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Histopathological features of the small intestines and quantification of lactic acid bacteria of broiler chickens supplemented with plectasin

Características histopatológicas do intestino delgado e quantificação de bactérias ácido-láticas de frangos de corte suplementados com plectasina

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Abstract: This study was conducted to determine the histopathological features of the small intestines and to quantify lactic acid bacterial population in the ceca of broiler chickens supplemented with varying doses of plectasin, an antimicrobial peptide with gut health- promoting potentials. This research was done by supplementing broiler chickens with varying doses (150 ppm, 300 ppm and 450 ppm) of plectasin alongside negative and positive control groups. Small intestinal samples showed decreased occurrence and severity of histopathological lesions from the duodenum to the ileum. The occurrence of duodenal lesions such villi necrosis, de-epithelization of villi, haemorrhage and inflammation, observed in the duodenum decreased with supplementation of plectasin, which demonstrated its potential in promoting the structural integrity of the small intestines. On the other hand, microbial enumeration resulted in lower total lactic acid bacteria count in treatment groups supplemented with plectasin, demonstrating its inability to enhance beneficial microbiota, but may be suggestive of improved intestinal absorptive capacity based on the concept of competitive exclusion.

Keywords: Histopathology; lactic acid bacteria; poultry; plate count; plectasin.

Resumo: Este estudo foi realizado para investigar as características histopatológicas do intestino delgado e quantificar a população de bactérias lácticas no ceco de frangos de corte suplementados com diferentes doses de plectasina, um peptídeo antimicrobiano com potencial para promover a saúde intestinal. O experimento incluiu dietas suplementadas com plectasina em doses de 150 ppm, 300 ppm e 450 ppm, além de um grupo controle negativo (sem antibiótico) e um controle positivo (com enramicina). A análise das lâminas de amostras do intestino delgado revelou uma diminuição na ocorrência e na gravidade das lesões histopatológicas, desde o duodeno até o íleo. Lesões no duodeno, como necrose, desepitelização das vilosidades, hemorragia e inflamação, foram reduzidas com a suplementação de plectasina, evidenciando seu potencial para promover a integridade estrutural do intestino delgado. No entanto, a avaliação da microbiota revelou uma contagem total de bactérias lácticas menor nos grupos tratados com plectasina, indicando sua incapacidade de aumentar a microbiota benéfica. Isso sugere uma melhor capacidade de absorção intestinal, com base no conceito de exclusão competitiva.

Palavras-chave: Histopatologia; bactérias lácticas; aves; contagem de placas; plectasina

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1. Introduction

Antibiotics have been widely used to combat antimicrobial infections, including infectious diseases in humans and animals. Antibiotics have been used to treat and prevent diseases and for efficient production in the livestock and poultry industries [1]. However, alongside the discovery of antimicrobial therapy came the overuse/misuse of antibiotics leading to the development of antibiotic resistance. Risks of antibiotic use in animal feeds have been increasingly documented comparing with benefits [2]. The continued rise of new and re-emerging infectious diseases in parallel with the decreasing efficacy of antibiotics necessitates research and development of new classes of antimicrobial drugs in order to address this dilemma.

A recently developed antibiotic of interest is plectasin, a host defense peptide (HDP) or antimicrobial peptide (AMP), harvested from the fungus *Pseudoplectania nigrella*. It has demonstrated antimicrobial effects, notably, against antibiotic-resistant strains of bacteria. Plectasin reportedly has the ability to enhance the population of beneficial bacteria in the gut and improve overall intestinal health [3]. The bactericidal mechanisms of AMPs are different from traditional antibiotics. Most AMPs act on bacterial cell membranes, resulting in increased membrane permeability to penetrate and kill bacteria. Therefore, AMPs greatly reduce the possibility of resistance developing [4].

However, the lack of locally generated information on the efficacy of plectasin as a feed additive requires the conduct of studies that may serve as the basis for its possible utilization in the local livestock industry. In poultry, enteric diseases have been associated with high morbidity and mortality resulting in significant economic losses[5]. Observing the histopathological features of the small intestines of broilers following supplementation with varying doses of plectasin will determine its potential beneficial or toxic effects in the gut while the observance of growth of lactic acid bacteria (LAB) shall provide basis for the capability of the drug to promote the proliferation of beneficial intestinal microflora.

2. Material and methods

A total of 300 day-old Ross chicks were used in the study. All procedures performed in broiler chickens were approved by the Institutional Animal Care and Use Committee (IACUC) number 2018-0032 of the College of Veterinary Medicine, University of the Philippines Los Baños. The birds were housed in single-tiered battery cages with wire flooring, and were vaccinated against transmune Infectious Bursal Disease (IBD) and Newcastle Disease (ND) HB1 during day 1, with booster vaccinations against ND given at days 10 and 18 via the nasal and ocular route. Booster diet was given from days 1 to 10, whereas starter mash was given from days 11 to 23. Access to finisher ration was then provided until day 35. Food and water were given ad libitum, while temperature and humidity were recorded daily.

2.1 Research design

Chicks were divided into three blocks according to initial weight, then were distributed into the five treatment groups. Each treatment group was given the same basal diet, alongside

varying doses of plectasin (Fun-Tide®, Guangdong Hinabiotech Co. Ltd, China) with T1 serving as the negative control group, while T2 served as positive control group, receiving 250 ppm of enramycin. T3, T4 and T5 received 150 ppm, 300 ppm, and 450 ppm of plectasin, respectively.

2.2 Histological analysis of the small intestines

On day 35, two representative chicks from each replicate were sacrificed via atlanto-occipital joint dislocation. Thereafter, 1-2 cm sections of the intestines (duodenum, jejunum and ileum) were obtained followed by gentle flushing of the lumen using buffered formalin. Samples were then fixed in 10% formalin and were submitted for routine tissue processing and staining. Sections were viewed using a microscope (EVOSTM XL Autoimaging Microscopy System, Fisher Scientific, USA) under high power objective (40X) where three random fields were chosen and subjected to lesion scoring.

Table 1. Lesion scoring system (adapted and modified from Gibson-Corley[6]).

Lesion Score	% Tissue Affected	Severity
0	0	None
1	<25	Mild
2	26-50	Moderate
3	51-75	Severe
4	76-100	Extremely severe

2.3 Microbiological enumeration of Lactic Acid Bacteria

One representative sample per replicate was obtained for Lactic Acid Bacteria (LAB) enumeration, wherein both ceca of each bird were ligated dissected, and transported in an ice cooler immediately after necropsy. Thereafter, approximately 1 g of cecal contents were collected aseptically in sterile sampling tubes. Cecal contents were serially diluted until a final dilution of 10^7 was reached and were inoculated in count plates for LAB (3M Petrifilm™, USA), which were placed inside an anaerobic jar, and incubated at 37°C for 48 hours. The resulting microbial growth were manually counted and expressed in colony-forming units (CFU) per gram.

2.4 Data analysis

Data on LAB were checked for normality, homogeneity of variances and independence of errors with Shapiro-Wilk test, Bartlett's Test, and Runs Test, respectively. Treatment means were compared using one-way analysis of variance (ANOVA) in Completely Randomized Design followed by pairwise comparison using Tukey's Honest Significant Difference (HSD) Test. The probability of significance was set at $P<0.05$. Statistical analyses were done using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA). Data on histopathological lesions were presented in terms of incidence and mean lesion score.

3. Results

Among the observed lesions, villi necrosis and denudation, and hemorrhage were the most common in the duodenum, which occurred with mild severity. Among treatments, T1 and T2 had the highest incidence of lesions while the least was noted in T3 (Table 3). Histological sections of the duodenal mucosa are shown in Figure 1. Based on the mean lesion score, T1 and T2 had more severe lesions, while T3 had the lowest. The incidence and severity of villi necrosis and enteritis was relatively higher in the duodenum compared to the other intestinal segments. Parasitic infection was also noted in two samples belonging to T1 and T2.

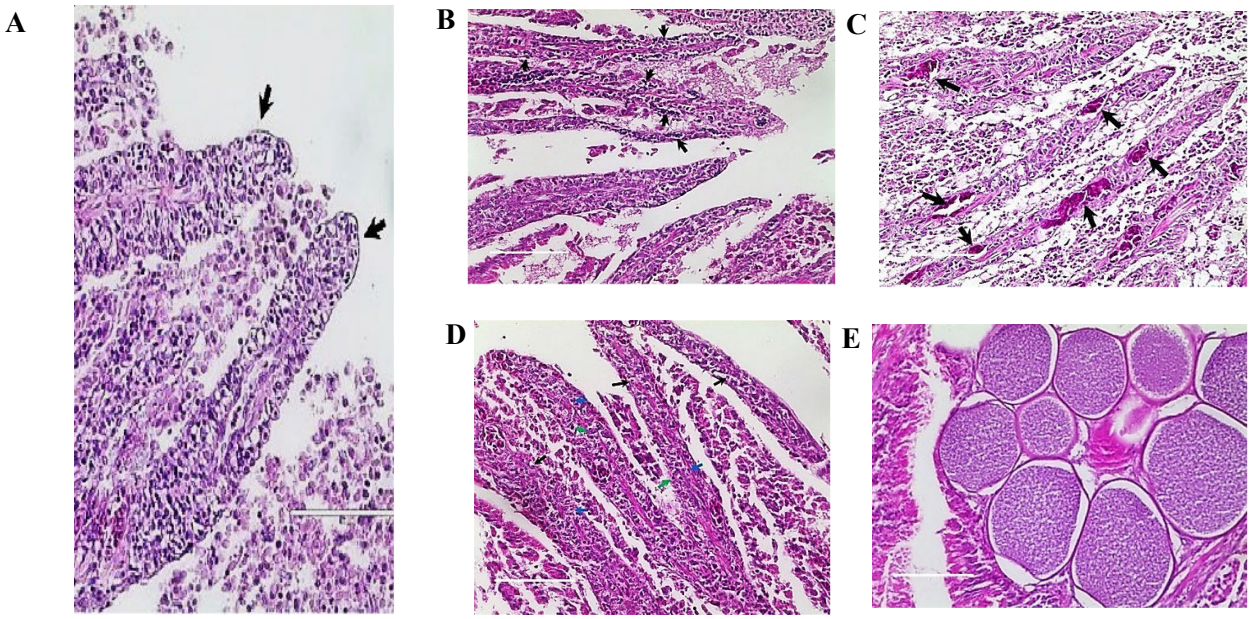

Figure 1. Histological sections of the duodenal mucosa showing (A) normal intact villi, (B) necrotic villi, (C) hemorrhage, (D) inflammation, and (E) parasitic infection. H&E stain. Scale bar = 1000µm.

Table 2. Incidence (%) and mean lesion score in the duodenum of broiler chickens supplemented with plectasin.

Lesion	T1	T2	T3	T4	T5
Crypt hyperplasia	8 (0.03)	17 (0.17)	0	17 (0.17)	25 (0.17)
Short/blunt villi	8 (0.03)	8 (0.08)	0	8 (0.03)	0
Nude villi	75 (1.25)	67 (0.69)	17 (0.06)	25 (0.08)	50 (0.67)
Fused villi	0	0	0	0	8 (0.06)
Villi necrosis	100 (1.17)	83 (1.44)	75 (0.69)	92 (1.06)	75 (0.78)
Hemorrhage	58 (0.56)	58 (0.39)	42 (0.25)	33 (0.19)	17 (0.08)
Inflammation	8 (0.06)	67 (0.53)	17 (0.17)	17 (0.17)	8 (0.03)
Total	52 (0.44)	60 (0.47)	30 (0.17)	38 (0.24)	37 (0.25)

[T1] negative control; [T2] positive control; [T3] 150 ppm plectasin; [T4] 300 ppm plectasin; [T5] 450 ppm plectasinThe lesions seen in the duodenum were likewise noted in the jejunum.

Histological sections of the jejunal mucosa are shown in Figure 2. However, the incidence and severity of lesions were significantly lower in the jejunum compared to the duodenum. T2 had the highest incidence of lesions among all treatment groups, with prominence of villi de-epithelization and shortening in T1 and T2, while crypt hyperplasia was most commonly observed in T2, T3 and T4. Hemorrhage was also frequently observed in T2. Overall, intact villi were more commonly observed in T3 and T4.

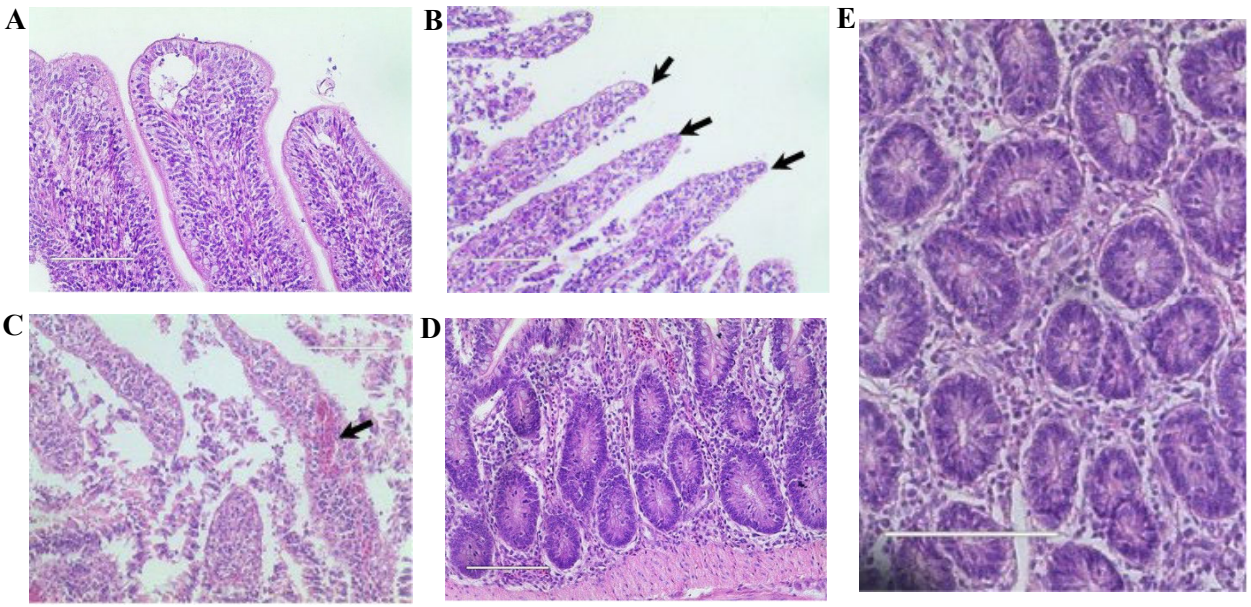

Figure 2. Histological sections of the jejunal mucosa showing (A) normal intact villi, (B) nude villi, (C) hemorrhage, (D) normal crypts, and (E) crypt hyperplasia. H&E stain. Scale bar = 1000µm.

Table 3. Incidence (%) and mean lesion score in the jejunum in broiler chickens supplemented with plectasin.

Lesion	T1	T2	T3	T4	T5
Crypt hyperplasia	8 (0.08)	33 (0.33)	33 (0.28)	33 (0.31)	17 (0.22)
Short/blunt villi	33 (0.31)	33 (0.17)	17 (0.14)	17 (0.11)	25 (0.39)
Nude villi	33 (0.64)	42 (0.61)	42 (0.58)	25 (0.47)	33 (0.42)
Fused villi	33 (0.19)	17 (0.11)	25 (0.17)	33 (0.25)	33 (0.22)
Villi necrosis	0	8 (0.22)	0	0	0
Hemorrhage	25 (0.17)	42 (0.31)	25 (0.08)	33 (0.19)	25 (0.14)
Inflammation	0	0	0	0	0
Total	27 (0.23)	35 (0.29)	28 (0.21)	28 (0.22)	27 (0.23)

[T1] negative control; [T2] positive control; [T3] 150 ppm plectasin; [T4] 300 ppm plectasin; [T5] 450 ppm plectasin

The ileum was observed to have the lowest incidence and mean lesion scores among all segments of the small intestines (Figure 3.). The most frequently observed lesions were hemorrhage and villi fusion. Hemorrhage was more commonly observed in T2 while villi fusion had the highest incidence in T4. Intact villi were most commonly observed in T3 (Table 5).

Table 4. Incidence (%) and mean lesion score in the ileum of broiler chickens supplemented with plectasin.

Lesion	T1	T2	T3	T4	T5
Crypt hyperplasia	0	17 (0.11)	17 (0.11)	8 (0.06)	17 (0.17)
Short/blunt villi	17 (0.25)	17 (0.19)	0	8 (0.06)	0
Nude villi	8 (0.17)	25 (0.22)	8 (0.17)	17 (0.17)	17 (0.25)
Fused villi	17 (0.11)	25 (0.19)	8 (0.11)	42 (0.31)	25 (0.19)
Villi necrosis	0	0	0	0	0
Hemorrhage	8 (0.03)	42 (0.22)	25 (0.11)	25 (0.08)	17 (0.06)
Inflammation	0	0	0	0	0
Total	12 (0.09)	25 (0.16)	12 (0.08)	20 (0.11)	15 (0.11)

[T1] negative control; [T2] positive control; [T3] 150 ppm plectasin; [T4] 300 ppm plectasin; [T5] 450 ppm plectasin

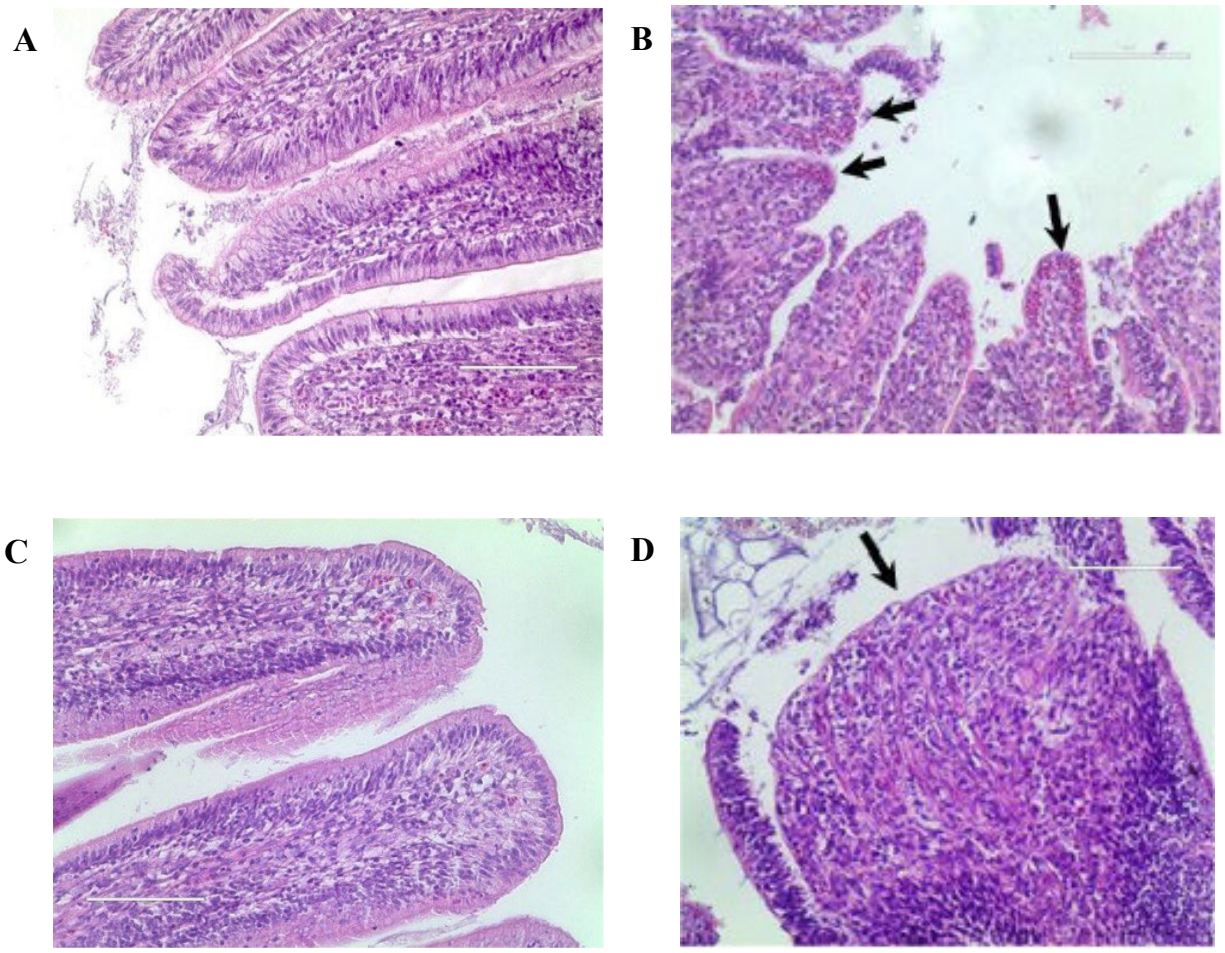

Figure 3. Histological sections of the ileal mucosa showing (A and B) normal intact villi, (C) fused villi and (D) hemorrhage. H&E stain. Scale bar = 1000µm.

Throughout the intestinal segments, the commonly observed lesions included crypt hyperplasia, hemorrhage, and shortening or blunting, denudation, fusion, and necrosis of villi. While enteritis and parasitic infection were observed specifically in the duodenum. Crypt hyperplasia occurs concurrently with villous atrophy as loss of mature enterocytes at the tips of the villi require production of new enterocytes, which are formed at the crypts. Hence,

crypts become hyperplastic as a result of increasing necessity to replace lost enterocytes. Villous atrophy may be observed in the form of villi shortening or blunting, fusion, and/or de-epithelization. In villi shortening, there is inability of the crypts to replace lost enterocytes at the tips, resulting to decrease in villus length [7]. Loss of villi epithelium is commonly observed in conditions where pathogenic causes, such as in bacterial infection, target enterocytes specifically at the villi epithelium. Villi necrosis is observed microscopically in the form of pyknosis, karyolysis and karyorrhexis of cells; all of which result from cellular damage caused by either hypoxia, ischemia or direct cell membrane injury [8,9]. These changes in intestinal morphology are related to poor nutrient absorption, increased gut secretion, reduced disease resistance, and impaired overall performance of broilers [10].

Occurrence of histopathological lesions across all treatment groups is suggestive of the effects of temperature and humidity as predisposing factors. Chronic exposure to increased temperature and humidity is known to cause heat stress in chickens. Across the 35 days of experimentation, both temperature and humidity recordings were noted to be higher than

recommended values of 30°C and 60-70%, respectively. In a study by Hu *et al.* [11], exposure of broilers to 35°C has led to changes in intestinal morphology wherein lesions such as broken villi, necrosis of epithelial cells, and edema were observed. In another study by Quinteiro-Filho *et al.* [12], enteritis characterized by infiltration of lymphocytes and plasma cells in the lamina propria of the jejunum were also observed. Song *et al.* [13] attributes the negative effects of heat stress on the digestive tracts of broilers to the disturbance of intestinal barrier function and intestinal microbiota, which increase the hosts' susceptibility to enteric pathogens. No existing studies have been carried out to elucidate the mechanism of heat stress in inducing observed intestinal lesions.

The lack of structural changes among treatment groups observed in both the jejunum and ileum suggest positive effects of plectasin in maintaining intestinal wall integrity. Good structural integrity of the small intestines is correlated with the protective function of the intestinal wall against leakage of unwanted substances from the lumen to the submucosa, as well as to its absorptive function [14,15]. An incidental finding of mild parasitic infection in the negative and positive control groups may suggest a possible antiparasitic potential of the drug. However, further studies are needed to confirm this. While previous reports have been established on the possible anticoccidial effects of antimicrobial peptides [16], its potential pharmacologic effect against other parasitic infections have not been explored.

Total lactic acid bacteria (LAB) count was highest in T2 and lowest in T3 ($p<0.05$) (Table 4). Although not statistically significant, an increasing trend in colony counts was observed with increasing inclusion rates of plectasin from T3 through T5. LAB without gas (homofermentative) were highest in T2 and lowest in T3, while no significant difference was detected in the colony counts of LAB with gas (heterofermentative).

Table 5. Mean (± SD) lactic acid bacteria (LAB) colony counts isolated from cecal contents of broiler chickens supplemented with plectasin.

LAB Count ($CFUx10^7/g$)	T1	T2	T3	T4	T5
Homofermentative	55.33 ± 27.01^{b}	102.67 ± 56.87^{a}	26.17 ± 24.80^{b}	31.00 ± 28.45^{b}	36.17 ± 20.83^{b}
Heterofermentative	11.67 ± 15.32	4.17 ± 5.23	0.33 ± 0.82	0.17 ± 0.41	3.33 ± 3.44
Total	67.00 ± 42.33^{ab}	106.84 ± 62.10^{a}	26.50 ± 25.62^{c}	31.17 ± 28.86^{bc}	39.50 ± 24.27^{bc}

Means with different superscripts in a column indicate significant difference ($p < 0.05$). [T1] negative control; [T2] positive control; [T3] 150 ppm plectasin; [T4] 300 ppm plectasin; [T5] 450 ppm plectasin

Total lactic acid bacteria (LAB) were enumerated by manually counting colonies that appeared on the Petrifilm™. Two types of colonies were counted, namely, heterofermentative and homofermentative LAB. Heterofermentative and homofermentative LAB differ mainly on the products they produce following fermentation of sugars. Homofermentative LAB mainly produce lactic acid, while heterofermentative LAB produce acetic acid, ethanol, mannitol, and carbon dioxide (CO2) along with lactic acid [17]. These two types of LAB are often distinguished through detection of CO2 production by heterofermentative LAB, which is the basis of distinction between colony growths in the Petrifilm™.

Studies by Lan *et al.* [18] and Nazef *et al*. [19] have described the general abundance of species of *Lactobacillus* and *Enterococcus* in the intestinal tract of poultry. However, species of homofermentative and heterofermentative LAB specifically of chickens have yet to be investigated. In this specific study, no further testing was performed to confirm the identity of the isolated colonies.

A significant increase in total lactic acid bacteria (LAB) count was observed in T2 compared to T1. Conversely, birds that received plectasin (T3, T4 and T5) had significantly decreased total LAB count. This may be suggestive of altered intestinal absorptive function based on the concept of competitive exclusion. The mechanism of microbial competition is highly based on available dietary protein in the intestinal tract. As dietary protein is digested, it is taken up by both the host cells and residential microflora throughout the portions of the intestinal tract. Thus, competitiveness of bacteria in the distal portion of the intestines is dependent on the amount of excess dietary protein following absorption in the proximal portions of the intestines. When amino acids in the distal tract is low, there is higher competitiveness amongst intestinal microbiota resulting in high amounts of bacteria that are dependent on externally available amino acid such as *Escherichia coli* and *Clostridium perfringens*, while those that are not, such as *Lactobacillus* sp., are expected to reduce in number. In contrast, when high amounts of excess amino acids are available in the distal tract, there is less competitiveness of bacteria that are dependent on external amino acids [20]. Hence, lower proportions of beneficial microbiota, such as LAB, in the distal intestines can be linked to improved absorptive capacity in the proximal intestines.

These results were in contrast to a recent study by Ma *et al*. [21] wherein supplementation of both low (100 mg/kg) and high dose (200 mg/kg) of plectasin resulted in the absence of

significant changes in *Lactobacillus* sp. count in cecal contents. However, the authors noticed an increase in *Lactobacillus* sp./*E. coli* ratio due to significant decrease in *E. coli* counts. This was accounted to LAB reaching their saturation point where the maximum amount of bacteria is present and able to grow with the available resources of energy in the intestines.

4. Conclusion

This study indicates that supplementation of plectasin promotes the structural integrity of the small intestines. The lower total LAB count in treatment groups supplemented with plectasin, demonstrates its inability to enhance beneficial microbiota, but may be suggestive of improved intestinal absorptive capacity based on the concept of competitive exclusion.

Statement of conflict of interest

The authors declare that there is no conflict of interest. All authors, except for NSVR (deceased), participated in the decision to publish the manuscript.

Author contributions

Conceptualization: M. G. V. Paraso; *Data curation*: N. S. V. Ramchandani; *Formal analysis*: M. G. V. Paraso, J. F. Dela Cruz, T. M. A. Collantes and N. S. V. Ramchandani; *Methodology*: M. G. V. Paraso and J. F. Dela Cruz; Project administration: J. F. Dela Cruz; *Supervision*: M. G. V. Paraso, T. M. A. Collantes and J. F. Dela Cruz; *Validation*: M. G. V. Paraso and J. F. Dela Cruz; Resources: T. M. A. Collantes; *Writing (original draft)*: N. S. V. Ramchandani. *Writing (proofreading & editing)*: M. G. V. Paraso, J. F. Dela Cruz and T. M. A. Collantes.

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