Research Article

The Influence of *Lactococcus* and *Bacillus species* Probiotics on Performance, Energy Utilization, Intestinal Ecosystem of Broiler Chickens

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Abstract | Antibiotics as growth promoters (AGP) has been prohibited, which has a detrimental impact on poultry performances. The prohibition is based on its negative impacts such as antibiotic residue and antimicrobial resistance. Alternative feed additives should be developed to replace the use of AGP in poultry production. Hence, the study aimed to evaluate the effects of combined dietary probiotics *L. lactis* D1813 and *B. licheniformis* D3270 on broiler performance, energy utilization, intestinal morphology, and microbial population. A total of 600 one-d-old Lohmann broiler chicks of 300 males and 300 females were used and reared for 35 days. Chicks were distributed into 6 groups (100 birds, 5 replicates of 20 birds) in a completely randomized design. The treatment diets were basal diet with 0.1% different supplementations: T1= $CaCO_3$ (w/w) (control), T2 = *L. lactis* at 108 CFU/g, T3 = *B. licheniformis* spore at 108 CFU/g, T4 = combination of *L. lactis* at 108 CFU/g and *B. licheniformis* spore at 108 CFU/g, T5 = *B. subtilis* spore at 108 CFU/g, and T6 = *B. amyloliquefaciens* spore at 10^9 CFU/g. At the pre-starter, the dietary combination T4 yielded lower ($p < 0.05$) feed conversion ratios compared to the T6 group. Broilers fed *L. lactis* showed higher body weight gains ($p < 0.05$) than those fed T5 and T6 groups. All treatments had no significant effect on broiler performance in starter, grower, cumulative periods, as well as *E. coli* and lactic acid bacteria populations. The combination of *L. lactis* and *B. licheniformis* improved ($p < 0.05$) villus height. The highest villi surface, true metabolizable energy (TME) value ($p < 0.05$) were recorded in the *L. lactis* group. In conclusion, the combination of *L. lactis* D1813 and *B. licheniformis* D3270 increases feed efficiency in the pre-starter period compared to the *B. amyloliquefaciens* group. This probiotic combination is unable to achieve the effectiveness of *L. lactis* in improving villi surface area, TME, and TMEn values. All probiotics have similar effects on the *E. coli*, LAB population, and broiler performance in the starter, grower, and cumulative periods.

Keywords | *Bacillus* sp.; Lohmann; Intestinal morphology; Probiotic; *Lactococcus lactis*

Received | October 26, 2021; **Accepted** | November 28, 2021; **Published** | February 15, 2022
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Citation | Lena M, Syahramadani DF, Gustya AN, Darmawan A, Sumiati, Winarsih W, Maeda M, Wiryawan KG (2022). The influence of *lactococcus* and *bacillus species* probiotics on performance, energy utilization, intestinal ecosystem of broiler chickens. Adv. Anim. Vet. Sci. 10(3): 651-658.
DOI | http://dx.doi.org/10.17582/journal.aavs/2022/10.3.651.658
ISSN (Online) | 2307-8316

INTRODUCTION

Antibiotics as growth promoters (AGP) are currently prohibited in Indonesia, which has negative consequences for livestock performance, such as decreased animal growth, feed efficiency, and increased mortality rates, all of which can result in significant financial losses (Khalique et al., 2020). The prohibition of AGP usage was based

on its negative impacts such as the development of antibiotic resistance and dangerous residues in animal products. Therefore, alternative feed additives must be developed to replace the use of antibiotics in animal production. Alternatives have been experimented with for a long such as bacteriophage (Tiwari et al., 2014), cinnamon (*Cinnamomum zeylanicum*) oil (Abd El-Hack et al., 2020a), inorganic and nano-selenium (Soliman et al., 2020**)**, inorganic selenium (Ali et al., 2020; Hassan et al., 2020), *Nigella sativa* Linn (Soliman et al., 2017), and probiotics (Aalaei et al., 2019; Zainuddin et al., 2020). Probiotics are non-pathogenic microorganisms that enhance the gastrointestinal microbiota to maintain a healthy digestive tract, thereby supporting poultry health status and growth **(**Abd El-Hack et al., 2020b). In general, dietary probiotics can effectively improve the immune system, intestinal morphology, digestive enzyme secretion, the presence of beneficial microorganisms as well as reduce ammonia excretion, and pathogen colonization (Jha et al., 2020).

Currently, many *Bacillus* bacteria species are utilized as probiotic supplements in broiler diets. *Bacillus* species are anaerobic gram-positive bacteria with endospores, which can survive at higher pressures and temperatures. In addition, spore bacteria can also tolerate acidic intestinal environments and reach the end of the digestive tract safely (Zaghari et al., 2020). Meanwhile, *L. lactis* bacteria are well characterized as food-grade lactic acid bacteria which can produce several metabolic substances, including acetic acid and lactic acid that shift the intestinal pH toward the acidic pH making it unfavorable for pathogenic bacteria survival. Supplementation of *B. licheniformi* (Trela et al., 2020), *B. subtilis* (Soliman et al., 2021; Bai et al., 2016), *B. coagulans* (Zainuddin et al., 2020), and *L. lactis* (Brzóska et al., 2012) improved daily gains, feed intakes, and feed efficiency of broiler chickens. *B. amyloliquefaciens* was able to inhibit the *E. coli* growth, improved villus height, and crypt depth (Ahmed et al., 2014; Hung et al., 2012).

There is limited information on the effect of dietary *L. lactis* and *B licheniformis* combination on broiler chicken performances.The study aims to evaluate the efficacy of combined dietary probiotics *L. lactis* D1813 and *B. licheniformis* D3270 compared with other single *Bacillus* Sp. (*B. subtilis* and *B. amyloliquefaciens*) on broiler performance, energy utilization, intestine morphology, and microbiology.

MATERIALS AND METHODS

Animal And Managements

All experimental procedures were approved by the Animal Ethics Committee of IPB University. A total of 600 one-d-old Lohmann broilers chicks of 300 males and 300 females with a mean initial weight of 40.36±0.61 g were used and reared for 35 days. Chicks were distributed into 6 dietary treatmentswith 5 replicates (20 birds per replicate) and raised on a floor pen of 2 m x 1 m x 1 m with 5 cm thick of rice husk litter under natural circumstances (opened house).

The animal house and all equipment were cleaned and washed with foam detergent. Rice husks litter and all equipment were sprayed with disinfectant. Ten days before the chicks arrive, the animal house was fumigated with 5% formalin (500 ml formalin in 10 liters of water) in closed condition. During the feeding trial, each floor was equipped with 100 watts an electrical lamp, 2 drinking water tubes and a feed tube to ensure *ad libitum* access. Lamps were turned on for 24 hours during the pre-starter to starter phases, and then just at night during the grower phase. Animal house temperature and humidity were recorded at 06.00 am, 12.00 pm, and 06.00 pm. The diameter of the brooder and the height of the lamps were adjusted to regulate the temperature. Pre-starter, starter, and grower phase ambient temperatures and humidity were 31.54°C, 68.41%; 29.68°C, 72.34% and 33.16°C, 79.12%, respectively. Removing wet litter and spraying the insecticide were carried out to control flies in the animal house.

Experimental Design And Diet

A completely randomized design was used in this experiment. The treatment diets were: T1= $CaCO_3$ 0.1% (w/w) (negative control), T2 = *L. lactis* D1813 at a rate of 108 CFU/g, T3 = *B. licheniformis* D3270 spore at a rate of 108 CFU/g, T4 = combination of *La. lactis* D1813 at a rate of 108 CFU/g and *B. licheniformis* D3270 spore at a rate of 108 CFU/g, T5 = *B. subtilis* spore at a rate of 108 CFU/g, and T6 = *B. amyloliquefaciens* spore at a rate of 10^9 CFU/g. The diet was prepared isoprotein and isocaloric in crumble form with 23% crude protein (CP) and 3200 kcal/kg metabolizable energy (ME) for pre-starter diet, 22% CP and 3050 ME kcal/kg ME for starter diet, and 20% CP and 3100 ME kcal/kg for grower diet as recommended by Leeson and Summers (2001) (Table 1). Probiotics preparation was provided by Kyushu Medical Co., Ltd., Japan. Parameters measured were body weight gains (BWG), feed intakes (FI), feed conversion ratios (FCR), the mortality rates (MR), metabolizable energy (ME), intestine microbial populations (*E. coli* and lactic acid bacteria (LAB)), and morphology (villus height and surface area).

Data Collection

Broiler Performances: At the beginning of the experiment, Day Old Chick (DOC) was weighed individually as the initial body weight. Then, the birds were weighed weekly until the end of the experiment for measuring the final weight. Bodyweight gains (g/bird) were measured as the difference between two consecutive weighing and feed

Table 1: Composition and nutrients content of the experimental diets (as-fed basis)

Ingredient (%)	Pre-starter	Starter	Grower
	(0-7 d)	(8-21 d)	(22-35 d)
Corn	61.00	60.00	63.00
Rice bran	-	4.10	4.70
Soybean meal	22.00	20.00	20.00
Meat bone meal	10.00	8.00	6.00
Corn gluten meal	3.40	2.00	-
Crude palm oil	2.00	4.00	4.30
$CaCO_3$	-	0.35	0.40
NaCl	0.20	0.20	0.20
Premix[1]	0.50	0.50	0.50
DL-Methionine	0.40	0.40	0.40
L-Lysine	0.40	0.40	0.40
Tryptophan	0.10	0.10	0.10
Probiotics	0.10	0.10	0.10
Total	100	100	100
Calculated nutritive value (%)			
Dry matter	89.10	90.31	89.56
Metabolizable energy (kcal/kg)	3219	3055	3105
Crude protein	23.17	22.17	20.24
Crude fiber	1.53	2.63	1.64
Crude fat	4.22	6.08	6.99
Digestible Methionine	0.69	0.65	0.61
Digestible Lysine	1.34	1.30	1.17
Digestible Methionine+ Cystine	0.98	0.90	0.88
Ash	6.27	6.34	4.92
Calcium	1.1	0.98	0.88
Available Phosphorus	0.66	0.58	0.49
Sodium	0.19	0.18	0.17
Chloride	0.22	0.21	0.20

[1]Premix (mg/kg) : vitamin A, 500,000 IU; vitamin D, 100,000 IU; vitamin E, 150 mg; vitamin K, 50 mg; vitamin B_2, 250 mg; vitamin B_1, 50 mg; vitamin B_{12}, 250 mcg; Ca-d-panthotenate, 125 mg; niacinamide, 375 mg; choline chloride, 5,000 mg; folic acid, 25 mg; Fe sulphate, 1,250 mg; Mn sulphate , 2,500 mg; Mg sulphate , 1,700 mg; Cu sulphate, 25 mg; K iodine, 5 mg; Zn sulphate , 500 mg.

intake was measured weekly. Conversion ratios (FCR) were calculated by dividing feed intakes by body weight gains (FI: BWG). The mortality rates (MR) were obtained by dividing the number of dead chickens by the number of chickens at the beginning of the experiment multiplied by 100%.

Metabolizable Energy And Nitrogen Retention: Thirty-five of 35 days old male broilers were used to determine diet digestibility using the modified Farrell method (Farrell, 1978). Birds were placed in individual metabolic cages and adapted for three days. Thirty birds were used for diets energy assay, while the other five birds were used to assess endogenous energy. After three days of adaptation, 35 birds fasted for 24 hours with *ad libitum* drinking water. Then, the experimental diet was offered to 30 birds, while 5 birds fasted continuously for 24 hours with *ad libitum* drinking water. During the trial, excreta was sprayed with 0.01 % H_2SO_4 for two hours. Then, the excreta of each bird was collected, weighed, and frozen for 24 hours. The excreta were thawed, dried at 60°C/48 hours, ground, and weighed before being analyzed for dry matter, gross energy, nitrogen, and crude protein. The metabolizable energy (ME) was calculated using the Sibbald and Wolynetz method (1985).

Table 2: The comparison of selected single *Bacillus* strains and combination of *Bacillus* and *Lactococcus* probiotic on the growth performance of broiler chickens

Treatments[1]	BWG (g/bird)	FI (g/bird)	FCR
	Pre-starter (1-7 days)		
T1	126.70 ± 5.69^{ab}	101.73 ± 7.59	0.80 ± 0.05^{ab}
T2	132.42 ± 4.60^{b}	97.92 ± 5.31	0.74 ± 0.03^{a}
T3	127.70 ± 6.87^{ab}	99.52 ± 7.50	0.78 ± 0.06^{ab}
T4	127.89 ± 4.42^{ab}	94.44 ± 4.92	0.74 ± 0.02^{a}
T5	124.82 ± 5.07^{a}	95.40 ± 5.67	0.76 ± 0.03^{a}
T6	124.34 ± 3.75^{a}	103.72 ± 8.31	0.84 ± 0.08^{b}
	Starter (8-21 days)		
T1	488.74 ± 38.79	658.73 ± 24.21	1.36 ± 0.15
T2	526.53 ± 19.00	676.48 ± 21.60	1.29 ± 0.06
T3	517.33 ± 27.46	655.85 ± 50.02	1.27 ± 0.11
T4	509.93 ± 29.17	644.05 ± 24.87	1.26 ± 0.04
T5	496.73 ± 48.24	684.64 ± 33.46	1.39 ± 0.10
T6	519.83 ± 33.70	654.70 ± 19.17	1.26 ± 0.05
	Grower (22-35 days)		
T1	877.13 ± 53.32	1374.33 ± 72.55	1.57 ± 0.12
T2	933.26 ± 72.65	1410.92 ± 71.90	1.52 ± 0.12
T3	945.46 ± 46.10	1368.99 ± 214.92	1.44 ± 0.16
T4	939.41 ± 152.62	1373.66 ± 100.47	1.48 ± 0.14
T5	896.36 ± 163.94	1376.37 ± 113.61	1.58 ± 0.31
T6	924.87 ± 76.19	1422.24 ± 105.26	1.54 ± 0.12
	Cumulative (1-35 days)		
T1	1492.57 ± 62.29	2134.79 ± 81.30	1.43 ± 0.07
T2	1592.21 ± 69.09	2185.32 ± 55.16	1.37 ± 0.06
T3	1590.49 ± 47.00	2124.36 ± 201.27	1.33 ± 0.09
T4	1577.22 ± 178.14	2112.15 ± 124.62	1.35 ± 0.08
T5	1517.91 ± 166.33	2156.42 ± 137.60	1.43 ± 0.15
T6	1569.04 ± 102.24	2180.66 ± 118.79	1.39 ± 0.06

[a,b,c] Means within a column with different superscripts differ significantly ($p < 0.05$)

[1]T1= CaCO3 0.1% (w/w) (negative control); T2 = *Lactococcus lactis* D1813 at 10 ^{8} CFU/g; T3 = *Bacillus licheniformis* D3270 (spore) at 10^{8} CFU/g; T4 = *Lactococcus lactis* D1813 at 10^{8} CFU/g + *Bacillus licheniformis* D3270 (spore) at 10^{8} CFU/g; T5 = *Bacillus subtilis* (spore) at 10^{8} CFU/g; T6 = *Bacillus amyloliquefaciens* (spore) at 10^{9}CFU/g

Table 3: The comparison of selected single *Bacillus* strains and combination of *Bacillus* and *Lactococcus* probiotic on the selected microbiota populations and intestinal morphology traits of 35-d-old broiler chickens

Treatment[1]	LAB (Log10 cfu/g)	E. coli (Log10 cfu/g)	Villus height (µm)	Villus Surface Area (µm^2)
T1	9.020 ± 0.623	7.041 ± 0.295	454.74 ± 8.87^{a}	987.82 ± 39.38^{a}
T2	9.405 ± 0.499	5.540 ± 0.088	486.50 ± 14.75^{c}	1090.99 ± 8.88^{c}
T3	8.722 ± 0.693	6.126 ± 1.505	483.38 ± 6.48^{bc}	1044.00 ± 22.98^{b}
T4	9.055 ± 0.736	5.192 ± 3.049	481.55 ± 15.28^{bc}	988.31 ± 12.79^{a}
T5	9.498 ± 0.309	6.495 ± 1.450	457.28 ± 16.53^{ab}	981.05 ± 13.21^{a}
T6	9.267 ± 0.639	6.613 ± 0.428	482.04 ± 16.40^{bc}	1003.27 ± 22.17^{ab}

[a,b,c] Means within a column with different superscripts differ significantly ($p < 0.05$)

[1]T1= CaCO3 0.1% (w/w) (negative control); T2 = *Lactococcus lactis* D1813 at 10 8 CFU/g; T3 = *Bacillus licheniformis* D3270 (spore) at 108 CFU/g; T4 = *Lactococcus lactis* D1813 at 108 CFU/g + *Bacillus licheniformis* D3270 (spore) at 108 CFU/g; T5 = *Bacillus subtilis* (spore) at 108 CFU/g; T6 = *Bacillus amyloliquefaciens* (spore) at 109 CFU/g

Table 4: The comparison of selected single *Bacillus* strains and combination of *Bacillus* and *Lactococcus* probiotic on the nitrogen retention, and energy utilization of 35-d-old broiler chickens

Treatments[1]	AME[2] (kcal/kg)	TME (kcal/kg)	AMEn (kcal/kg)	TMEn (kcal/kg)	N Retention (%)
T1	3225.00±189.75	3706.63±112.81^{ab}	3121.97±173.47	3603.60±118.49^{ab}	57.87±13.32
T2	3485.43±207.43	3848.07±196.69^{b}	3345.41±182.14	3708.05±172.95^{b}	64.80±12.05
T3	3421.73±153.17	3736.87±135.48^{ab}	3285.70±141.48	3600.84±124.65^{ab}	62.95±6.65
T4	3222.74±104.15	3570.34±59.94^{a}	3097.22±84.15	3444.81±51.57^{a}	58.09±9.98
T5	3214.72±126.63	3522.14±98.25^{a}	3092.43±118.37	3399.85±89.84^{a}	56.59±4.23
T6	3251.26±301.45	3564.67±244.07^{a}	3125.10±266.13	3438.51±208.95^{a}	59.75±14.76

[1]T1= CaCO3 0.1% (w/w) (negative control); T2 = *Lactococcus lactis* D1813 at 10^{8} CFU/g; T3 = *Bacillus licheniformis* D3270 (spore) at 10^{8} CFU/g; T4 = *Lactococcus lactis* D1813 at 10^{8} CFU/g + *Bacillus licheniformis* D3270 (spore) at 10^{8} CFU/g; T5 = *Bacillus subtilis* (spore) at 10^{8} CFU/g; T6 = *Bacillus amyloliquefaciens* (spore) at 10^{9} CFU/g

[2] AME= Apparent Metabolizable Energy; AMEn =Apparent Metabolizable Energy corrected by nitrogen; TME=True Metabolizable Energy; TMEn=True Metabolizable Energy corrected by nitrogen; N= Nitrogen

Microbial, Population, And Intestinal Morphology: Two birds from each replicate were slaughtered at the end of the feeding trial. Small intestines were removed and the digesta content was collected. A total of 1 g sample was homogenized with 0.9% normal saline in a sterile tube (1:1). Then, the solution was mixed at a vortex. Serial dilution was carried out until the sixth dilution. A total of 0.1 ml of each dilution was poured and spread uniformly onto Mac Conkey's agar and incubated at 37°C for 48 hours. The typical convex pink colonies were counted by the Total Plate Count Method (Quinn et al., 1992). The result was expressed as CFU/g of content by multiplying the average number of colonies by the reciprocal of the dilution factor. Meanwhile, two broilers from each replicate were randomly selected and slaughtered at 35 days of broiler age for intestinal morphology measurements. Duodenal samples were collected immediately after slaughtering and immersed in 10% neutral buffered formalin (NBF). Duodenal samples 2 cm long were dissected and dehydrated with the addition of ethyl alcohol concentrations of 70%, 90%, 96%, and 100%, then the samples were cleaned in xylene and immersed in the paraffin. The tissue was sectioned with a thickness of 6 µm using a microtome and attached to a glass object with albumin adhesive and stained with hematoxylin-eosin (H & E). Height, basal and apical width of the duodenal villi were calculated using a microscope (Olympus) on objective magnification 4 times, meanwhile representative fields were captured by a video microscope (Video measuring gauge IV-560, Company Limited). The villi's surface area (µm^2) was calculated using the formula:
(b + c)/c x a
where a = villi height, b = basal width and c = apical width) (Iji et al., 2001).

Statistical Analysis

Data were analyzed using SPSS ver. 26.0 and Duncan's multiple comparisons were used to separate differences between groups. The experimental data in the tables are mean values with standard deviation. Differences were considered to be significant at values of $p < 0.05$.
The statistical model used was:
$Y_{ij} = \mu + T_j + e_{ij}$
Where: Y_{ij} = Observation value, μ = Overall mean, T_j = Effect of treatments(probiotics) e_{ij} = Random error

RESULTS

Broiler Performances

BWG, FI, and FCR of broiler chickens fed with different probiotics are shown in Table 2. In the pre-starter phase, there was a significant effect ($p < 0.05$) in BWG, and FCR, but not in starter, grower, and cumulative experiments. Dietary combination of *L. lactis* and *B. licheniformis*, *L. lactis*, and *B. subtilis* had lower FCR compared to *B. amyloliquefaciens* group. Broilers fed *L. lactis* at the pre-starter phase showed higher BWG ($p < 0.05$) than those fed *B. subtilis* and *B. amyloliquefaciens* groups. Considering that the highest mortality rate was recorded in the control group (7%).

E. Coli, Lab Population, And Intestinal Morphology

E. coli, LAB population, and intestinal morphology of 35 days old broiler chickens fed probiotics are presented in Table 3. Dietary probiotics had no significant effect on the population of *E. coli* and LAB population. The dietary combination of *L. lactis* and *B. licheniformis* had a significantly higher ($p < 0.05$) villus height compared to the control group. The highest surface of villi ($p < 0.05$) was found in *L. lactis* treatment (1090.99 µm^2) ($p < 0.001$).

Metabolizable Energy And Nitrogen Retention

The all experimental diets had similar values of Apparent Metabolizable Energy (AME), Apparent Metabolizable

Energy corrected by nitrogen (AMEn), Nitrogen (N) retention percentages. However, *L. lactis* had significantly higher ($p < 0.05$) True Metabolizable Energy (TME) and True Metabolizable Energy corrected nitrogen (TMEn) compared to those in the combination of *L. lactis* and *B. licheniformis*, *Bacillus subtilis*, and *B. amyloliquefaciens* (Table 4).

DISCUSSION

Probiotics had tremendous beneficial influences on broiler productivity related to improving nutrient utilization efficiency (Lambo et al., 2021). Moreover, there is a clear acceptance that dietary probiotics effectively increase the immune system, intestinal morphology, digestive enzyme secretion, the presence of beneficial microorganisms, and reduce ammonia excretion, and inhibit pathogen colonization (Jha et al., 2020).

In the present study, broilers fed a combination of *L. lactis* and *B. licheniformis* had lower FCR values compared to *B. amyloliquefaciens* group, meanwhile, broilers fed *L. lactis* had significantly higher BWG than those fed *B. subtilis* and *B. amyloliquefaciens* at the pre-starter phase (0-7d). However, FI BWG and FCR were not different in starter, grower, and cumulative periods. This was in line with Fajardo, (2012) who reported that feeding *L. lactis* CECT 539 at 6.68×10^{10} CFU/kg resulted in lower FCR than that of *L. casei* CECT 4043, as well as the control group. In addition, adding *L. lactis* in the broiler diet did not affect body weight, FI, and FCR in 1- 42 days (Brzóska et al., 2012), which agrees with the present study. In contrast to this study, the addition of 1.5×10^{8} CFU/kg of *B. Licheniformis* increased BWG and feed efficiency in the finisher period (22- 42d) and cumulative experiment (Trela et al., 2020). The administration of *B. subtilis* ($2–4 \times 10^{10}$ CFU/kg) in the broiler diet improved BWG, FI, and feed efficiency during all phases (Bai et al., 2016).

The lack of response in performance was likely due to the adequate management practice applied in this study. According to Bitterncourt et al. (2011), the efficacy of probiotics was directly affected by rearing conditions such as the presence of health challenges and a stressful environment. Moreover, Lee et al. (2010) stated that the ineffectiveness of a probiotic treatment can be associated with microbial composition and survivability, feeding management, bird age, dosage, facility hygiene, synergism, or antagonism to microbial in feed components, and environmental stress.

In our study, the dietary combination of *L. lactis* and *B. licheniformis*, *L. lactis*, *B. licheniformis*, and *B. amyloliquefaciens* had a significantly higher villus height compared to the control group. Meanwhile, the highest villus surface area resulted from the *L. lactis* group. Hung et al. (2012) revealed that supplementation of *B. amyloliquefaciens* and *B. licheniformis* in broiler diets improved intestinal health by increasing crypt depth and villus height, as well as lowering the pH of the intestine, which promoted lactobacilli colonization and *E. coli* suppression. Better surface area and villus height as an indicator of intestinal villus function proposed the improvement of nutrient digestibility and absorption capacity (Lei et al., 2015). N retention also had a positive correlation with a healthy intestinal environment (Wealleans et al., 2017). Moreover, dietary *B. licheniformis* (Zhou et al., 2016) and *L. lactis* (Adel et al., 2016) improved nutrient digestion and absorption by increasing various enzymes including proteases, amylase, and lipases which were revealed by high TME, TMEn, and N retention in this study. This beneficial effect of probiotics on gut health may result in very impressive broiler growth from the starter phase, particularly in the *B. subtilis* and *B. amyloliquefaciens* groups, which were able to slightly exceed the control BWG despite having low performances in the pre-starter phase.

In our study, adding probiotics to broiler diets reduced mortality which could be attributed to the probiotics' ability to eliminate pathogenic bacteria and strengthen the immune system. Some studies have explained some mechanisms of probiotics in inhibiting intestine pathogen bacteria, such as increasing the ratio of LAB to pathogen bacteria, competition for colonization attachment site and nutrient, and antimicrobe peptide production (Wu et al., 2011). *L. lactis* is a lactic acid bacteria that is commonly found in foods. Lactic acid bacteria produce metabolic compounds such as acetic acid and lactic acid, which cause the gut pH to decrease below the minimal pH required for pathogenic bacterial viability (Mathipa et al., 2017). In addition, *L. lactis* LMG2081 generates two types of bacteriocins, one of which belongs to class I (lantibiotics; lacticin LMG) and the other to class IIb (lactococcin G) (Song et al., 2017). Furthermore, *L. lactis* subsp. lactis ATCC 1 1454 inhibited gram-positive and negative bacteria including *E. coli* bacteria through nisin production and secretion as a proteinaceous metabolite (Mirkovic et al., 2016). Some *B.* species including *B. subtilis* (Suzuki et al., 2021), *B. amyloliquefaciens* (Zhou et al., 2016) were able to generate bacteriocins substances that exhibit antibacterial activity. Bacteriocin is categorized as either bacteriostatic or bactericidal with the ability to alleviate pathogenic bacteria in the gastrointestinal tract Ahmed et al., 2014. Mousavi et al. (2018) reported that probiotics can eliminate pathogenic bacteria by competing for adhesion to the small intestinal wall. As a result, it prevents mucosal infection and protects the structural integrity of the intestine. The intestinal microbiota is required for intestinal epithelial maintenance as well as immune system development. Pathogenic

bacteria can promote epithelial damage in the absence of intestinal microbiota (Sethiya et al., 2016). Markovic et al. (2009) also stated that protecting villi from pathogens lowered the requirement for intestinal cell turnover. When the villi are destroyed, enterocyte regeneration requires more energy and protein, which inhibits tissue and organ system growth.

CONCLUSION

The combination of *L. lactis* D1813 and *B. licheniformis* D3270 increases feed efficiency in the pre-starter period compared to the *B. amyloliquefaciens* group. However, this probiotic combination is unable to achieve the effectiveness of *L. lactis* D1813 in improving villi surface area, TME, and TMEn values. All probiotics have similar effects on the *E. coli*, LAB population, and broiler chicken performance in the starter, grower, and cumulative periods.

ACKNOWLEDGMENTS

The authors are grateful to Kyushu Medical Co. Ltd., for funding and collaboration with the Faculty of Animal Science, IPB University.

CONFLICT OF INTEREST

All authors declare that there are no conflicts of interest.

NOVELTY STATEMENT

Our study is the first study that emphasizes the evaluation of the effect of combined dietary probiotics L. lactis D1813 and B. licheniformis D3270 compared to single Bacillus Sp. (B. subtilis and B. amyloliquefaciens) on broiler performance, energy utilization, intestine morphology, and microbiology.

AUTHORS CONTRIBUTION

ML, DFS, ANG, AD assisted in data collecting, analysis, interpretation, and designing of the manuscript. S, WW, MM, KGW conceptualized the experiment, supervised and evaluated the content of manuscript.

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