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## Performance and Histological Changes of the Intestinal Villi in Chickens Fed Dietary Natural Zeolite Including Plant Extract

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**Abstract:** To investigate the effects of dietary combinations of zeolite, plant extract and vermiculite (ZEM<sup>®</sup>) on growth performance and histological intestinal changes, male Sanuki Cochin chickens were allotted to 3 groups of 12 birds each. They were fed *ad libitum* on a basal commercial mash diet with 0 (control), 1 and 2 g kg<sup>-1</sup> dietary ZEM<sup>®</sup>. A starter diet (CP, 21%; ME, 3,000 kcal kg<sup>-1</sup>) was fed to chickens until 28 day of age, at which point a grower diet (CP, 18%; ME, 2,850 kcal kg<sup>-1</sup>) was fed until 70 day of age. Feed intake and body weight gain tended to increase with increasing levels of dietary ZEM<sup>®</sup> and feed efficiency tended to be highest in the 1 g kg<sup>-1</sup> ZEM<sup>®</sup> group. Most intestinal villus height, villus area, cell area and cell mitosis numbers were better than those of the control group and the duodenal cell areas and cell mitosis numbers of all intestinal parts were greater (p<0.05) in the ZEM<sup>®</sup> groups than in the control group. Protuberated cells were observed on the villus tip of the ZEM<sup>®</sup> groups. These histological changes indicate that the intestinal villi and epithelial cells were hypertrophied by ZEM<sup>®</sup>. The present results suggest that chicken growth performance would be improved due to hypertrophied intestinal villi and epithelial cells after feeding ZEM<sup>®</sup> and that ZEM<sup>®</sup> is effective alimentionation for chicken diets.

**Key words:** Chicken performance, natural zeolite, plant extract, histological intestinal changes

### INTRODUCTION

Zeolites and vermiculite are frequently considered as supplements that reduce odour emission and enhance growth due to hypertrophied functions of intestinal villi (Khambualai *et al.*, 2009). Zeolites are crystalline, hydrated aluminosilicates of alkali (e.g., Na<sup>+</sup>, K<sup>+</sup>) and alkaline (e.g., Mg<sup>2+</sup>, Ca<sup>2+</sup>) earth cations, having infinite three-dimensional structures (Ozturk *et al.*, 1998). Vermiculite is a clay mineral, magnesium alumino-silicate, which has a high cation-exchanging capacity (Evangelou and Lumbanraja, 2002). Zeolite has been shown to be a beneficial feed additive that exhibits a strong preference for binding nitrogenous cations like NH<sup>4+</sup> (Nakaue *et al.*, 1981). Dietary zeolite has been shown to reduce the toxicity of litter ammonia (Gupta *et al.*, 1997), as well as aflatoxins, which are contaminants in feed stuffs such as corn, wheat and soybeans (Miazzo *et al.*, 2000). Some researchers found that adding zeolites to broiler diets improved body weight gain, feed conversion ratio and tibia ash content (Zhang and Hung 1992; Debeic, 1994). In a earlier study,

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Khambualai *et al.* (2009) reported that dietary natural zeolite including plant extract (ZEM<sup>®</sup>) showed hypertrophied intestinal villus height, villus area, cell area, cell mitosis and absorptive epithelial cells in Aigamo ducks, resulting in improved body weight gain. However, data on the effects of ZEM<sup>®</sup> on the growth performance and histological changes of the intestinal villi in chickens are lacking.

Therefore, the aim of this study was to investigate the effects of dietary ZEM<sup>®</sup> on the performance and morphological changes of the duodenum, jejunum and ileum of male Sanuki Cochin chickens. Histologically, the intestinal villus height, villus area, cell area and cell mitosis numbers in each intestinal segment of these birds were observed using a light microscope. Additionally, epithelial cell changes were compared using a scanning electron microscope.

## MATERIALS AND METHODS

### Birds, Housing and Diets

One-day-old male Sanuki Cochin chickens were obtained from the Animal Science Farm in Kagawa prefecture. They were housed in an electrically heated brooder and given access *ad libitum* to water and diet. At 7 day of age, all birds were weighed and randomly allotted to 3 groups of 12 birds each. Chickens were maintained in individual cages in an environmentally controlled room under a photo-period of 14 h of light. From July 2007 to September 2007, experiment was conducted at the Faculty of Agriculture, Kagawa University.

The basal diet was supplemented with commercial ZEM<sup>®</sup> (ZEM Association, Kanagawa Prefecture, Japan), which is composed of zeolite (70%), enzymes extracted from plants such as pineapple and papaya (20%) and vermiculite (10%), at 0 (control group), 1 and 2 g kg<sup>-1</sup>. A commercial starter mash diet was fed to chickens up to 28 day of age and then changed to a grower mash diet until 70 day of age (Table 1). All birds were allowed *ad libitum* access to water and feed throughout the experimental period. Feed intake and body weight were measured weekly. All animal management and sampling procedures were in accordance with the Guidelines and Rules for Animal Experiments of Kagawa University, Japan.

Table 1: Feed ingredients and chemical compositions of the basal diet

Items	Starter	Grower
<b>Ingredients (%)</b>		
Maize	59.0	53.0
Milo	2.00	5.00
Soybean meal	27.0	20.0
Rapeseed meal	-	3.00
Gluten meal	2.00	13.0
Fish meal	7.00	3.00
Animal fat	1.10	0.80
Calcium carbonate	1.00	1.20
Dicalcium phosphate	0.30	0.40
Salt	0.20	0.20
Concentrate mixture <sup>1</sup>	0.40	0.40
<b>Chemical composition</b>		
Crude protein (%)	21.0	18.0
Crude fat (%)	3.00	3.00
Crude fiber (%)	6.00	6.00
Crude ash (%)	8.00	9.00
Calcium (%)	0.70	0.70
Available phosphorus (%)	0.50	0.50
Metabolizable energy (kcal kg <sup>-1</sup> )	3,000	2,850

<sup>1</sup>Concentrate mixture including (per kg of diet): vitamin A, 10,000 IU; vitamin D<sub>3</sub>, 2,000 IU; vitamin E, 15.63 mg; vitamin K, 2.6 mg; vitamin B<sub>1</sub>, 2.88 mg; vitamin B<sub>2</sub>, 3.7 mg; vitamin B<sub>6</sub>, 7.48 mg; vitamin B<sub>12</sub>, 0.01 mg; biotin, 0.14 mg; pantothenic acid, 16.12 mg; folic acid, 1.0 mg; niacin, 53.68 mg; choline, 1,400 mg; zinc, 75.00 mg; copper, 15.79 mg; manganese, 81.75 mg; iron, 170.76 mg; iodine, 0.35 mg; selenium, 0.15 mg

### **Small Intestine Sampling**

At the end of the experimental period, 4 chickens from each treatment were chosen and killed by decapitation under light diethyl ether anesthesia. After that, the entire intestine was removed and placed immediately into a mixture of 3% glutaraldehyde and 4% paraformaldehyde fixative solution in 0.1 M cacodylate buffer (pH 7.4). The midpoint of the duodenum, the midpoint between the entry of the bile duct and Meckel's diverticulum (jejunum) and the midpoint between Meckel's diverticulum and the ileo-caecal junction (ileum) were cut and prepared for light and scanning electron microscopy.

### **Specimen Preparation for Light Microscope**

The segment (2 to 3 cm in length) was washed with 0.1 M phosphate buffered saline (pH 7.4), fixed in Bouin's solution overnight and dehydrated in a graded ethanol series. Each segment was embedded in paraffin wax using standard techniques. All samples were performed on 4  $\mu$ m sections (8 sections/sample) and stained with haematoxylin and eosin and the resulting values were measured using an image analyzer (Nikon Cosmozone IzS, Nikon Co., Tokyo, Japan).

### **Measurement of Villus Height and Area**

Villi were selected from each section (2 villi per section) that demonstrated the longitudinal view of a villus from base to tip. The villus height was measured from the tip to the bottom excluding the crypt, while the villus width was measured at the basal and apical parts. The average villus height from 4 birds (16 villi from 8 different sections in each segment per bird) was expressed as a mean villus height for one group. The value of villus area was calculated from the villus height, basal width and apical width according to the method described by Iji *et al.* (2001). The average villus area from 4 birds (16 calculations of villus area from 8 different sections in each segment per bird) was expressed as a mean villus area for one group.

### **Measurement of Cell Area**

Villi were randomly selected from each section (2 villi per section). The villus area was measured at the middle part of the epithelial cell layer and then the cell nuclei within the cell layer were counted. Next, the area of the epithelial cell layer was divided by the number of cell nuclei. The average cell area from 4 birds (8 calculations of cell area from 4 different sections in each segment per bird) was expressed as a mean cell area for one treatment group.

### **Measurement of Cell Mitosis**

Mitoses were identified as darkly stained basophilic nuclei with haematoxylin. The number of mitoses within the entire crypt was counted from 4 different sections per bird. The average of these values was expressed as a mean cell mitosis number for each bird. These 4 mean cell mitosis numbers from 4 birds were expressed as a mean cell mitosis number for one group.

### **Scanning Electron Microscope**

The intestinal segment (length, 2 cm) was cut longitudinally, opened and washed with 0.1 M phosphate buffered saline (pH 7.4). Each segment was placed in a mixture of 3% glutaraldehyde and 4% paraformaldehyde in 0.1 mol L<sup>-1</sup> cacodylate buffer (pH 7.4) at room temperature for 1 h. Afterward, the segment was cut into 5×6 mm squares and fixed in the same solution for a further 1 h. The specimens were stored in 0.1 mol L<sup>-1</sup> sodium cacodylate

buffer (pH 7.4) and post-fixed with 1% osmium tetroxide in an ice-cold buffer for 2 h. Next, these specimens were rinsed with deionized distilled water and kept in 70% ethanol. After freeze-drying, the dried samples were mounted on aluminium stubs with electrically conducting carbon paste, coated (E-1030 ion sputter, Hitachi Ltd., Tokyo, Japan) and the epithelial cells on the villus tip surface observed under a scanning electron microscope (Hitachi S-4300SE/N, Hitachi Ltd., Tokyo, Japan) at 8 kV.

### Statistical Analysis

Data on growth performance and light microscopic examination were subjected to statistical analysis by one-way analysis of variance (ANOVA) of SPSS statistical software package (version 10.0 for windows, SPSS, Inc., Chicago, IL). The means of different treatments were compared with Duncan's multiple range tests. Significance was considered at  $p < 0.05$  level.

## RESULTS

### Growth Performance

Feed intake, body weight gain and feed efficiency did not show a significant difference among groups (Table 2). However, they tended to increase with increasing levels of dietary ZEM<sup>®</sup>, with feed efficiency highest in the 1 g kg<sup>-1</sup> ZEM<sup>®</sup> group.

### Light Microscopic Observations

The intestinal villus height and villus area of all segments did not vary among groups (Fig. 1A-D). The cell area and cell mitosis numbers of all intestinal parts tended to slightly increase with increasing levels of ZEM<sup>®</sup> and the cell area of the duodenum and cell mitosis numbers of the all intestinal parts were greater ( $p < 0.05$ ) in the ZEM<sup>®</sup> groups than in the control group.

### Morphological Changes of the Epithelial Cells

On the duodenal villus apical surface of the control, flat (small arrows) and protuberant cells (large arrows) were seen around the central sulcus (Fig. 2A). In the 1 g kg<sup>-1</sup> ZEM<sup>®</sup> group (Fig. 2B), in addition to protuberating cells (large arrows), some cells devoid of any microvilli (arrowheads) were found among intact cells that were totally covered with microvilli. In the 2 g kg<sup>-1</sup> ZEM<sup>®</sup> group (Fig. 2C), clearly protuberated cells (large arrows) and cell clusters (stars) composed of many epithelial cells were visible.

The jejunal villus apical surface of the control was covered by flat cell areas (small arrows) (Fig. 3A). In the experimental groups (Fig. 3B, C), such flat cells developed into faintly protuberated cells (large arrows). In the 1 g kg<sup>-1</sup> ZEM group, areas having cells with no microvilli and deeper cells at sites of recently exfoliated cells (arrowheads) were observed in the exfoliative zone of the villus tip.

Table 2: Effects of dietary natural zeolite including plant extract on feed intake, body weight gain and feed efficiency in chickens (n = 4, Mean±SE)

Items	ZEM levels (g kg <sup>-1</sup> )		
	0	1	2
Feed intake (g)	3571.36±42.65	3618.86±51.05	3750.63±37.18
Body weight gain (g)	1239.79±25.80	1320.31±21.57	1323.96±16.14
Feed efficiency	0.34710±0.0056	0.36480±0.0012	0.35300±0.0027

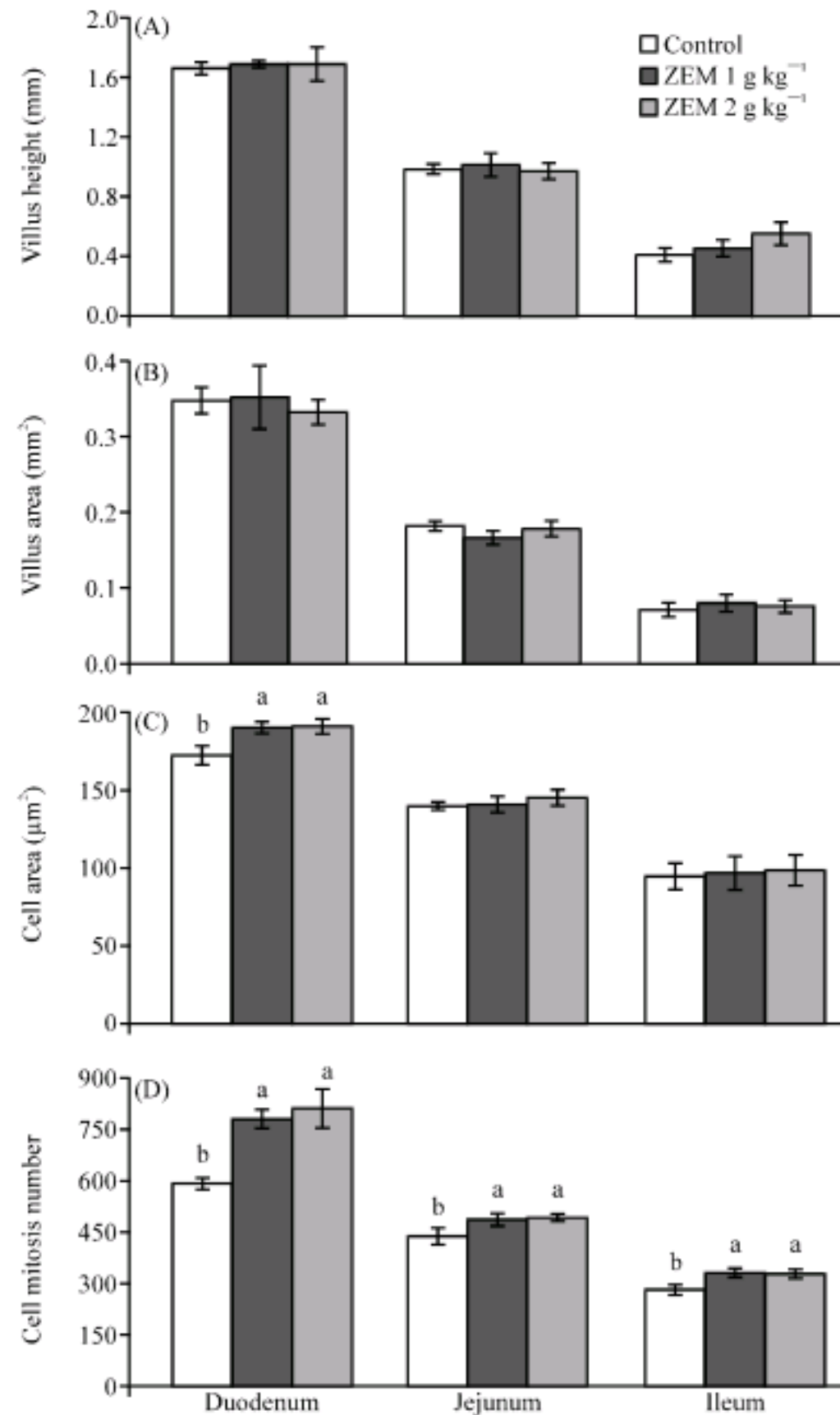


Fig. 1: (A-D) Intestinal villus height, villus area, cell area and cell mitosis number of duodenal, jejunal and ileal segments in chickens fed a basal commercial mash diet with 0 (control), 1 and 2 g kg<sup>-1</sup> dietary ZEM. (Mean±SE, n = 4). Mean with different letters significantly differ (p<0.05) from each other

On the ileal villus tip surface, faintly protuberating cells (small arrow) were observed in the control (Fig. 4A) and the ZEM groups (Fig. 4B, C). In addition, many intestinal sulci were observed.

## DISCUSSION

In the present study, feed intake, body weight gain and feed efficiency were greater with increasing levels of a mixture of zeolite and plant extract (protease). Total weight gain was improved in the 1 and 2 g kg<sup>-1</sup> ZEM<sup>®</sup> groups by approximately 6.49 and 6.79%, respectively,

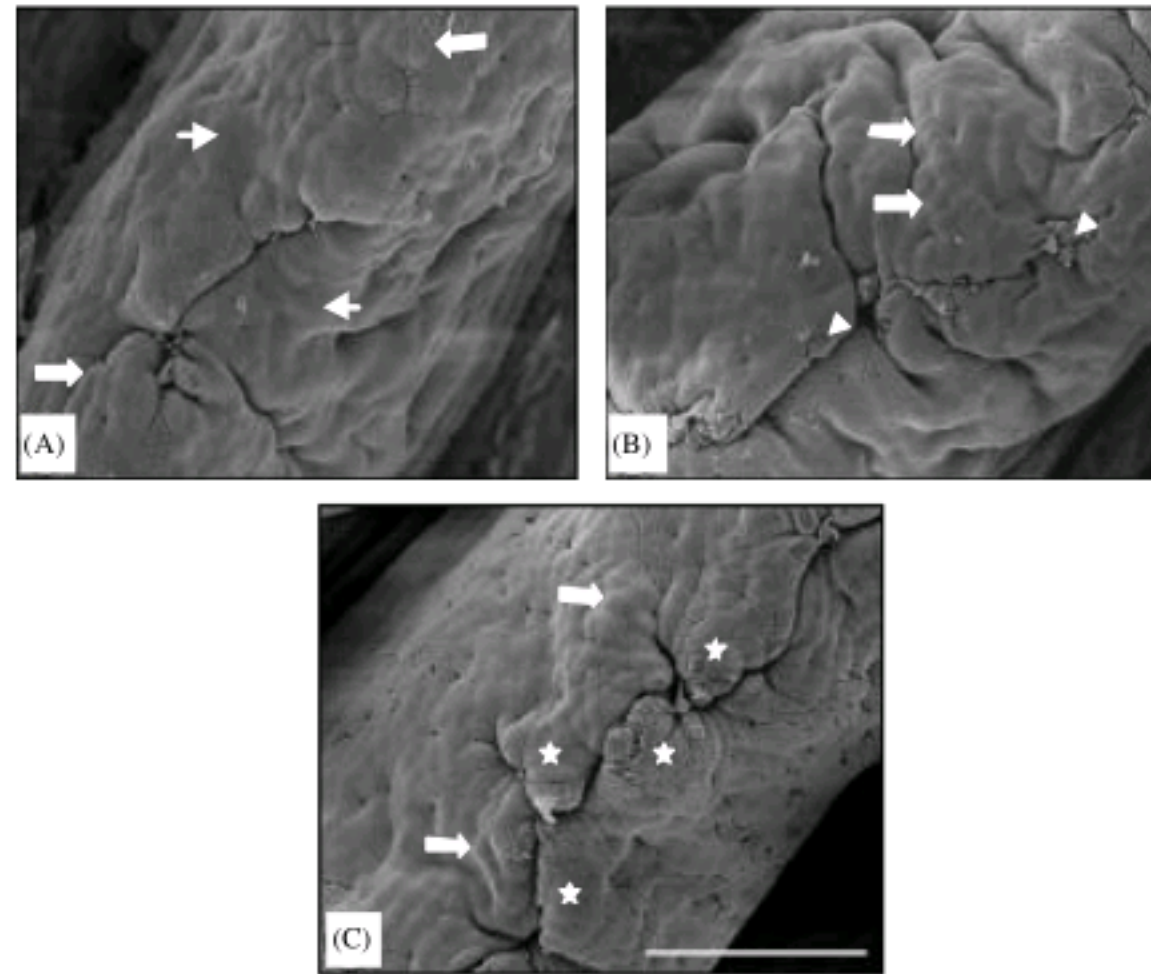


Fig. 2: Duodenal villus apical surface in chickens fed a basal commercial mash diet with 0 (A; small arrow, flat cell; large arrows, protuberant cell), 1 (B; large arrows, protuberant cell; arrowheads, exfoliated cells) and 2 g kg<sup>-1</sup> dietary ZEM (C; large arrows, protuberant cell, star, cell cluster). Scale bar = 50  $\mu$ m (x600)

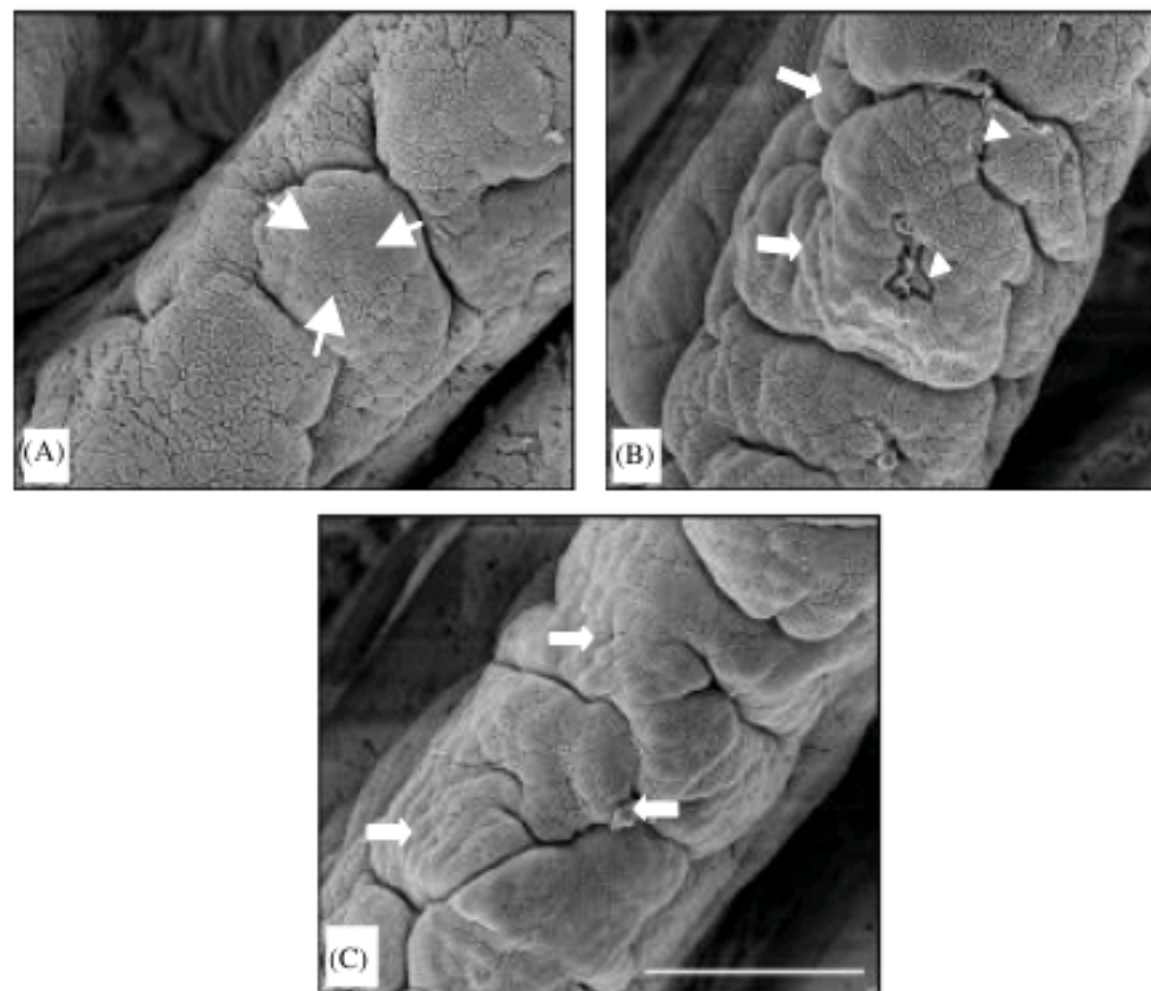


Fig. 3: Jejunal villus apical surface in chickens fed a basal commercial mash diet with 0 (A; small arrow, flat cell area), 1 (B; large arrows, protuberant cell; arrowheads, exfoliated cells) and 2 g kg<sup>-1</sup> dietary ZEM (C; large arrows, protuberant cell). Scale bar = 50  $\mu$ m (x600)

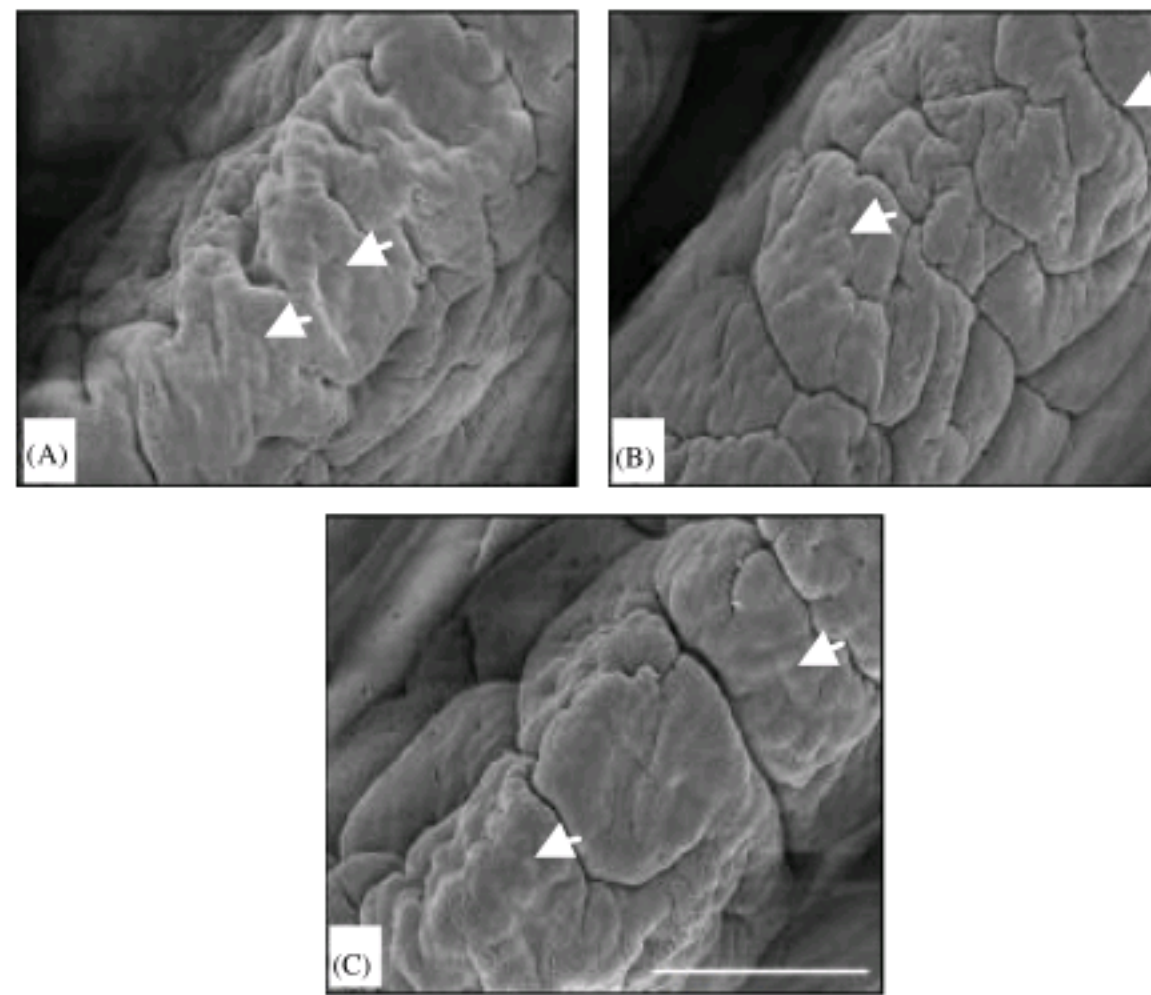


Fig. 4: Ileal villus apical surface in chickens fed a basal commercial mash diet with 0 (A; small arrow, flat protuberating cell), 1 (B; small arrow, flat protuberating cell) and 2 g kg<sup>-1</sup> dietary ZEM (C; small arrow, flat protuberating cell). Scale bar = 50 μm (x600)

as compared to the control. Dietary zeolite improved body weight gain, feed conversion ratio and tibia ash content (Zhang and Hung, 1992; Debeic, 1994) and feed efficiency (Elliot and Edwards Jr., 1991). Also, dietary natural zeolite improved weight gain and feed efficiency in cattle (Koknaroglu *et al.*, 2006). The addition of natural zeolite at 25 g kg<sup>-1</sup> to the aflatoxin-containing diet also provided a slight improvement in body weight gain. In addition, many enzymes, such as protease, induced body weight gain and feed conversion (Cowieson and Adeola, 2005), due to improvements in total protein digestibility (Zanella *et al.*, 1999). These reports suggest that the present slightly better growth performance might be the result of zeolite and plant extract. This is in agreement with the findings that dietary ZEM<sup>®</sup> (0.1, 0.5 and 1.0 g kg<sup>-1</sup>) improved weight gain in Aigamo ducks (Khambualai *et al.*, 2009).

In these ZEM<sup>®</sup> groups, most light microscopic parameters were higher than those of the controls and the duodenal cell area and the cell mitosis numbers in all intestinal parts were increased. Most of the digestive and absorptive processes of ingested feeds occur in the intestine. It has been reported that intestinal villus and epithelial cell morphology is associated with intestine function and growth rate (Ruttanavut *et al.*, 2009). Large-sized villi have been associated with activated cell proliferation (Lauronen *et al.*, 1998). Narrow and long villi indicate a faster multiplication of the base of the crypt which migrated faster to the tip of villi and that the turnover of the epithelial cells would, therefore, be shorter (Nordstrom and Dahlqvist, 1973). Greater villus height and numerous mitotic cells in the intestine are indicators that the function of the intestinal villi is activated (Langhout *et al.*, 1999; Yasar and Forbes, 1999). Furthermore, the present epithelial cells on the villus apical surface were protuberated in the duodenum and jejunum of the ZEM<sup>®</sup> groups. Areas having cells with no microvilli and deeper cells at sites of recently exfoliated cells were also observed on the duodenal and jejunal villus surface in the 1 g kg<sup>-1</sup> ZEM group. Absorptive epithelial cells originate at the base of the crypts as immature proliferative cells, differentiate as they



migrate up the villus tip and are finally extruded from the villous tips (Cheng and Leblond, 1974). The cell cycle of intestinal absorptive cells from origin to shedding at the villus tip is 2 to 3 days (Leblond, 1981). Thus, that the epithelial cells seem to show dramatic morphological changes at the exfoliative zone might be due to the villus function. Numerous epithelial cells were observed on the villus apical surface in chickens with increased body weight (Yamauchi *et al.*, 2006) and cell protuberance into the intestinal lumen has been demonstrated to show hypertrophied cells (Shamoto and Yamauchi, 2000; Tarachai and Yamauchi, 2000). These histological reports suggest that the present intestinal villi and epithelial cells can be considered hypertrophied.

Wood charcoal could induce the hypertrophied villi and epithelial cells due to its many pores adsorption in chickens (Ruttanavut *et al.*, 2009) and pigs (Mekbungwan *et al.*, 2008). Zeolite also has many framework structures (Coombs *et al.*, 1997) and is able to absorb water and exchange nitrogen molecules (Mumpton, 1999). Dietary zeolite reduced the toxicity of ammonia in the litter (Gupta *et al.*, 1997) and aflatoxins in corn, wheat and soybeans (Miazzo *et al.*, 2000). Exogenous enzymes, such as carbohydrases, proteases and phytases, have been used in poultry to improve growth performance (Bi and Chung, 2004; Kidd *et al.*, 2001) and nutrient digestibility (Cowieson and Adeola, 2005) and to reduce the excretion of phosphorus (Maguire *et al.*, 2004). These functions of zeolite and enzymes might induce hypertrophied intestinal villi and epithelial cells.

In conclusion, as ZEM could improve the growth performance of chickens due to the hypertrophy of the intestinal villi and epithelial cells, ZEM is an effective alimentation for chicken diets.

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