disk Ø 0.6 mm (Oxoid) and applied to agar plate containing pathogenic bacteria as an indicator of microbe, following the agar diffusion method by Bonev *et al.* (2008). The supernatant was obtained by centrifugation of inoculum at 4136 x g for 15 minutes at 4°C. The parameters measured were diameter (mm) of the visible clear zone of pathogenic bacteria namely *Escherichia coli* FNCC 0194, *Staphylococcus aureus* FNCC 6049, *Pseudomonas aeruginosa* FNCC 0063, and *Salmonella pulorum* ATCC 13036 in nutrient agar/NA media (Merck).

Measurement of Nutrients Utilization

The value of nutritional utilization (ME and NR) was estimated, according to Santos *et al.* (2015). The experiment began with 40-day-old chickens for three days. On the first day, all birds were fasted for 24 hours so that the gut was empty (Sibbald & Wolynets, 1985). On the second day, all birds were given probiotic (1.45 mL) before they were fed and provided drinking water. On the third day, all excreta samples were collected, dried, milled, and weighed. Subsequently, the samples were analyzed for dry matter and chemicals. Additionally, four birds were fasted for 48 hours to estimate endogenous energy and nitrogen (N) losses. Parr ® 6200 Oxygen Bomb Calorimeter (Parr Instrument Company, USA) was used as an instrument to measure GE samples and the Kjeldahl method (AOAC, 2005) for analyzing nitrogen samples. ME and NR were estimated according to Sibbald & Wolynets (1985), following the formula:

$$AME = [(Ei - Ex) / Fi] \times 1000$$

$$AMEn = \{(Ei - [Ex + (8.22 \times NR)]) / Fi\} \times 1000$$

$$TME = [(Ei - (Ex + E end)) / Fi] \times 1000$$

$$TMEn = \{(Ei - [Ex - E end + (8.22 \times NR)]) / Fi\} \times 1000$$

$$NR = \{[Ni - (Nex - N end)] / Ni\} \times 100\%$$

where AME was apparent ME (kcal kg⁻¹), AMEn was apparent ME with nitrogen correction (kcal kg⁻¹), TME was true ME (kcal kg⁻¹), TMEn was true ME with nitrogen correction (kcal kg⁻¹), Fi was feed intake (g), Ei was

energy intake (g), Eexc was energy excreta (g), Eend was energy endogenous (g), Ni was nitrogen intake (g), Nex was nitrogen excreta (g), Nend is nitrogen endogenous (g), NR was nitrogen retention (%), and 8.22 was coefficient of energy value from uric acid (kcal g⁻¹ RN).

Intestinal Morphology Analysis

Scanning electron microscopy (Hitachi- SU3500) was used to analyze the ultrastructure of the intestine. Fifteen broiler chickens were randomly sampled and necropsied. Intestine (jejunum) was taken and sliced 4 cm. The jejunum samples were prepared using the biological sample preparation method for scanning electron microscope (Titze & Christel, 2016).

The preparation process of the intestinal sample includes a cleaning process involving soaking in a phosphate buffer pH 7 for 5 hours, followed by an agitation process in an ultrasonic cleaner for 7 min. The sample was prefixed into 2.5% glutaraldehyde solution for 24 hours and continued the fixation process in 2% tannic acids solution for 5 hours, then washed with phosphate buffer pH 7 for 15 min 4 times. The next process was the dehydration of the sample by soaking it in alcohol with graded concentrations (50, 70, 85, 95, and absolute). After passing through the dehydration process, the sample was dried by soaking in tetra-butanol, placed in the freezer until frozen, and then put into a freeze-drier for 24 hours. The prepared sample was subsequently placed on a stub specimen and coated with Au using an ion coater tool. Further, the length of jejunum villi was evaluated by using an image processing from a scanning electron microscope (Hitachi SU-3500).

Statistical Analysis

The collected data from ME and NR were statistically analyzed by using analysis of variance (ANOVA). The Duncan's post hoc test was applied to determine the effects of treatments on the parameters measured by

using the CoSTAT statistical software (Cohort, 2008). To evaluate the relationship between parameters from all the treatments, the data were analyzed using the principal component analysis (PCA) (Rotaru *et al.*, 2012).

RESULTS

In vitro Assay: Chemical Composition of Synbiotic Metabolites

The results showed that all treatments contained essential fatty acids (Table 2, Figure 1) wherein the content of myristic acid and palmitate acid in treatment D (inoculum in MRSB + inulin 0.5%) and treatment E (inoculum in MRSB + MOS 0.5%) were higher than the other treatments, but oleic acid was higher in treatment A (inoculum in MRSB without oligosaccharides). Treatments A, D, and E contained a small amount of CLA while treatments B (MRSB + 0.5% inulin) and C (MRSB + MOS 0.5%) did not contain CLA. Essential amino acids such as threonine and iso leucine were found with the higher concentrations in the treatment E compared to the other treatments. It was detected that treatment A contained the highest lysine and valine (Table 2). The essential amino acids index (EAAI) value (Table 2) showed that treatments A, D, and E had similar EAAI values i.e., 32.99%, 32.48%, and 32.37%, respectively. However, the EAAI values in treatments A, D, and E were better than treatments B and C with EAAI values of 21.2% and 20.99%, respectively. The EAAI values of treatments A, D, and E were similar ($p > 0.05$) that were significantly higher ($p < 0.05$) compared to those of treatments B and C. These results indicated that the amino acid in probiotic could not be replaced by prebiotics.

The values of optical density of *L. plantarum* in treatments D and E were 0.9979 and 1.0062, respectively that were higher compared to that in treatment A having a low optical density of 0.0014 at the 18th hour. At the 24th hour, the values were similar, and it was detected that the value of optical density of *L. plantarum* in treatments D and E were 1.0090 and 1.0034, respectively, that

were not significantly different but both of them were significantly higher than that in treatment A, i.e., 0.0079. These results showed that the addition of inulin and MOS had a better effect on the growth of *L. plantarum* compared to controls.

Table 2 showed that there were inhibitory zones on treatments A, D, and E groups, but antibacterial activity was not detected on treatments B and C. These results indicated that *L. plantarum* still had antibacterial activity even though oligosaccharides were added. Bacteriocins of *L. plantarum* AKK30 inhibited the growth of *Staphylococcus aureus*, *Salmonella pullorum*, *Escherichia coli*, and *Pseudomonas aeruginosa*. In contrast, the antibacterial activity of *L. plantarum* AKK30 was not affected by inulin and MOS (Figure 2).

In vivo Assay: Nutrients Utilization and Intestinal Morphology

According to Table 3, the weight of nitrogen excreta and gross energy excreta in broilers treated with inoculum in MRSB + inulin 0.5% (Group D) were lower than those in broilers treated with the other treatments. The results of ME and NR showed that the experimental broilers treated with inoculum in MRSB + inulin 0.5% (Group D), inoculum in MRSB + MOS 0.5% (Group E), and MRSB + inulin 0.5% (Group B) had similar AME, TME, and TME_n that were higher ($p < 0.05$) compared to those in Groups C and A.

The NR values in broilers treated with inoculum in group D, E, and B were similar i.e., 70.83%, 67.08%, and 66.41%, respectively. These NR values were higher ($p < 0.05$) compared to those in broilers treated with MRSB + MOS 0.5% (Group C) and inoculum in MRSB without oligosaccharides (Group A) having NR values of 56.35% and 61.85%, respectively. The results showed that broilers treated with inoculum in group D appeared to be superior compared to the other treatments.

The effects of various treatments on the heights of jejunum villus are shown in Table 3 and explained in Figure 3. Broilers treated with inoculum in group D, E,

Table 2. Probiotic growth and metabolites profile of broilers treated by *Lactobacillus plantarum* AKK30 – Oligosaccharides

Variables	Treatments				
	A	B	C	D	E
Optical density at 18 h	1.0009 ± 0.2 ^b	0.3193 ± 0.1 ^a	0.3303 ± 0.2 ^a	0.9979 ± 0.25 ^b	1.0026 ± 0.85 ^b
Optical density at 24 h	1.0024 ± 0.1 ^a	0.3247 ± 0.1 ^a	0.3314 ± 0.1 ^a	1.0090 ± 0.1 ^b	1.0034 ± 0.25 ^b
pH	3.13 ± 0.15 ^b	5.9 ± 3.0 ^a	6.2 ± 2.5 ^a	3.07 ± 2.4 ^b	3.11 ± 4.8 ^b
Myristic (C14) (%)	2.00	1.81	1.45	3.13	2.64
Palmitic (C16:1) (%)	17.05	10.15	9.82	21.94	17.79
Oleic (C18:1) (%)	51.32	30.22	31.05	29.07	41.7
CLA (C18:2) (%)	6.2	nd	nd	1.92	2.81
Lysine (mg kg ⁻¹)	1026.82	268.25	789.24	560.91	566.13
Iso-Leucine (mg kg ⁻¹)	381.84	218	36.84	341.19	489.35
Threonine (mg kg ⁻¹)	406.26	200.34	403.02	410.73	414.19
Valine (mg kg ⁻¹)	555.71	25.50	212.34	342.71	488.49
EAAI	32.99	21.12	20.99	32.48	32.37

Note: A= control, B= *de Maan Rogosa Sharpe Broth* (MRSB) + inulin 0.5%, C= MRSB + mannan oligosaccharides (MOS), D= *L. plantarum* AKK30 (10⁷ cfu g⁻¹) + MRSB + inulin 0.5%, and E= *L. plantarum* AKK30 (10⁷ cfu g⁻¹) + MRSB + MOS 0.5%. nd= not detected, CLA= conjugated linoleic acid, EAAI= essential amino acid index. Means in the same row with different superscripts differ significantly ($p < 0.05$).

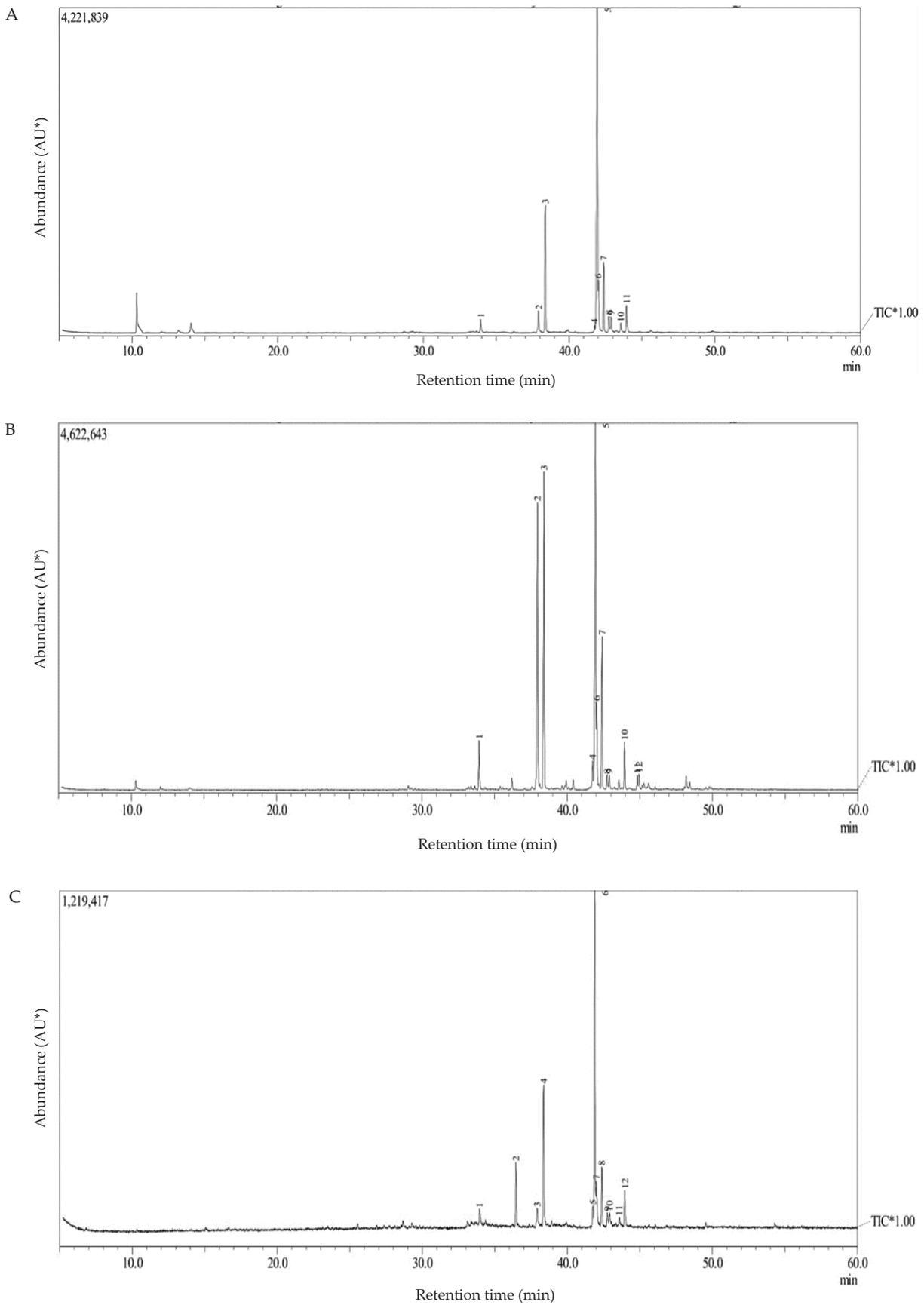


Figure 1. Chromatogram of control group (culture) (a), culture with inulin 0.5% ($w v^{-1}$) (b), and culture with MOS 0.5% ($w v^{-1}$) (c). * = Arbitrary unit, 1 = Myristic acid, 2 = Palmitoleic acid, 3 = Palmitic acid, 4 = Palmitolinoleic acid, 5 = Oleic acid, 6 = Vaccenic acid, 7 = Stearic acid, 8-9 = Conjugated linoleic acids.

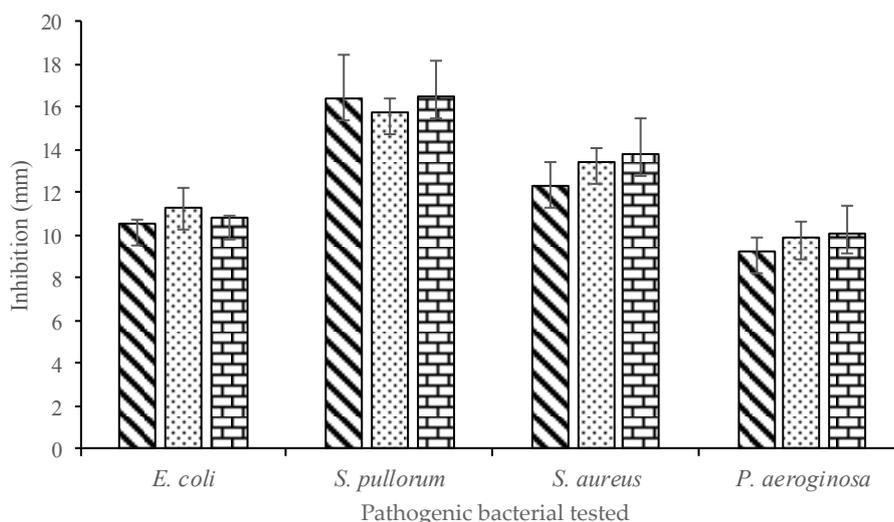


Figure 2. Inhibition of pathogenic bacterial growth by symbiotic consisted of *L. plantarum* and oligosaccharides, ▨ = LpAKK30, ▩ = LpAKK30 + Inulin, ▤ = LpAKK30 + MOS.

Table 3. Nutrient utilizations and villous height of broilers treated by *Lactobacillus plantarum* AKK30 – Oligosaccharides

Variables	Treatments				
	A	B	C	D	E
Weight of excreta (g DM)	64.10 ± 23.32 ^a	64.39 ± 13.00 ^{ab}	72.27 ± 21.94 ^a	45.84 ± 18.13 ^b	56.05 ± 21.69 ^b
N-excreta (g DM)	9.68 ± 12.61 ^a	11.44 ± 17.73 ^a	9.11 ± 11.51 ^a	8.78 ± 13.58 ^b	9.70 ± 16.59 ^a
GE-excreta (kcal kg ⁻¹)	2289.27 ± 24.13 ^a	2293.39 ± 18.41 ^a	2598.72 ± 20.04 ^a	1708.23 ± 15.62 ^b	1938.60 ± 23.08 ^b
AME (kcal kg ⁻¹)	2876.34 ± 2.55 ^a	3015.24 ± 4.64 ^{ab}	2910.29 ± 1.65 ^a	3119.44 ± 4.42 ^b	3106.53 ± 3.07 ^b
TME (kcal kg ⁻¹)	2988.50 ± 2.45 ^a	3121.83 ± 4.44 ^{ab}	3015.10 ± 1.61 ^a	3217.19 ± 4.37 ^b	3202.86 ± 2.93 ^b
TMEn (kcal kg ⁻¹)	2989.37 ± 2.45 ^a	3122.80 ± 4.44 ^{ab}	3015.89 ± 1.60 ^a	3218.19 ± 4.37 ^b	3203.81 ± 2.93 ^b
NR (%)	61.85 ± 9.78 ^{ab}	66.41 ± 8.17 ^{ab}	56.36 ± 12.45 ^a	70.83 ± 6.07 ^b	67.07 ± 10.96 ^b
Villous height (µm)	582.00 ± 18.15 ^a	769.30 ± 3.98 ^b	729.30 ± 11.30 ^b	877.60 ± 4.46 ^b	841.60 ± 11.58 ^b

Note: A= control, B= *de Maan Rogosa Sharpe Broth* (MRSB) + inulin 0.5%, C= MRSB + mannan oligosaccharides (MOS), D= *L. plantarum* AKK30 (10⁷ cfu g⁻¹) + MRSB + inulin 0.5%, and E= *L. plantarum* AKK30 (10⁷ cfu g⁻¹) + MRSB + MOS 0.5%. GE = gross energy, AME = apparent metabolizable energy, TME = true metabolizable energy, TMEn = true metabolizable energy with nitrogen correction, NR= nitrogen retention. Means in the same row with different superscripts differ significantly (p<0.05).

B, and C had the jejunum villus with the heights of 841.6 µm, 877.6 µm, 769.3 µm, and 729.3 µm, respectively, that were significantly higher (p<0.05) compared to control broilers treated with inoculum in MRSB without oligosaccharides (Group A) having the jejunum villus at the height of 582 µm. Those results indicated that the treatment significantly (p<0.05) increased the intestinal morphology of broiler, compared to the control broiler without oligosaccharides treatment.

Interrelationship Between Parameters

The results of PCA (Figure 4) showed that the effects of treatment with *L. plantarum* AKK30 added with oligosaccharides (inulin or MOS) on some parameters were significantly different. There was interaction between *L. plantarum* AKK30 and enrichment with oligosaccharides on nutrients utilization and intestinal morphology. As shown in Figure 4, the contribution of oligosaccharides to *L. plantarum* AKK30 was relatively strong. The majority of parameters approached the main axis. These results imply that the smaller the cosine

angle formed then the closer the relationship between inulin and MOS with *L. plantarum* AKK30. Factors affecting *L. plantarum* AKK30 were energy metabolism, nitrogen retention, optical density, pH, fatty acids, and amino acids. Antibacterial activity did not require additional inulin and MOS although the viability of *L. plantarum* AKK30 was increased.

DISCUSSION

Some important fatty acids such as oleic, palmitic, myristic, and CLA were found in the culture media *L. plantarum* AKK30 with inulin and MOS, but CLA was not found (Figure 1). The difference in fatty acid contents possibly correlated with the composition of culture media from oligosaccharides such as inulin and MOS, especially the carbon source and glucose source, as were previously reported by Soto (2013). Inulin (Birmani et al., 2019) and MOS (Rageb et al., 2018) can supply enough glucose to *L. plantarum*. CLA is not found in MRSB media containing oligosaccharides and this condition is interesting to discuss in more detail. This result

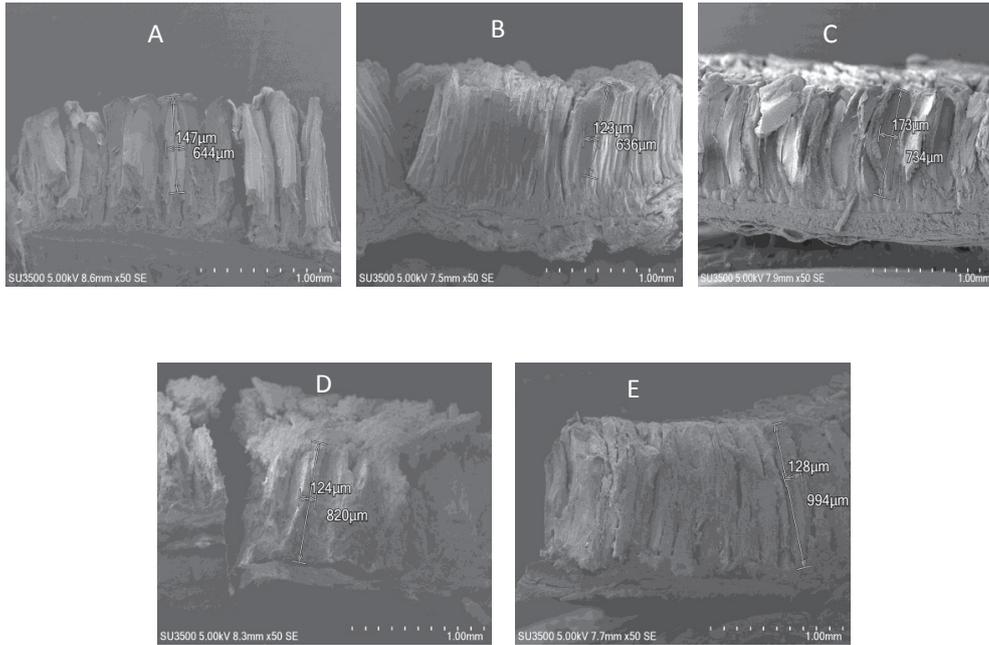


Figure 3. Scanning electron microscopy image of jejunum villi of broilers treated with symbiotic consisted of *L. plantarum* and oligosaccharides. A= control, B= mannan oligosaccharides (MOS), C= inulin, D= *L. plantarum* AKK30 (10^7 cfu g^{-1})-inulin, and E= *L. plantarum* AKK30 (10^7 cfu g^{-1})-MOS.

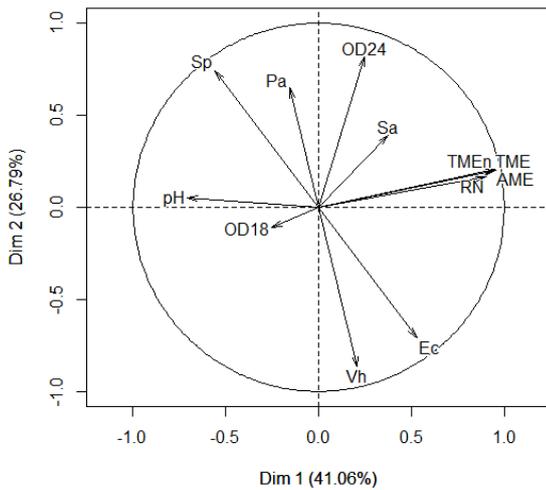


Figure 4. Principal component analysis visualizing the relationship between inhibition activity, optical density, and nutrient utilization. Dim 1= the first principal component explained by 41.06% of total variations, Dim 2= the second principal component explained by 26.79% of total variations AME= apparent metabolism energy, TME= true metabolism energy, TME_n= true metabolism energy nitrogen correction, RN= nitrogen retention, OD= optical density, Sa= *S. aerus*, Sp= *S. pullorum*, Ec= *E. coli*, Pa= *P. aeruginosa*.

showed that *L. plantarum* could metabolize CLA and the possible potency of enzymes in *L. plantarum* AKK30 to hydrolyze inulin and MOS to produce different levels of fatty acids. Inulin and MOS are water-soluble substances but cannot be enzymatically hydrolyzed in the

intestine (Adebola *et al.*, 2014) or hydrolyzed by probiotics (Pranckute *et al.*, 2016).

Table 2 showed that the *L. plantarum* AKK30 culture media with MOS altered the composition of the amino acids, i.e., threonine and isoleucine. For synthesizing amino acids, *L. plantarum* AKK30 requires MOS to balance the GIT microflora (Rageb *et al.*, 2018) because the amino acid synthesis depended on intestinal microflora conditions such as pH and proteolytic action of endogenous microbial enzymes (Wang *et al.*, 2016). Index of essential amino acid (EAAI) in *L. plantarum* AKK30 is evidenced that the synbiotic contains amino acids and affect the nutrient utilization. Amino acids in synbiotic improved intra-cellular proteolytic activity (Zhang *et al.*, 2016) to increase nutrient utilization in chicken (Alagawany *et al.*, 2018).

Optical density of *L. plantarum* AKK30 in combination with MOS and inulin indicated that the stationary phase occurred at 18 and 24 hours, respectively, which was different from control. There was an influence of oligosaccharides addition on the growth of *L. plantarum* AKK30, while there was no significant difference between inulin and MOS additions. It might indicate the associative effect between the growth of *L. plantarum* AKK30 and oligosaccharides. This effect occurred because monosaccharides from fermented carbohydrates in the intestine are used as an energy source for the growth of *L. plantarum* AKK30. Inulin can be hydrolyzed by β -fructan fructose hydrolases enzyme, its enzyme could remove the end fructose residue and acts on the internal link (De Oliveira *et al.*, 2011). The solubility of inulin at room temperature was only 10% and inulin and MOS were not hydrolyzed by digestive enzymes

in monogastric animals (Gibson *et al.*, 2017). MOS is an indigestible short-chain polymer that can improve the utilization of energy and improve the modulatory of the GIT microflora in broilers (Yang *et al.*, 2008). Thus, inulin and MOS as glucose sources for the lactic acid bacteria and can assimilate the process of fermentation by lactic acid bacteria.

L. plantarum AKK30 has antibacterial activity against gram-negative and positive pathogenic bacteria (Table 2). There was no effect of oligosaccharides addition on the antibacterial activity of *L. plantarum* AKK30 even though the viability of *L. plantarum* AKK30 increased with the addition of inulin and MOS. Palaniyandi *et al.* (2017) reported that lactic acid bacteria have inhibition activity against gram-positive pathogenic bacteria (i.e. *E. coli* and *P. aeruginosa*). Lactic acid bacteria such as *L. plantarum* produced bacteriocins antimicrobial peptides 'plantaricin' and it can kill pathogenic bacteria at the cellular and molecular levels (Wang *et al.*, 2019). Bacteriocins of *L. plantarum* AKK30 inhibited the growth of *Staphylococcus aureus*, *Salmonella pullorum*, *Escherichia coli*, and *Pseudomonas aeruginosa*. In contrast, the antibacterial activity of *L. plantarum* AKK30 was not affected by inulin and MOS (Figure 2). This effect was possibly caused by oligosaccharides such as MOS and inulin have no antibacterial activity, but it increased the growth of probiotic bacteria (Pranckute *et al.*, 2016) because oligosaccharides are used as a nutrient for lactic acid bacteria (Gibson *et al.*, 2017).

This study proved that nitrogen and energy content in excreta of broiler offered synbiotic *L. plantarum* AKK 30 + inulin was significantly different ($p < 0.05$) from the other treatments (Table 2). The amount of feed consumed might be linear with the amount of feed digested but not necessarily linear with the nutrients absorbed, because residual feed intake is always present in the digesta (Metzler-Zebeli *et al.*, 2018). Residual feed intake in the digesta is food that was not absorbed by the body that was subsequently secreted into the excreta. The un-optimal nutritional absorption might be associated with the high population of pathogenic bacteria, which affects the health of the intestinal microflora of the broiler (Alonge *et al.*, 2017). This proves that the synergy of *L. plantarum* AKK 30 and inulin has functioned as a probiotic because it could nourish the intestinal microflora, since the absorption of nutrients is affected by the intestinal microflora (Yan *et al.*, 2017) and villous performance (Coskun *et al.*, 2017). These pathogenic bacteria could be suppressed by synbiotic *Lactobacillus* and oligosaccharides so that the environment of the intestinal microflora and development of villi would be optimized by allowing the increase levels of absorbed nutrients (Yan *et al.*, 2017).

Probiotics that provide a beneficial effect on nutritional absorption is still unclear (Roberts *et al.*, 2015). This study showed that the administration of synbiotic could increase TME and TME_n compared to control and MOS. There was a tendency for synbiotic *L. plantarum* AKK-30+inulin to increase ME value compared with the other treatments that was similar to the observations previously reported by Ahmed *et al.* (2018).

Monosaccharides from fermented carbohydrates in the intestine directly stimulate the proliferation of epithelial cells (Roberfroid *et al.*, 2010; Ajuwon, 2016). Intestinal epithelial cells and enzyme activity in the digestive tract are maintained by probiotics, so that stimulates the absorption of the increased nutrients (Wang *et al.*, 2016), including feed energy sources (Santos *et al.*, 2015).

Oligosaccharides are needed by probiotics to increase the viability and population of *L. plantarum* (Ravangard *et al.*, 2017) and to provide a healthy effect on the host (Cao *et al.*, 2019). Fermentations of oligosaccharides are sources of nutrients for probiotic bacteria, thus increasing its viability (Pranckute *et al.*, 2016). Oligosaccharides, including MOS and inulin are used selectively by certain microorganisms (Gibson *et al.*, 2017). While intestinal pH is reduced by the increasing bacterial population, the viability of pathogenic bacteria can be potentially reduced (Zhu *et al.*, 2006). Therefore, synbiotic probiotics and oligosaccharides are needed so that the *Lactobacillus* population will be higher and potentially live longer in the chicken intestine. The antibacterial activity could be enhanced by the supplementation of synbiotic (oligosaccharides and probiotic) (Pranckute *et al.*, 2016).

Metabolic energy parameters (AME, TME, and TME_n) and nitrogen retention (NR) in broilers treated with synbiotic *L. plantarum* AKK30-inulin were higher than those in *L. plantarum* AKK30-MOS, even though both treatments were significantly different from the control ($p < 0.05$). This result showed that there was a relationship between lactic acid bacteria and oligosaccharides, because oligosaccharides as a source of nutrition for the growth of lactic acid bacteria. Moreover, Baurhoo *et al.* (2007) reported that MOS could inhibit the growth of pathogenic bacteria in the intestine. Some oligosaccharides, such as inulin and MOS, can be synergized with *Lactobacillus* (Schrezenmeir & deVrese, 2001). Synbiotic (*Lactobacillus*-oligosaccharides) can increase the production of bacterial metabolites that influence the environment of intestinal microbiota and immunity (Wilson & Whelan, 2016). Roberfroid *et al.* (2010) revealed that the presence of oligosaccharides promotes the growth of lactic acid bacteria i.e., *Bifidobacteria* and *Lactobacilli*. Inulin was more likely to be synbiotic with *Lactobacilli* because inulin could be hydrolyzed by the β -fructofuranosidase enzyme so that it increases the growth of *Lactobacilli* (Wilson & Whelan, 2016).

In this study, nitrogen retention in all treatments was positive, meaning that the quality and protein content of the feed was treated well, as was recommended by Jimenez *et al.* (2013). The high NR was possibly correlated with the oligosaccharides content of the feed (Thorat *et al.*, 2015) so that the existence of synbiotic in the intestine improved proteolysis activity and created a healthier intestinal environment (Wealleans *et al.*, 2017). The improved nitrogen retention by the supplementation of synbiotic in the present study may be contributed by the improved gut health by the synbiotic by reducing the load of pathogenic bacteria thereby improving nutrition utilization. It was proven that syn-

biotic *L. plantarum* with oligosaccharides activity, such as MOS and inulin, could improve food digestion and nutrient absorption.

The improvement of NR in the synbiotic group could be due to the partial hydrolysis of non-starch polysaccharides, thereby reducing the viscosity of gut contents (Thorat *et al.*, 2015) and resulting in the improved nutrients utilization (Alagawany *et al.*, 2018). Nitrogen retention possibly associated with a healthier intestinal environment (Thorat *et al.*, 2015). Effect of synbiotic treatments on villus height was shown in Table 3 and explained in Figure 2.

Using the SEM (Figure 2), we observed that synbiotic *L. plantarum* with oligosaccharides (inulin and MOS) showed the increased villus height in jejunum epithelia. The positive effect of synbiotic is possibly due to the modulating and maintaining effects on cell functions and integrities, in accordance with the observations in the previous study (Franz *et al.*, 2017). In this study, *L. plantarum* improved the morphology of intestine in broiler chickens administered with different oligosaccharides, compared with the control. The symbiosis of *L. plantarum* AKK30-inulin and *L. plantarum* AKK30-MOS has antibacterial abilities in the chicken intestine, which potentially replace the use of antibiotic growth promoters. *Lactobacillus plantarum* could effectively regulate the immune response of intestinal mucosal and improve the structures of villi in broiler chickens (Cao *et al.*, 2019). The population of pathogenic bacteria in the intestinal mucosa can be reduced by treatment with synbiotic *L. plantarum* AKK30-inulin and *L. plantarum* AKK30-MOS, thereby increasing the performance of villi in the intestine. The density, length, and surface area of villi in the small intestine can be influenced by probiotics (Olnood *et al.*, 2015) and protein of cytoskeleton might affect the height of intestinal villi and associated with intestinal lumen (Franz *et al.*, 2017; Cao *et al.*, 2019). Shortening villi imply the reduction of surface area in the digestion tract and possibly impaired the digestive system and nutrients absorption (Kavoi *et al.*, 2016). *L. plantarum* effectively regulated the immune responses of the intestinal mucosal system and improved the structures of villi in broiler chickens (Cao *et al.*, 2019).

Overall, synbiotic affected energy metabolism and protein digestibility in the intestine, because synbiotic inhibited pathogenic bacteria in the intestine. There is a correlation in the digestibility and the growth of pathogenic bacteria in the intestine, i.e., the higher the population of pathogenic bacteria the higher the number of undigested nutrients. These results suggested that supplementation of *L. plantarum* AKK30 in quails diet increased nutrient digestibility mainly nitrogen retention as previously reported by Sofyan *et al.* (2019).

CONCLUSION

Inclusion of oligosaccharides inulin or mannan oligosaccharide (MOS) affects the metabolite profiles of *Lactobacillus plantarum* AKK-30 by in vitro assays. Supplementation of *Lactobacillus plantarum* AKK-30 + inulin improved the intestinal morphology, metabolizable energy, and nitrogen retention in broilers chicken.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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