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EFFECT OF DIETARY RED YEAST (*Sporidiobolus pararoseus*) SUPPLEMENTATION ON SMALL INTESTINAL HISTOMORPHOMETRY OF LAYING HENS

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ABSTRACT

This study aimed to evaluate the effect of dietary supplementation with red yeast (*Sporidiobolus paraeoseus*) on small intestinal morphology and determine the correlation of red yeast supplementation on intestinal morphology in laying hens. One hundred sixty laying hens were randomly assigned into four dietary groups as follows: basal diet (control) and basal diet with red yeast supplementation at 0.5 (R1), 1.0(R2), and 2.0(R3) g/kg feed. After 12 weeks of feeding, 16 hens (4from each group) were randomly euthanized and the small intestines collected to determines mall intestinal morphology. The villus height of the duodenum was higher in R1, R2, and R3 red yeast-supplemented groups compared to the control-treated group. However, dietary treatments did not differ significantly in terms of jejunal and ilealmorphology data (villus height and width, crypt depth, villus height/crypt depth ratio, crypt area, and muscularis mucosae thickness). A positive linear correlation was also observed between the level of red yeast supplementation and the width of jejunal villi (R^2=0.8992).In conclusion, red yeast supplementation in the diet improved duodenal lumen health of laying hens by enhancing duodenal villus height.

Key words: red yeast, laying hen, small intestinal morphology, prebiotic.

INTRODUCTION

For several years, animals have been fed yeast products, including live or dried yeast supplementation, yeast fermented mash production, yeast byproducts from brewery and bakery industries, and yeast products commercially produced for animal feed. Typically, yeast cell walls are made of 30-60% polysaccharides (15-30% beta-glucanand, 15-30% mannan sugar polymers), 15-30% proteins, 5-20% lipids, and a small amount of chitin (Aguilar-Uscanga and François, 2003). Most of the protein is linked to mannan-oligosaccharides (MOS), and is referred to as the mannoprotein complex.

Red yeast (*Sporidiobolus pararoseus*) is a novel yeast that grows on glycerol waste. The red yeast genus *Sporidiobolus* has been shown to naturally produce carotene pigment (Valduga *et al*., 2014) and its cell wall functions as a prebiotic. Many of these prebiotics are carbohydrates, including fructo oligosaccharide, galacto oligosaccharide, transgalacto oligosaccharide, and mannan oligosaccharide (Shashidhara and Devegowda, 2003). The literature has focused on the prebiotic activities of the MOS present in yeast cell walls, with a competitive binding site for pathogenic intestinal bacteria with mannose-specific type-1 fimbriae (Ferket and Gernat, 2002; Zopf and Roth, 1996) and increased villus height of digestive mucosa (Yasar and Forbes, 1999). Furthermore, Baurhoo *et al*. (2009) observed that dietary supplementation with MOS improved morphological development of the small intestines of broilers, conferring intestinal health benefits. Supplementation with MOS led to increased villus height and width, as well as a decrease in crypt depth in all parts of the small intestine in broilers (Markovc *et al*., 2009).All these studies were conducted on broiler chickens; in contrast, little information is available about the effect of dietary supplementation with *Sporidiobolus pararoseus* on small intestinal morphology and intestinal lumen health in laying hens. We hypothesized that supplementing feed with *Sporidiobolus pararoseus* increases development of small intestinal morphologyin laying hens. Therefore, this experiment aimed to evaluate the effect of dietary supplementation with red yeast (*Sporidiobolus paraeoseus*) on small intestinal morphology and determine the correlation of red yeast supplementation on small intestinal morphology in laying hens.

MATERIALS AND METHODS

Meteorological data, animal, and experimental design: This experiment was conducted at a Poultry farm, Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University situated at longitude 98°55' 54.3" E, latitude 18°45' 40.3" N and an altitude of 312 m above sea level. A completely randomized design (CRD) was used with four nutritional treatments. This study used 160 ISA-Brown laying hens. At the age of 23 weeks, hens were allocated randomly into four treatment groups of 40 hens each, and housed in laying cages in a windowed poultry house. Each treatment group was distributed into 10 replicates, with four hens per replicate. The climatic conditions and

lighting program were computer-operated and followed commercial recommendations. Room temperature was thermostatically controlled at 25-27 °C, with 12 h of light/day.

Dietary treatments: The Division of Biotechnology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, Thailand provided the dried red yeast (*Sporidiobolus paraeoseus*). The four treatment groups received the following diets for 12 weeks (from 23 to 34 weeks of age): basal diet (control group) and basal diet with red yeast supplementations at 0.5(R1 group), 1.0 (R2 group), and 2.0 g/kg feed (R3 group). Feeding occurred at 08:30 h and the feeding management remained the same throughout the experimental period. The laying hens had *ad libitum* access to the selected diets and water (from nipple drinkers) throughout the experimental period. The basal diet was balanced to meet the nutrient requirements for laying hens (NRC, 1994).The ingredient and chemical composition are demonstrated in Table 1.The metabolisable energy (ME) of the basal diet was estimated using the Carpenter and Clegg equation (Leeson and Summers, 2001).

ME (kcal kg^{-1}) = 53 + 38 × [crude protein (%) + 2.25 × ether extract (%) + 1.1 × starch (%) + sugar (%)]

Samples collection and preparation of tissue: At the end of the experiment (34 weeks of age), four hens from each treatment group were euthanized and their small intestines collected. The small intestines were divided into three parts: duodenum (from the gizzard outlet to the end of the pancreaticloop), jejunum (from the pancreatic loop to Meckel's diverticulum), and ileum (from Meckel's diverticulum to the ileo-caeco-colic junction) (Giannenas *et al*., 2010).Immediately after euthanasia, intestinal tissue samples were quickly removed, immersed in a solution of phosphate-buffered saline (PBS; pH = 7) at 4 °C, and transported to the laboratory within 2h. In the laboratory, each intestinal segment was flushed clean with ice-cold buffered PBS (pH = 7). Then, use forceps to remove any fat tissue and blood vessels form the exterior of intestinal tissue samples. Four centimeters of each segment were fixed in a 4.0% solution of formaldehyde (formalin) for 24 h. Immediately after fixation, small intestinal tissues were dehydrated by immersing through a series of alcohols of increasing concentrations (from 70% to absolute), infiltrated with xylene, embedded in paraffin wax, cut by a microtome (5 µm thick), and fixed on slides.A routine staining procedure was carried out using haematoxylin and eosin(Awad*et al*., 2011). After staining, coverslips were placed on all sections with the use of mounting medium to visualize morphology of small intestines.

Image analysis and histomorphometric examinations: Histomorphometric analysis of the small intestinal tissues was performed at the Histology Laboratory of Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University. Histological sections were examined using an Olympus CX21 microscope (Olympus Cooperation, Tokyo, Japan) fitted with a digital video camera (Motic MC 2000), at 10X magnification. From the best stained sections, intestinal images were taken from villus area, crypt length as well as muscularis mucosa of each intestinal segment. Intestinal histomorphometry were quantified with the use of the Motic Images Plus 2.0 software (Motic China Group Co, Fujian, China).The villus height and width, crypt depth and area, and muscularis mucosae thickness were calculated for each intestinal image. On the basis of cellular morphology, the villus height was measured from the tip of the villus to the villus-crypt junction, and the crypt depth was defined as the depth of the invagination between adjacent villi. The villus width was measured at the middle of the villi (Awad *et al*., 2011; Choe *et al*., 2012).The thickness of muscularis mucosae (thin layer of smooth muscle)was measured from the base of the crypt to the base of the muscularis mucosae. The crypt depth, and muscularis mucosae thickness, and crypt area (intestinal gland) were also measured. The ratio of villus height to crypt depth (villus height/crypt ratio) was then measured. Four intact, well-oriented, small intestine morphologic units were randomly selected per hen for each intestinal section and calculated as the mean values of the histomorphometric variables for further analysis. The histomorphometric variables evaluated included villus height(µm), villus width(µm), crypt depth(µm), muscularis mucosae thickness (µm), crypt area(μm^2), and villus height/crypt ratio for each hen. Moreover, all the histomorphometric analyses were conducted by the same person.

Statistical analysis: Data were presented as mean ± SD. Villus height and width, crypt depth, villus height/crypt depth ratio, crypt area, and muscularis mucosae thickness were analyzed with ANOVA procedure of SAS (SAS Institute, Cary, NC, USA). Differences among means were assessed by Duncan's New Multiple Range Test (Steel *et al*., 1997); $P<0.05$ was considered statistically significant. Simple linear correlation between the level of the red yeast supplementation and the morphology data for the small intestine were evaluated using PROC REG (regression analysis) procedure of SAS (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Until now, little research has focused on explaining the effect of dietary red yeast (*Sporidiobolus pararoseus*) supplementation on small intestinal morphology of laying hens. The present study provides the first description of the positive effect of dietary red yeast on duodenal morphology in laying hens. Figure 1

shows photomicrographs of duodenal tissue sections for control diet-treated (Fig. 1A) and red yeast-treated(Fig. 1B)laying hens. Interestingly, the villus height of the duodenum was greater (P<0.05) in the red yeast-supplemented group(R1, R2, and R3) than in the control group (Table 2). However, we found that duodenal villus width, crypt depth, villus height/crypt depth ratio, muscularis mucosae thickness, and crypt area were not significantly different among dietary treatments (P>0.05; Table 2). Figures 2 and 3 show photomicrographs of jejunal and ileal tissue sections for control diet-treated (Fig.2A and 3A) and red yeast-treated (Fig. 2B and 3B) laying hens, respectively. Overall, the morphology results for the jejunum and ileum demonstrated no significant differences (P>0.05) forvillus height, villus width, crypt depth, villus height/crypt depth ratio, crypt area, and muscularis mucosae thickness (Tables 3 and 4). Although red yeast supplementation appeared to increase the jejunal villus height, jejunal villus width, ileal villus height, and ileal villus width compared to the control group, the differences were not statistically significant. In addition to the morphology data for the small intestine, a positive linear correlation was also observed between the level of the red yeast supplementation and the jejunal villus width (R^2=0.8992, P<0.05) (Fig. 4).

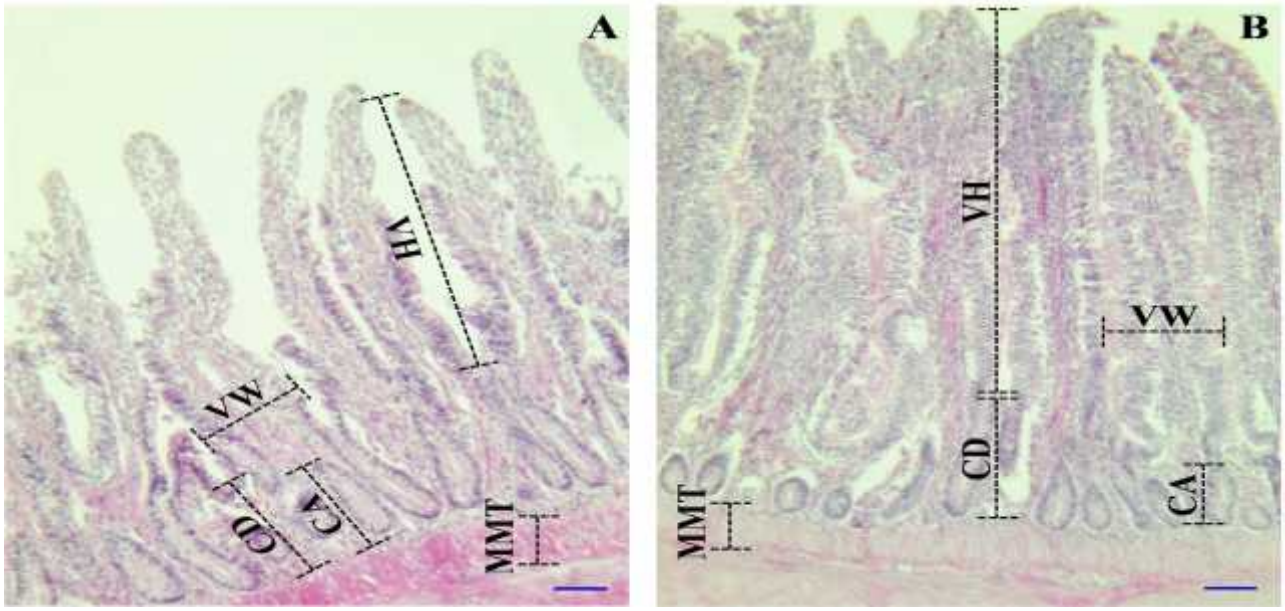

Fig. 1.Photomicrographs of haematoxylin and eosin staining of duodenal tissues in sections from control-treated (A) and red yeast-supplemented (B) laying hens. Magnification was 10 × objective lens. Scale bars represent 100 µm. VH = villus height; VW = villus width; CD = crypt depth; CA = crypt area; MMT = muscularis mucosae thickness

Laying hens of all dietary treatments had a normal gut structure; the highest villi were in duodenal segment followed by lower villi in jejunal segment and the lowest in ileal segment (Giannenas *et al*., 2010). The results of this study support the hypothesis that supplementing feed with red yeast (*Sporidiobolus pararoseus*) can increase development of duodenal morphology in laying hens, as demonstrated by the increase in the height of duodenal villi. This is consistent with results obtained by Ghosh *et al*. (2012), who demonstrated that supplementation with yeast cell walls in broiler diets significantly increased duodenal villus height. In fact, the increasing villus height was paralleled by an enhanced digestive and absorptive performance of the small intestine, due to increased absorptive surface area and expression of brush border enzymes and nutrient transport systems (Pluske *et al*., 1996; Awad *et al*., 2009). Taken together, the present study showed that adding red yeast (*Sporidiobolus pararoseus*) to the diets of laying hens increased duodenal villus height. It has been suggested that the activated cell proliferation in the duodenal epithelium increased absorptive surface area (Samanya and Yamauchi, 2002) and consequently lead to greater nutrient absorption (Caspary, 1992). This finding is supported by a recent study in boiler chickens, which demonstrated that supplementation of brewer's/baker's yeast (*Saccharomyces cerevisiae*) and MOS improved duodenal villus height by increasing cell proliferation (Padihari *et al*., 2014). In fact, cell proliferation and the epithelial cell turnover rate had important factors on the protein and energy requirements of the small intestinal mucosa (Simon, 1989). As previously suggested, yeast cell walls contain yeast metabolite (e.g., peptide, flavor substances, and some unidentified growth factor), which

might be offered the protein and energy requirements for small intestinal proliferation (Gao *et al*., 2008). Based on this morphological data of the small intestine, we suggest that the enhanced villus height obtained in the duodenal segment of red yeast-supplemented laying hens could be due to improved duodenal lumen health. Measuring the height of duodenal villi is another well-known way to examine the duodenal lumen health of poultry (Jeurissen *et al*., 2002; Chen *et al*., 2015). Moreover, in our study, a positive linear correlation was observed between the level of red yeast supplementation and the enlargement of the width of jejunal villi. Similarly, in broiler chickens, expansion of the height and width of the small intestinal villi increased the absorptive surface area when alternative growth promoters were adapted (Markovic *et al*., 2009).

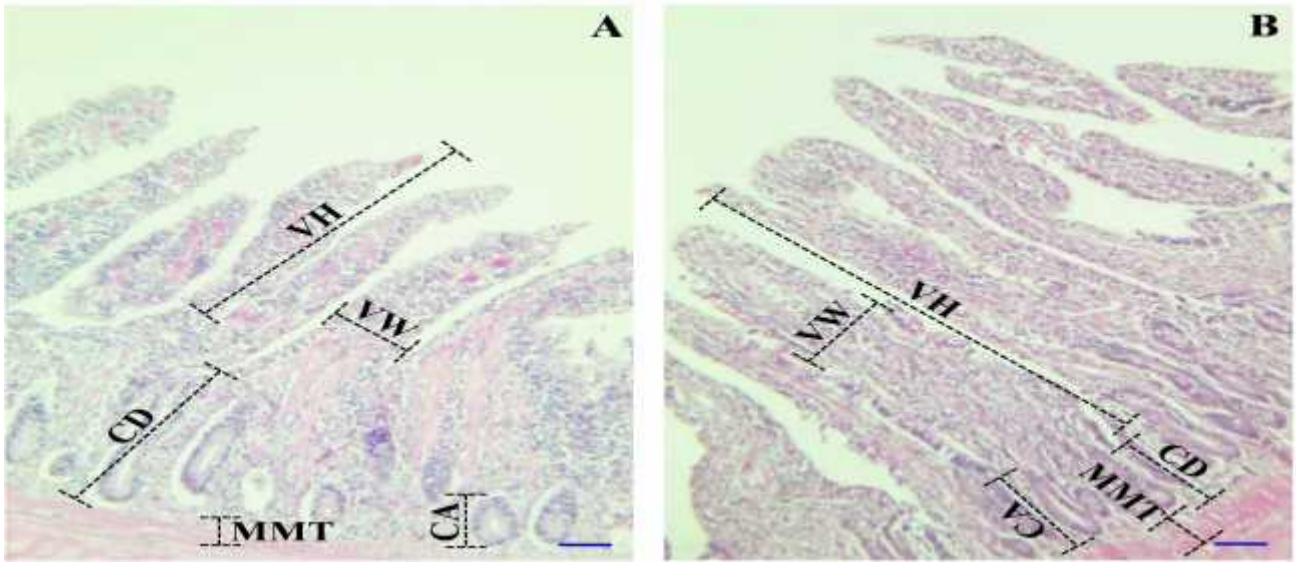

Fig. 2.Photomicrographs of haematoxylin and eosin staining of jejunal tissues in the sections from control-treated (A) and red yeast-supplemented (B) laying hens. Magnification was 10 × objective lens. Scale bars represent 100 µm. VH = villus height; VW = villus width; CD = crypt depth; CA = crypt area; MMT = muscularis mucosae thickness.

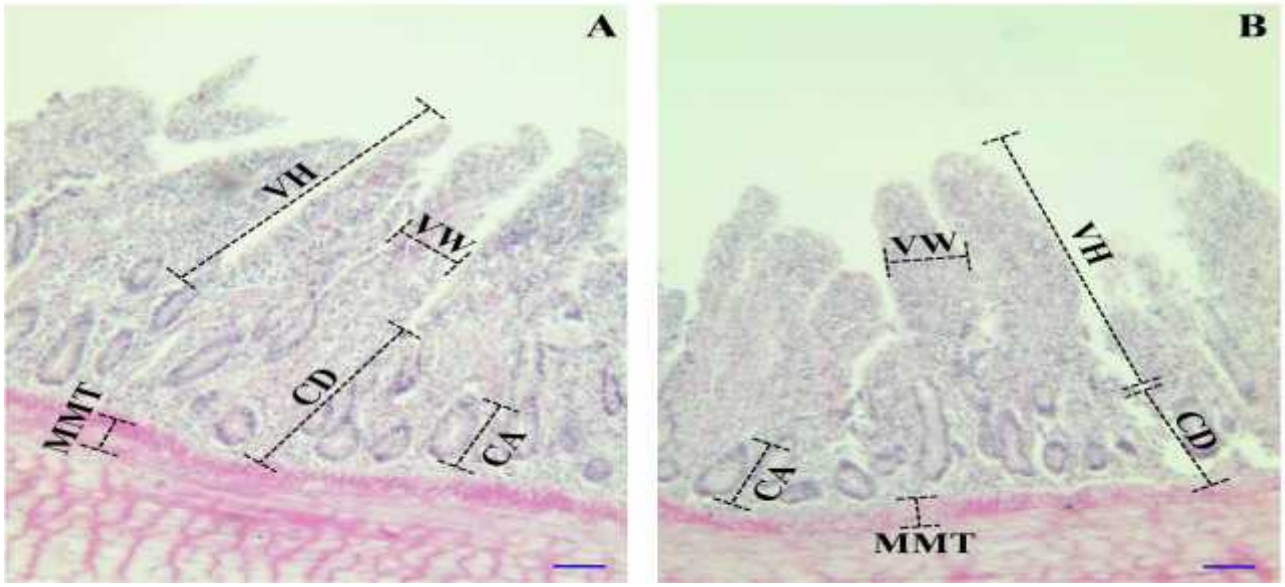

Fig. 3.Photomicrographs of haematoxylin and eosin staining of ileal tissues in sections from control-treated (A) and red yeast-supplemented (B) laying hens. Magnification was 10 × objective lens. Scale bars represent 100 µm. VH = villus height; VW = villus width; CD = crypt depth; CA = crypt area; MMT = muscularis mucosae thickness.

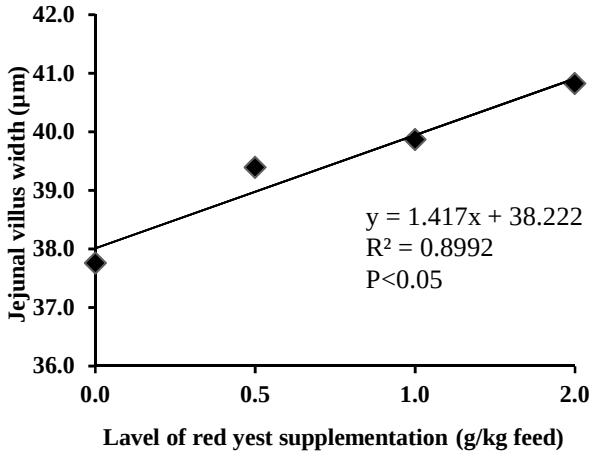

Fig. 4. A positive linear correlation indicates a positive association between the level of red yeast (*Sporidiobolus paraeoseus*) supplementation and the jejunal villus width.

Table 1.Ingredients and chemical composition of diets.

Ingredients (g/kg)	
Maize	546.5
Soybean meal, 440 g crude protein/kg	205.2
Fish meal	40.0
Rice bran	90.0
Soybean oil	13.0
Limestone	80.0
Dicalcium phosphate	12.0
Sodium chloride	2.5
DL-methionine	0.8
Vitamin-mineral premix*	10.0
Chemical composition (g/kg DM)	
Metabolisable energy† (MJ/kg)	2761.0
Crude protein	168.4
Crude fat	62.0
Crude fiber	36.0
Calcium	38.0
Total phosphorus	7.8
Lysine	9.5
Methionine	3.2

* Supplied per kilogram of diet: vitamin A 12400 IU, vitamin D_3 3100 ICU, vitamin E 17 IU, vitamin K 5.2 mg, riboflavin 5.5 mg, niacin 40 mg, pantothenic acid 15 mg, vitamin B_{12} 0.05 mg, folic acid 1 mg, pyridoxine 1.5 mg, Fe 30 mg, Mn 30 mg, Zn 45 mg, Cu 7.5 mg.
† Estimated according to the Carpenter and Clegg equation.

Table 2.Effect of dietary red yeast (*Sporidiobolus paraeoseus*) supplementation on the morphology of duodenum in laying hens.

Parameters	**Treatments**			
	Control*	**R1†**	**R2‡**	**R3§**
Villus height (µm)	1069.38±155.38[b]	1316.38 ± 98.42[a]	1391.42 ± 48.46[a]	1396.44 ± 126.43[a]
Villus width (µm)	43.71 ± 6.26	43.17 ± 5.08	41.46 ± 7.08	41.21 ± 11.89
Crypt depth (µm)	74.89 ± 7.37	70.47 ± 9.09	83.92 ± 21.14	82.38 ± 16.54
Villus height/crypt depth ratio	14.47 ± 1.41	19.35 ± 2.95	17.58 ± 4.04	18.95 ± 5.46
Crypt area (µm^2)	2972.36 ±378.84	2984.37 ± 391.11	3119.72 ± 535.69	2959.84 ± 971.10
Muscularis mucosae thickness (µm)	69.04 ± 12.95	71.27 ± 8.23	82.80 ± 12.72	79.55 ± 15.28

[a,b] Values in the same row not sharing a common superscript differ significantly at P<0.05.
* Laying hens were fed a basal diet.
† Laying hens were fed a basal diet supplemented with red yeast at 0.5 g/kg feed.
‡ Laying hens were fed a basal diet supplemented with red yeast at 1.0 g/kg feed.
§ Laying hens were fed a basal diet supplemented with red yeast at 2.0 g/kg feed.

Table 3.Effect of dietary red yeast (*Sporidiobolus paraeoseus*) supplementation on the morphology of jejunum in laying hens

Parameters	**Treatments**			
	Control*	**R1†**	**R2‡**	**R3§**
Villus height (µm)	729.04 ± 37.29	778.33 ± 60.66	856.29 ± 137.17	844.18 ± 108.12
Villus width (µm)	37.76 ± 3.25	39.39 ± 7.38	39.87 ± 10.80	40.83 ± 7.84
Crypt depth (µm)	92.14 ± 10.55	102.94 ± 19.35	90.83 ± 21.41	93.01 ± 7.88
Villus height/crypt depth ratio	8.61 ± 2.19	7.97 ± 1.55	10.23 ± 3.43	9.60 ± 1.72
Crypt area (µm^2)	2906.69 ± 682.90	3007.78 ± 260.20	2686.21 ± 933.84	2493.33 ± 290.20
Muscularis mucosae thickness (µm)	84.36 ± 16.72	85.19 ± 25.78	81.53 ± 16.16	76.53 ± 18.60

* Laying hens were fed a basal diet.
† Laying hens were fed a basal diet supplemented with red yeast at 0.5 g/kg feed.
‡ Laying hens were fed a basal diet supplemented with red yeast at 1.0 g/kg feed.
§ Laying hens were fed a basal diet supplemented with red yeast at 2.0 g/kg feed.

Table 4. Effect of dietary red yeast (*Sporidiobolus paraeoseus*) supplementation on the morphology of ileum in laying hens

Parameters	Treatments			
	Control*	R1†	R2‡	R3§
Villus height (µm)	557.41 ± 70.97	595.60 ± 47.11	613.43 ± 73.78	611.18 ± 64.75
Villus width (µm)	31.57 ± 8.08	36.64 ± 9.91	34.39 ± 6.04	40.15 ± 9.04
Crypt depth (µm)	100.63 ± 7.49	90.41 ± 19.16	101.33 ± 19.17	98.93 ± 13.06
Villus height/crypt depth ratio	5.70 ± 0.60	7.21 ± 2.34	6.30 ± 1.58	7.14± 2.00
Crypt area (μm^2)	2752.48±574.68	2602.92 ± 682.14	3086.75 ± 1063.67	2876.60 ± 446.15
Muscularis mucosae thickness (µm)	89.72 ± 23.51	91.14 ± 21.24	93.12 ± 10.20	89.78 ± 18.25

* Laying hens were fed a basal diet.
† Laying hens were fed a basal diet supplemented with red yeast at 0.5 g/kg feed.
‡ Laying hens were fed a basal diet supplemented with red yeast at 1.0 g/kg feed.
§ Laying hens were fed a basal diet supplemented with red yeast at 2.0 g/kg feed.

Conclusion: Dietary supplementation with red yeast (*Sporidiobolus paraeoseus*) at levels of 0.5, 1.0, and 2.0 g/kg feed increased duodenal villus height in laying hens, which might be associated with improved duodenal lumen health.

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