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ORIGINAL ARTICLE

Feeding of different levels of metabolite combinations produced by *Lactobacillus plantarum* on growth performance, fecal microflora, volatile fatty acids and villi height in broilers

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ABSTRACT

The effects of feeding different dosages of metabolite combination of *L. plantarum* RS5, RI11, RG14 and RG11 strains (Com3456) on the performance of broiler chickens was studied. A total of 504 male Ross broilers were grouped into 7 treatments and offered different diets: (i) standard corn-soybean based diet (negative control); (ii) standard corn-soybean based diet +100 ppm neomycin and oxytetracycline (positive control); (iii) standard corn-soybean based diet + 0.1% metabolite combination of *L. plantarum* RS5, RI11, RG14 and RG11 strains (Com3456); (iv) standard corn-soybean based diet + 0.2% of Com3456; (v) standard corn-soybean based diet + 0.3% of Com3456 (vi) standard corn-soybean based diet + 0.4% of Com3456 and (vii) standard corn-soybean based diet + 0.5% of Com3456. Supplementation of Com3456 with different dosages improved growth performance, reduced *Enterobacteriaceae* and increased lactic acid bacteria count, and increased villi height of small intestine and fecal volatile fatty acid concentration. Treatment with 0.4% and 0.2% Com3456 had the best results, especially in terms of growth performance, feed conversion ratio and villi height among other dosages. However, the dosage of 0.2% was recommended due to its lower concentration yielding a similar effect as 0.4% supplementation. These results indicate that 0.2% is an optimum level to be included in the diets of broiler in order to replace antibiotic growth promoters.

Key words: *broilers, L. plantarum, metabolite combination, performance.*

INTRODUCTION

The developing resistance to antibiotics in clinical use is recognized as a worldwide problem. Most risk assessors consider that reservoirs of resistance determinants found in food-producing animals are largely attributable to the use of antibiotic growth promoters (AGPs). These contribute to the development and the spread of resistance to the extent which threatens the capacity to deal with many diseases (Chesson 2006). Although AGPs possess growth-promoting effects, feed efficiency improvement and gastrointestinal infections prevention, antibiotic resistance is a major concern. Pathogenic bacteria resistant to several antimicrobial agents emerged globally in the 1980s (Aarestrup 1995). WHO has drawn attention to the potential hazards of AGP and recommends prudence in their use (WHO 1998). Infections caused by enterococci, streptococci, *Salmonella*, *Campylobacter* and *E. coli* are the current treatment problems that are potentially attributable to animal use of antimicrobials (Wegener 2006). It has

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also been shown that fecal *E. coli* isolated from pigs was resistant to antibiotics such as neomycin, oxytetracycline, nalidixic acid and chloramphenicol (Loh *et al.* 2006).

Thus, finding an alternative to AGPs in animal production is urgent. Investigating the optimal dosages of natural metabolites is particularly important in poultry production. Compared to live microorganisms, metabolites have advantages in storage, transportation and handling. Therefore, an alternative substance that is environmentally friendly and can easily be used on a farm needs to be found. This study was conducted using different levels of metabolite combinations produced by 4 strains of *L. plantarum* in order to evaluate growth performance of broilers, fecal lactic acid bacteria (LAB) and *Enterobacteriaceae* (ENT) counts, fecal volatile fatty acids (VFA), and gut morphology. The aim of the study was to identify the optimal dosage of the metabolite combinations supplemented in the diets of broiler chickens.

MATERIALS AND METHODS

Broiler chicks and experimental design

A total of 504 male Ross broilers from a local company were raised from 1 day old to 42 days of age in a deep litter house. Each pen consisted of 12 chicks and was randomly allocated to the open house with wood shavings litter. Upon arrival, the birds were vaccinated against infectious bronchitis (IB) and Newcastle disease (ND) (IB-ND Fort Dodge, Collegeville, USA) by intraocular route. The IBD vaccine (UPM93; MyVac, Bangi, Malaysia) against infectious bursal disease (IBD) was applied on day 14 of the rearing period. After vaccination, the birds were wing banded for monitoring of individual weight. Water and feed were provided *ad libitum*. The starter and finisher diets were offered to the birds from 0–21 and 22–42 days of age, respectively. The dietary treatments consisted of: (i) corn-soybean basal diet without antibiotic (−ve control); (ii) basal diet with 100 ppm neomycin and oxytetracycline (+ve control); (iii) basal diet supplemented with 0.1% of metabolite combination from 4 strains of *L. plantarum* RS5, RI11, RG14 and RG11 (Com3456); (iv) basal diet supplemented with 0.2% metabolite combinations of Com3456; (v) basal diet supplemented with 0.3% metabolite combinations of Com3456; (vi) basal diet supplemented with 0.4% metabolite combinations of Com 3456 and (vii) basal diet supplemented with 0.5% metabolite combination of Com3456. The metabolites without bacterial cells were produced as described by Loh *et al.* (2008, 2009). The diets were formulated to meet the requirements of all nutrients for broiler chickens. The percentage composition of starter and finisher diets are presented in Tables 1 and 2, respectively.

Data and sample collection

The individual body weight (BW) and pen feed intake (FI) were recorded weekly and live weight gain (WG), feed conversion ratio (FCR) and average daily gain (ADG) were calculated. Twelve birds at week 6 of each treatment were randomly selected and slaughtered for sampling as feces and small intestines were taken for further analysis. All procedures were approved by Research Advisory Committee, University Putra Malaysia.

Fecal lactic acid bacteria and *Enterobacteriaceae* count

The fecal LAB and ENT population was determined using the method as described by Foo *et al.* (2003a). Ten percent (w/v) of fecal samples were diluted in sterile peptone water and left at room temperature for an hour prior to further ten-fold serial dilutions (v/v). Enumerations of LAB were performed on MRS-agar (*Lactobacillus*-Agar De Man, ROGOSA and SHAPE) (Merck®, KgaA, Darmstadt). The plates were incubated in an anaerobic jar at 30°C for 48 h. ENT were spread and counted on EMB-Agar (Eosin-methylene-blue Lactose Sucrose Agar (Merck®, KgaA, Darmstadt) and incubated aerobically for 24 h at 37°C. The number of colony-forming units (CFU) was expressed as the base 10 logarithm of CFU (logCFU) per gram. All samples were repeated in triplicate.

Small intestine morphology

The procedure was a modified method as described by Hair-Bejo (1990). Segments of 5 to 6 cm long were removed from the duodenum, jejunum, and ileum as follows: (i) the middle part of the duodenal loop, (ii) midway between the end point of duodenal loop and Meckel's diverticulum (jejunum), and (iii) midway between the Meckel's diverticulum and the ileo-cecal junction (ileum). The intestinal segments were flushed with 10% neutral buffered formalin solution and were then used for morphometric analysis. For morphometric analysis, segments were fixed in 10% neutral buffered formalin solution overnight. Intestinal samples were then excised, dehydrated in a tissue processing machine (Leica Microsystems K. K., Tokyo, Japan) and embedded in paraffin wax. Sections of 4 μm were cut from each of the sample, fixed on slides, stained with haematoxylin and eosin, mounted and examined under light microscopes. The morphometric variables examined included villi height (from the tip of the villi to the villi crypt junction) and crypt depth (defined as the depth of the invagination between adjacent villi). The villi height and crypt depth were measured using an image analyzer. Values are means from the best 20 villi and only vertically oriented villi and crypts from each slide were measured.

Volatile fatty acid determination

The VFA concentration in the feces was determined using a method modified from Jin *et al.* (1998). One gram of feces (stored at −20°C) from each sample was weighed in a sampling tube. One mL of 24% metaphosphoric acid diluted in 1.5 mol/L sulphuric acid (BHD Laboratories, Poole, UK) was added. The mixture was kept at room temperature overnight and centrifuged at 10 000 rpm for 20 min at 4°C. The collected supernatant was kept in a 2-mL screw-capped vial (Kimble Glass Inc., USA). The internal standard 20 mmol/L 4-methyl-valeric acid (Sigma Chemical Co., St. Louis,

Table 1 Percentage composition of starter diet

Ingredients	Dietary treatments†						
	−ve control	+ve control	0.1% Com3456	0.2% Com3456	0.3% Com3456	0.4% Com3456	0.5% Com3456
Corn	50.600	50.600	50.600	50.600	50.600	50.600	50.600
Soybean meal	29.382	29.382	29.382	29.382	29.382	29.382	29.382
Wheat Pollard	6.072	6.062	5.972	5.872	5.772	5.672	5.572
Crude palm oil	3.600	3.600	3.600	3.600	3.600	3.600	3.600
Fish Meal 55%	7.600	7.600	7.600	7.600	7.600	7.600	7.600
L-Lysine	0.250	0.250	0.250	0.250	0.250	0.250	0.250
DL-Methionine	0.200	0.200	0.200	0.200	0.200	0.200	0.200
Monodicalcium-phosphate 21	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Calcium carbonate	0.680	0.680	0.680	0.680	0.680	0.680	0.680
Choline chloride	0.060	0.060	0.060	0.060	0.060	0.060	0.060
Salt	0.250	0.250	0.250	0.250	0.250	0.250	0.250
Mineral Mix‡	0.100	0.100	0.100	0.100	0.100	0.100	0.100
Vitamin Mix§	0.060	0.060	0.060	0.060	0.060	0.060	0.060
Antioxidant	0.010	0.010	0.010	0.010	0.010	0.010	0.010
Toxinbinder	0.135	0.135	0.135	0.135	0.135	0.135	0.135
Antibiotic¶		0.010					
Metabolite powder			0.100	0.200	0.300	0.400	0.500
Total	100	100	100	100	100	100	100
Calculated analysis:							
Crude protein, %	22.50	22.48	22.48	22.47	22.45	22.44	22.50
ME, kcal/kg	2920	2919	2919	2917	2916	2915	2920

†Diets supplemented with different dosages (0.1–0.5%, w/w) of metabolite powder of Com3456 (a combination of 4 strains RS5, RI11, RG11 and RG14). ‡Mineral mix that provided per kilogram of the diet: Fe 100 mg; Mn 110 mg; Cu 20 mg; Zn 100 mg; I 2 mg; Se 0.2 mg; Co 0.6 mg. §vitamin mix that provided per kilogram of the diet: Vitamin A 6667 IU; vitamin D 1000 IU; vitamin E 23 IU; vitamin K3 1.33 mg; cobalamin 0.03 mg; Thiamin 0.83 mg; riboflavin 2 mg; folic acid 0.33 mg; biotin 0.03 mg; pantothenic acid 3.75 mg; niacin 23.3 mg; pyridoxine 1.33 mg. ¶a combination of oxytetracyclin and neomycin at the concentration of 100 ppm (w/w).

Table 2 Percentage composition of finisher diet

Ingredients	Dietary treatments†						
	−ve control	+ve control	0.1% Com3456	0.2% Com3456	0.3% Com3456	0.4% Com3456	0.5% Com3456
Corn	54.900	54.900	54.900	54.900	54.900	54.900	54.900
Soybean meal	26.900	26.900	26.900	26.900	26.900	26.900	26.900
Wheat Pollard	6.534	6.524	6.434	6.334	6.234	6.134	6.034
Crude palm oil	3.200	3.200	3.200	3.200	3.200	3.200	3.200
Fish Meal 55%	5.000	5.000	5.000	5.000	5.000	5.000	5.000
L-Lysine	0.250	0.250	0.250	0.250	0.250	0.250	0.250
DL-Methionine	0.200	0.200	0.200	0.200	0.200	0.200	0.200
Monodicalcium-phosphate 21	1.400	1.400	1.400	1.400	1.400	1.400	1.400
Calcium carbonate	0.992	0.992	0.992	0.992	0.992	0.992	0.992
Choline chloride	0.058	0.058	0.058	0.058	0.058	0.058	0.058
Salt	0.250	0.250	0.250	0.250	0.250	0.250	0.250
Mineral Mix‡	0.100	0.100	0.100	0.100	0.100	0.100	0.100
Vitamin Mix§	0.058	0.058	0.058	0.058	0.058	0.058	0.058
Antioxidant	0.008	0.008	0.008	0.008	0.008	0.008	0.008
Toxinbinder	0.150	0.150	0.150	0.150	0.150	0.150	0.150
Antibiotic¶		0.010					
Metabolite powder			0.100	0.200	0.300	0.400	0.500
Total	100	100	100	100	100	100	100
Calculated analysis:							
Crude protein, %	20.34	20.34	20.34	20.31	20.29	20.28	20.26
ME, kcal/kg	2912	2912	2912	2910	2909	2907	2906

†Diets supplemented with different dosages (0.1–0.5%, w/w) of metabolite powder of Com3456 (a combination of 4 strains RS5, RI11, RG11 and RG14). ‡Mineral mix that provided per kilogram of the diet: Fe 100 mg; Mn 110 mg; Cu 20 mg; Zn 100 mg; I 2 mg; Se 0.2 mg; Co 0.6 mg. §vitamin mix that provided per kilogram of the diet: Vitamin A 6667 IU; vitamin D 1000 IU; vitamin E 23 IU; vitamin K3 1.33 mg; cobalamin 0.03 mg; Thiamin 0.83 mg; riboflavin 2 mg; folic acid 0.33 mg; biotin 0.03 mg; pantothenic acid 3.75 mg; niacin 23.3 mg; pyridoxine 1.33 mg. ¶a combination of oxytetracyclin and neomycin at the concentration of 100 ppm (w/w).

Missouri, USA) was added to the supernatant to achieve 10 mmol/L in the combination and stored at −20°C until GLC analysis. VFA were separated by a Quadrex 007 Series (Quadrex Corp., New Haven, CT 06525 USA) bonded phase fused silica capillary column (15 m, 0.32 mm ID, 0.25 μm film thickness) in a 6890N (Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector. The purified nitrogen functioned as a carrier gas with a flow rate of 60 mL/min. The temperature of the injector and detector was 230°C. The column temperature was set at 200°C in an isothermal status. The commercial standards of 20 mmol/L acetic, and 10 mmol/L each of propionic, butyric, isobutiric, valeric, isovaleric and 4-methyl-valeric acids from Sigma (Sigma chemical Co., St. Louis, Missouri, USA) were used as external standards to identify the peaks.

Data analysis

Data were analyzed as a complete randomized design using the General Linear Models procedure of the Statistical Analysis System (SAS 1998). Duncan's Multiple Range Test was used to compare means of treatments. The data were presented as the mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Growth performance

The growth performance is shown in Table 3. The BW, total WG and ADG of chickens at 42 days of age in the −ve control treatment were the lowest ($P < 0.05$) among the treatments, while the chickens in treatment, supplemented with 0.4% Com3456 from 4 strains of *L. plantarum* had the highest ($P < 0.05$), followed by birds in the +ve control group and 0.2% Com3456 in third place. However, there was no BW and WG difference ($P > 0.05$) among chickens in the +ve control and treatments supplemented with Com3456, except for treatment supplemented with 0.5% Com3456, which was the lowest among treated groups. Feed intake means were not significantly different ($P > 0.05$) among the treatments. Relating to the FCR, the lowest result ($P < 0.05$) was found in treatment supplemented with 0.4% Com3456 as compared with the remaining of the treatments.

The results showed higher live BW, total WG and ADG for the +ve control group and the 5 groups supplemented with different dosages of Com3456 compared to that of birds in −ve control, which was fed with corn-soybean based diet. The metabolite combinations from the 4 strains of *L. plantarum* (Com3456) at different levels of dosages could partially replace antibiotic growth promoter. The optimal dosage in terms of growth performance was 0.4% Com3456, which gave the highest growth performance, followed by 0.2% Com3456. In terms of improvement

Table 3 Growth performance at week 6 of treatments supplemented with different dosages of metabolites from Com3456

Parameters	Dietary treatments†						
	−ve control	+ve control	0.1% Com3456	0.2% Com3456	0.3% Com3456	0.4% Com3456	0.5% Com3456
Body weight, kg	2.23 ± 0.04^{d}	2.45 ± 0.04^{ba}	2.38 ± 0.03^{bc}	2.40 ± 0.03^{bac}	2.37 ± 0.03^{bc}	2.49 ± 0.03^{a}	2.32 ± 0.03^{c}
Weight gain, kg	2.19 ± 0.04^{d}	2.41 ± 0.04^{ba}	2.34 ± 0.03^{bc}	2.36 ± 0.03^{bac}	2.33 ± 0.03^{bc}	2.44 ± 0.03^{a}	2.28 ± 0.02^{c}
Average daily gain, g	52.12 ± 0.98^{d}	57.29 ± 0.85^{ba}	55.65 ± 0.70^{bc}	56.08 ± 0.74^{bac}	55.52 ± 0.71^{bc}	58.21 ± 0.66^{a}	54.38 ± 0.59^{c}
Feed intake, kg	3.80 ± 0.08^{a}	3.92 ± 0.08^{a}	3.98 ± 0.08^{a}	3.96 ± 0.06^{a}	3.94 ± 0.06^{a}	4.00 ± 0.09^{a}	3.84 ± 0.03^{a}
Feed conversion ratio	1.73 ± 0.04^{a}	1.66 ± 0.02^{ba}	1.73 ± 0.02^{a}	1.70 ± 0.02^{ba}	1.72 ± 0.02^{a}	1.63 ± 0.04^{b}	1.70 ± 0.02^{ba}

$^{a-d}$Row means SEM with different superscripts are significantly different ($P < 0.05$). †Diets supplemented with different dosages (0.1–0.5%, w/w) of metabolite powder of Com3456 (a combination of 4 strains RS5, RI11, RG11 and RG14). Each treatment consisted of 6 pens with 12 birds per pen.

percentage of performance compared to –ve control, birds supplemented with different dosages of metabolite combinations had 4–12% higher live BW at week 6 than –ve control birds.

The study showed that metabolite combinations are potential replacements of AGP due to their antimicrobial effects. The results were in line with the findings of Loh *et al.* (2003a) and Foo *et al.* (2003a,b) who reported feeding *Lactobacillus* species enhanced growth performance in post-weaning rats. Ogunbanwo *et al.* (2004) also reported that bacteriocin from *L. plantarum* F1 improved the growth rate of broilers. The supplementation of *Bacillus coagulans* in broiler diets significantly improved growth performance (Cavazzoni *et al.* 1998). The FCR improvement result was in agreement with the findings of Yu *et al.* (2007) who reported that supplementation of *L. reuteri* improved 5% FCR in broilers.

The main effects of antimicrobials in improving growth rate were due to their bacteriostatic and bactericidal effect to inhibit and kill pathogenic bacterial load in gastrointestinal microflora. The antibacterial activity of AGP has been proven in germ free animals. The important mechanisms of AGP in improving growth performance include the decrease of the toxins produced by the bacteria, the increase of the available nutrients, the increase in the absorption of nutrients, and the reduction in the incidence of subclinical infections (Butaye *et al.* 2003). Additionally, AGP reduces opportunistic pathogens and subclinical infection and stimulates synthesis of vitamins by bacteria and improve gastrointestinal enzyme activities (Page 2006).

Fecal lactic acid bacteria and *Enterobacteriaceae* count

Fecal LAB and ENT count is presented in Table 4. The fecal LAB count from –ve control birds was the lowest ($P < 0.05$), while no significant difference was found among the treatment groups. With regard to ENT count at week 6, the –ve and +ve control birds had the highest ($P < 0.05$) results. In contrast, the lowest ($P < 0.05$) ENT count was observed in 0.2% and 0.5% Com3456. ENT count from other treatment groups of 0.1%, 0.3% and 0.4% Com3456 were also significantly lower ($P < 0.05$) than that of the –ve and +ve control groups.

The results of the study proved the effect of metabolite combinations in reducing gastrointestinal ENT. Additionally, the metabolite combinations increased gastrointestinal LAB. Although with varying degrees,

Table 4 Fecal LAB and ENT count, and VFA at week 6 of treatments supplemented with different dosages of metabolites from Com3456

Parameters	Dietary treatments†					
	–ve control	+ve control	0.1% Com3456	0.2% Com3456	0.3% Com3456	0.4% Com3456	0.5% Com3456
LAB and ENT count, logCFU/g							
LAB	6.04 ± 0.11^{b}	6.80 ± 0.15^{a}	6.74 ± 0.12^{a}	6.91 ± 0.14^{a}	6.65 ± 0.15^{a}	6.72 ± 0.11^{a}	6.43 ± 0.10^{a}
ENT	4.88 ± 0.10^{a}	4.81 ± 0.18^{a}	4.28 ± 0.12^{b}	3.94 ± 0.19^{b}	4.10 ± 0.11^{b}	4.32 ± 0.14^{b}	3.94 ± 0.11^{b}
VFA, mM							
Acetic acid	50.39 ± 1.96^{c}	59.56 ± 4.51^{bc}	62.77 ± 7.51^{bc}	62.33 ± 4.87^{bc}	69.88 ± 5.74^{ba}	81.27 ± 5.51^{a}	64.21 ± 3.77^{bc}
Propionic	1.96 ± 0.43^{a}	2.09 ± 0.76^{a}	3.33 ± 1.13^{a}	1.80 ± 0.51^{a}	1.83 ± 0.28^{a}	2.01 ± 0.21^{a}	1.44 ± 0.15^{a}
Butyric	2.22 ± 1.12^{ba}	1.96 ± 0.90^{ba}	2.53 ± 0.86^{a}	1.13 ± 0.43^{ba}	0.64 ± 0.07^{b}	0.86 ± 0.08^{b}	0.68 ± 0.05^{b}
Others	0.55 ± 0.10^{a}	0.46 ± 0.12^{a}	0.83 ± 0.25^{a}	0.73 ± 0.14^{a}	0.46 ± 0.08^{a}	0.54 ± 0.07^{a}	0.65 ± 0.12^{a}
Total	52.29 ± 2.08^{c}	62.71 ± 5.68^{bc}	68.32 ± 8.79^{bac}	65.68 ± 5.71^{bac}	72.74 ± 6.04^{ba}	84.23 ± 5.51^{a}	66.63 ± 3.77^{bac}

$^{a–c}$Row ± means SEM with different superscript are significantly different ($P < 0.05$). †Diets supplemented with different dosages (0.1–0.5%, w/w) of metabolite powder of Com3456 (a combination of 4 strains RS5, RI11, RG11 and RG14). Each treatment consisted of 6 replicates.

all dosage levels of metabolite combination had effects in increasing gastrointestinal LAB. All treatments supplemented with Com3456 had positive effects in reducing ENT count. The results were in line with the findings of Savadogo *et al.* (2004), as they reported that metabolites exhibited broad antagonistic activity, which has capability to inhibit pathogens from various species such as Gram-positive (e.g. *Bacillus cereus*, *Staphylococcus aureus*, *L. monocytogenes*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Enterococcus faecium* and *P. acidilactici*) and Gram-negative (e.g. *E. coli* and *S. typhimurium*) bacteria. The same effect of *Lactobacillus* on fecal ENT count was reported in rats by Loh *et al.* (2003a) and Foo *et al.* (2003a,b). Feeding of fermented products that contained LAB also reduces *Enterobacteriaceae* population numbers in feces of pigs and layers (Loh *et al.* 2003b, 2007).

The ability of LAB in reducing ENT is mainly due to competitive exclusion and the ability to produce and secrete antimicrobial substances such as bacteriocin and VFA to reduce pH and inhibit viability of ENT (Reid 2001). The effect of metabolite combination in reducing gastrointestinal ENT gave better chances for LAB to increase their population in intestinal microflora via competitive exclusion. Several studies have proved the effect of LAB on competitive exclusion of pathogens in different animals such as in chickens (Pascual *et al.* 1999), pigs (Harvey *et al.* 2005), and in humans (Tuomola *et al.* 1999). Competition of attachment sites and nutrients are the two key fundamentals of competitive exclusion. Besides the physical barrier, LAB are also involved in making an unfavorable environment for many of the unnecessary bacteria such as *Salmonella* and *E. coli* as LAB are capable of producing and secreting antimicrobial substances such as bacteriocin and VFA to reduce pH and inhibit viability of ENT (Reid 2001). The bacteriostatic effects of VFA in the caeca (van der Wielen *et al.* 2000) also play an additional role in reducing ENT. The negative correlation between ENT and LAB once again proved the competitive exclusion of LAB against ENT. The increased number of LAB in treated groups reduced the ENT count.

Fecal volatile fatty acids

The fecal VFA is presented in Table 4. The main VFA was acetic acid, followed by propionic and butyric acids. The treatments supplemented with 0.4% and 0.3% Com3456 had the highest ($P < 0.05$) acetic acid and total VFA levels. In contrast, the lowest ($P < 0.05$) results were found in −ve control birds. However, no significant difference ($P > 0.05$) of acetic acid and total VFA concentration was found among the rest of the treatments. With regard to propionic acid and other VFA, no significant difference ($P > 0.05$) was observed among all of the treatments. The butyric acid level of birds fed with 0.1% Com3456 was significantly different ($P < 0.05$) with that of treatments supplemented with 0.3%, 0.4% and 0.5% Com3456. However, no significant difference ($P > 0.05$) was found among the other 6 treatments.

The supplementation of metabolite combinations increased fecal VFA. There was an increase of VFA in some dosages of Com3456, especially in 0.3% and 0.5% Com3456 at week 3 and 0.4% Com3456 at week 6. One of the main reasons of the increase of VFA in treated birds may be the increase of LAB in treatments supplemented with metabolite combinations as LAB and other gut microbiota ferments various substrates like lactose, biogenic amines and allergenic compounds into short-chain fatty acids and other organic acids and gases (Gibson & Fuller 2000).

Intestinal villi height and crypt depth

The intestinal villi height and crypt depth of chickens from different treatments are presented in Table 5. The highest ($P < 0.05$) duodenal villi were found in birds fed with 0.4% Com3456, followed by birds in the 0.3% and 0.2% Com3456 groups, while −ve control birds had the shortest ($P < 0.05$). The similar ($P > 0.05$) duodenal villi height was found in +ve control, 0.1% and 0.5% Com3456, and three of them were significantly higher ($P < 0.05$) than that of the −ve control birds. Relating to the jejunal villi, the birds fed with 0.4% metabolites also had the highest ($P < 0.05$) results. However, the −ve control birds had the shortest ($P < 0.05$) jejunal villi and were significantly different from all of the other treatment groups. For ileal villi, the highest results belonged to the birds fed with 0.1% Com3456, followed by 0.5%, 0.4% and 0.3% Com3456, respectively. The similar ileal villi height was found between +ve control and 0.2% Com3456. The shortest ileal villi and significantly different ($P < 0.05$) with all of the remaining treatments was observed in −ve control birds.

The +ve control birds had the deepest ($P < 0.05$) duodenal crypt, followed by groups of 0.2% and 0.3% Com3456 and −ve control. Similar results ($P > 0.05$) were observed in groups supplemented with 0.1%, 0.4% and 0.5% Com3456 and they were the shallowest ($P < 0.05$) among the treatment groups. For jejunal crypts, the +ve control and treatment supplemented

Table 5 Villi height and crypt depth at week 6 of treatments supplemented with different dosages of metabolites from Com3456

Parameters	Dietary treatments†						
	−ve control	+ve control	0.1% Com3456	0.2% Com3456	0.3% Com3456	0.4% Com3456	0.5% Com3456
Villi height, μm							
Duodenal	1683 ± 19^{a}	1756 ± 26^{b}	1752 ± 16^{b}	1865 ± 22^{c}	1871 ± 15^{c}	1984 ± 26^{d}	1765 ± 18^{b}
Jejunal	1157 ± 9^{a}	1303 ± 13^{c}	1373 ± 21^{d}	1390 ± 19^{d}	1272 ± 19^{bc}	1532 ± 24^{e}	1224 ± 20^{b}
Ileal	719 ± 12^{a}	788 ± 13^{b}	913 ± 14^{d}	778 ± 9^{b}	826 ± 12^{c}	832 ± 12^{c}	849 ± 12^{c}
Crypt depth, μm							
Duodenal	275 ± 10^{b}	306 ± 8^{c}	247 ± 8^{a}	296 ± 7^{bc}	289 ± 7^{bc}	237 ± 4^{a}	237 ± 7^{a}
Jejunal	219 ± 7^{a}	276 ± 9^{c}	276 ± 7^{c}	235 ± 7^{a}	218 ± 5^{a}	226 ± 3^{a}	255 ± 4^{b}
Ileal	134 ± 4^{bc}	115 ± 3^{a}	185 ± 3^{f}	165 ± 5^{e}	137 ± 3^{c}	124 ± 3^{ab}	151 ± 2^{d}

$^{a–f}$Row means ± SEM with different superscript are significantly different ($P < 0.05$). †Diets supplemented with different dosages (0.1–0.5%, w/w) of metabolite powder of Com3456 (a combination of 4 strains RS5, RI11, RG11 and G14). Each treatment consisted of 20 replicates.

with 0.1% Com3456 had the highest ($P < 0.05$) results, followed by group of 0.5% Com3456. However, no significant difference ($P > 0.05$) was found for the rest of the treatment groups. The deepest ($P < 0.05$) ileal crypt belonged to treatment supplemented with 0.1% Com3456, followed by groups of 0.2% and 0.5% Com3456. In contrast, the +ve control had the shallowest ($P < 0.05$) ileal crypt. The ileal crypts of −ve control birds did not significantly differ ($P > 0.05$) with that of groups supplemented with 0.3% and 0.4% Com3456.

Villi are the key components taking part in nutrients' absorption in small intestine. High villi enlarge the surface area of the intestinal epithelium for greater absorption of necessary nutrients (Caspary 1992). The increase of villi height and crypt depth was found in birds treated with different dosages of Com3456. The most prominent villi height increase was observed in treatment supplemented with 0.4% Com3456, which also had the highest growth performance among all the treatments. The results of the present study was in agreement with the findings of Samanya and Yamauchi (2002) who reported a significant increase of villi height of duodenum and ileum in 28-day-old chicks fed with *Bacillus subtilis*. The results was also supported by Miles *et al.* (2006) who reported the increase of absorptive surface in small intestine of chicken fed with antimicrobials.

The increase of villi height from feeding broilers with Com3456 metabolite combinations may be attributed to their antimicrobial activity of bacteriocin and organic acid. The role of villi as a protective barrier against pathogenic bacteria also has positive effects on the increase of villi height (Paul *et al.* 2007). Pathogens in the normal microflora in the intestinal epithelium may contribute to altering the permeability of the villi surface. This may lead to invasion of pathogens, modifying metabolism and absorption of nutrients, resulting in chronic inflammation in the intestinal epithelium (Podolsky 1993) and lead to the decrease of villi height (Visek 1978). The metabolites with their important components bacteriocin and organic acids inhibit growth of many pathogens. This may reduce the colonization of bacteria in the epithelium and result in reducing inflammation and infection at the intestinal mucosa. Finally, taller villi exhibit enhanced secretion and absorption (Paul *et al.* 2007). On the contrary, reduced absorptive functions were occurred in short villi with the reduction in the villi surface area (Park *et al.* 1998). Additionally, reduction of enzyme activities, such as mucosal lactase and sucrase (Park *et al.* 1998), lactase and alkaline phosphatase (Zijlstra *et al.* 1997), alkaline phosphatase and disaccharidase (Lopez-Pedrosa *et al.* 1998), and total lactase phlorizin hydrolase and mucosal protein concentration (Dudley *et al.* 1998) were also observed in these short villi. Additionally, the present study also showed the positive correlation between live BW of birds and their duodenal and jejunal villi height. The results were consistent with the findings of others, who reported the positive association between an increase in villi height and BW in piglets (Zijlstra *et al.* 1996), chickens (Samanya & Yamauchi 2002), and turkeys (Ritz *et al.* 1995).

In conclusion, supplementation of Com3456 in the diet of broiler chickens at different dosages improved growth performance, reduced ENT and increased LAB count, and increased small intestine villi height, fecal

VFA. Treatment with 0.4% and 0.2% Com3456 gave the best results, especially in terms of growth performance, FCR and villi height. Therefore, metabolite combinations from *Lactobacillus plantarum* are potential replacements of AGP in the poultry industry.

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