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A fluorescence-based demonstration of intestinal villi and epithelial cell in chickens fed dietary silicic acid powder including bamboo vinegar compound liquid

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Summary. This study investigates the combined effect of silicic acid and bamboo vinegar compound liquid (SPV) on the growth and intestinal histological alterations in poultry. Forty-eight 7-day-old male Sanuki Cochin chickens were fed a commercial mash diet supplemented with SPV at 0, 0.1, 0.2, and 0.3% level *ad libitum* for 112 days. Body weight gain tended to improve with increased concentrations of dietary SPV, although these results were not statistically significant ($P<0.1$). Tissue observation by light microscopy revealed that the jejunal villus height ($P<0.01$) and duodenal and jejunal villus area ($P<0.05$) increased in the 0.2 and 0.3% SPV groups, respectively, compared with the control. Cell mitosis within the duodenum and jejunum also increased in the 0.2 and 0.3% SPV groups. Scanning electron microscopy revealed a prominent increase in the number of protuberant cells on the villus apical surface of the duodenum and jejunum for the 0.2 and 0.3% SPV groups compared with the control. Poultry in the 0.3% SPV group had the highest body weight gain and hypertrophied histological alterations of intestinal villi. Fluorescent microscopic images of cell mitosis and protuberant cells in the duodenal crypt clearly confirmed positive reactions for the activator protein 2α (AP-2α) and proliferating cell nuclear antigen (PCNA), compared with the control. The present results indicate that dietary SPV stimulates adsorption by the epithelial cells, which activate cell proliferation and self-renewal and regulate the expression of cell cycle regulators AP-2α and PCNA, resulting in higher body weight gain. Thus, we can conclude that a concentration of 0.3% dietary SPV is ideal for promoting growth in poultry.

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Introduction

Sanuki Cochin poultry meat is one of the most commonly consumed protein source in Japan. It has high nutritional value and is reasonably priced. However, with bans on the use of antibiotics as growth promoters in animal feed and increase in food safety concerns, it is necessary to find alternatives to antibiotics. Acidifiers are an alternative to antibiotics in poultry for promoting growth and improving intestinal function and feed efficiency. They are added to feed or water to lower the pH of the feed/water, gut, and microbial cytoplasm (Griggs and Jacob, 2005). An important objective of dietary acidification is to inhibit intestinal bacteria that compete with the host for available nutrients, thereby improving weight gain of the host animal (Samik et al., 2007). Furthermore, inhibition of the growth of pathogenic and zoonotic bacteria is beneficial with respect to animal health. Bamboo vinegar compound liquid is an acidic by-product of bamboo charcoal production containing organic acids, phenols, ketones, and aldehydes (Akakabe et al., 2006). Acetic acid comprises about 80% of the total organic compounds found in bamboo vinegar. The acid in wood vinegar enhances the growth of *Bifidobacterium* and *Enterococcus* but inhibits the growth of *Salmonella* species (Watarai and Tana, 2005). Bamboo vinegar also acts as an insecticide, bactericide, deodorant for treating pet malodor, and as a component of folk medicine (Akakabe et al., 2006).

Alternatively, silicic acid, a weak acid found in plants and animals, is known to be the active form of silicon. Silicon (50-500 ppm) is essential for growth and skeletal development in chicks (McDowell, 1992). In

addition, it is required to increase the activity of prolyl hydroxylase and is a measure of the rate of collagen biosynthesis (Carlisle et al., 1981).

A mixture of silicic acid and bamboo vinegar compound liquid (SPV) has been recently produced in Japan as additives to animal feed. However, no literatures concerning the effects of SPV on growth performance are found in animals. The histology of intestinal villi and epithelial cells located on the villus apical surface is affected by dietary feed components. Hypertrophied intestinal histological alterations, such as increased villus height and area, cell mitosis, and protuberated epithelial cells, have been reported in conjunction with feed additives (Yamauchi, 2007). These alterations seem to be induced by increased cell count due to activated cell mitosis in the intestinal crypt. The AP-2 family of transcription factors modulates apoptosis, cell cycle regulation, and tissue differentiation and development. This suggests that AP-2 proteins have been competing with transcriptional co-activators and cell cycle stabilizers, which are evolutionarily conserved roles for several tissues in poultry and mammals (Eckert et al., 2005; Stabach et al., 2006).

The PCNA gene found in poultry also has 94% homology with humans. In the case of human gastrointestinal lymphoma, PCNA expression in the cell population has been shown to enhance survival rates of cells in the S, G_2, and M cell cycle phases (Woods et al., 1991). However, no immunohistochemical correlation has been observed between AP-2 and PCNA expression on the intestinal crypt cell cycle. In this study, the effects of SPV treatment on growth performance and histological intestinal alterations were observed using light, fluorescence, and scanning electron microscopy.

Materials and methods

Animals and diets

All experiments were performed according to the humane care guidelines for the use of animals for experimentation as provided by Kagawa University, Japan (Kagawa University, 2006). In a preliminary experiment, we observed that Sanuki Cochin chickens fed 0.3% dietary SPV showed the most improved growth performance among 0.3, 0.4 and 0.5% dietary SPV groups (unpublished observation). Therefore, in this study, 0.1, 0.2 and 0.3 % dietary SPV groups were designed. Male Sanuki Cochin chickens were obtained from the animal science farm of Kagawa prefecture at 1 d of age. The chicks were housed in electrically heated brooder cages under continuous light for one week, and provided with *ad libitum* access to water and conventional starter mash diet. At 7 d of age, the birds were weighed individually; 5% of the heaviest and lightest were discarded, and the remaining birds were randomly divided into 4 groups with 4 replicates of 3 birds on the basis of similar body weight. The birds were housed in an environmentally controlled room under a photoperiod of 14 h of light.

The basal diets (Table 1) were supplemented with SPV at 0, 0.1, 0.2 and 0.3%. Commercial SPV (pH 4) was produced by the company (Shikoku Tekuno Co., Ltd, Kagawa, Japan) as follows: bamboo vinegar compound liquid (Table 2), obtained after cooling smoke during the making of bamboo charcoal from moso bamboo (*Phyllostachys pubescens*) by dry distillation at 700°C in an airless condition, was kept for one year. Then, the skimmed solution was distilled to remove harmful substances such as tar. This bamboo vinegar compound of 3 L was adsorbed into commercial silicic acid powder (particle size, 50 mesh) of 5 kg. The birds were fed a starter diet until 28 d of age followed by a

Table 1. Composition of the experimental diets (%).

Ingredients	Starter 1 to 28 d	Grower 29 to 70 d	Finisher 71 to 112 d
Maize	59.0	53.0	63.0
Milo	2.0	5.0	2.0
Soybean meal	27.0	20.0	15.0
Rapeseed meal	-	3.0	4.0
Gluten meal	2.0	13.0	6.0
Fish meal	7.0	3.0	1.0
Rice bran	-	-	6.0
Animal fat	1.1	0.8	0.5
Calcium carbonate	1.0	1.2	1.5
Dicalcium phosphate	0.3	0.4	0.4
Salt	0.2	0.2	0.2
Vitamin/mineral premix*	0.4	0.4	0.4
Calculated composition			
Crude protein	21.0	18.0	15.0
Metabolisable energy (kcal/kg)	3000	2850	2800
Crude fat	3.0	3.0	2.5
Crude fiber	6.0	6.0	8.0
Crude ash	8.0	9.0	9.0
Calcium	0.7	0.7	0.55
Available phosphorus	0.5	0.5	0.45

*Vitamin and mineral premix included per kg of diet: retinyl acetate, 2106 μg; cholecalciferol, 35 μg; DL-α-tocopherol acetate, 12.5 mg; menadione, 1.5 mg; thiamine, 2.6 mg; riboflavin, 2.7 mg; pyridoxine, 6 mg; cobalamine, 9 μg; biotin, 0.2 mg; folic acid, 0.5 mg; pantothenic acid, 15 mg; niacin, 22 mg; choline, 1000 mg; iodine, 1.05 mg; manganese, 50 mg; iron, 160 mg; zinc, 70 mg; copper, 8 mg.

Table 2. Chemical properties of bamboo vinegar compound liquid.

Items	Composition (%)
Total organic content	11.37
Acetic acid	2.87
Methanol	0.07
Formaldehyde	0.003
Phenol	0.177
Cresol	0.043
Tar	0.73
pH	3.25

grower diet from 29 to 70 d old and a finisher diet from 71 to 112 d old. Feed and water were provided *ad libitum*. Feed intake and body weight were measured every week.

Tissue sampling and preparation

At the end of the feeding period, 4 birds with the similar average body weight were selected from each treatment and killed by decapitation. In these birds, the entire small intestine was excised and fixed in a mixture of 3% glutaraldehyde and 4% paraformaldehyde fixative solution (pH 7.4). The same fixative solution was injected into the intestinal lumen of the middle part of each intestinal segment. The intestinal segment from the gizzard to pancreatic and bile ducts were regarded as duodenum, jejunum from the duct to Meckel's diverticulum, and ileum from the diverticulum to the ileo-cecal-colonic junction. The tissue samples were taken from the middle part of each intestinal segment. A 2 cm length of each intestinal segment was excised for scanning electron microscopic observations, and placed in the same fixative solution described above. Another 2 cm length of each intestinal segment was cut for light microscopic observations and kept in Bouin's solution. Light microscopic samples were taken immediately proximal to the block collected for scanning electron microscopy.

Light microscopic examination

After dehydration in graded alcohol, each intestinal segment was embedded in paraplast. Transverse sections were cut into 4 μm sections, and every 10th section was collected. After staining with haematoxylin-eosin, the following values were measured using an image analyzer (Nikon Labophot-2, Tokyo, Japan).

Measurement of villus height

The highest 2 villi having the lamina propria were selected per transverse section and length was measured from the tip to the bottom. Such a measurement was done using eight different sections (16 villi from eight sections) per one bird. The mean values from four birds was expressed as the mean villus height for that treatment group.

Measurement of villus area

The width of villus was measured at the basal and apical parts and two villi were selected from each section. Villus area was calculated from the villus height, basal width and apical width. A total of 16 calculations of the villus area were made for each bird. The average of these was expressed as the mean for each bird. Finally, the 4 bird means were expressed as a mean villus area for one group.

Measurement of epithelial cell area

The area of the epithelial cell layer was randomly measured at the middle part of the villus and then the cell nuclei within this measured epithelial cell layer were counted. Finally, the area of the layer was divided by the number of cell nuclei to obtain an epithelial cell area. A total of 16 samples per bird were counted in each group.

Measurement of cell mitosis in the crypt

Mitotic cells with homogenous, intensely stained basophilic nuclei with haematoxylin were counted as cell mitosis numbers as previously described (Tarachai and Yamauchi, 2000). In the case of cells in late stages of division, cell mitosis number was counted as one mitotic event. All instances of cell mitosis observed in each transverse section were counted for the four transverse sections obtained from each bird. Values from these sections were used to calculate the mean cell mitosis for each bird. Finally, mean cell mitosis from four randomly selected birds from each treatment group was expressed as the mean cell mitosis for that treatment group.

Immunofluorescence microscopy

The jejunum and ileum were selected to investigate the reaction activity of cell proliferation in the duodenal crypt. The 0.3% SPV treatment group demonstrated the highest body weight gain with highest values of all light microscopic parameters and clearer protuberant cells. Preliminary testing using real-time reverse transcription-polymerase chain reaction (RT-PCR) revealed that AP-2α mRNA expression levels in the duodenal tissue differed between the control and 0.3% SPV groups. However, AP-2α mRNA expression levels in the jejunum and ileum did not differ from the control. For this reason, the duodenal segments were used to identify the reaction activity of cell proliferation in this study. The duodenal sections (4 μm) from the control and 0.3% SPV groups (4 birds in each group) were used for immuno-fluorescence staining. After deparafinization, the tissue samples on glass slides were rehydrated, with treated heat-induced epitope retrieval with sodium citrate buffer using the microwave for 8 min, blocked in 1% bovine serum albumin in phosphate buffered saline (PBS) for 2 h, and incubated with primary antibodies overnight in a moist chamber; anti-monoclonal proliferating cell nuclear antigen (PCNA) (M0879, Dako Cytomation, Denmark) and anti-rabbit activator protein 2α (AP-2α) (SC-184, Santa Cruz Biotechnology, CA) were used for S-phase or G1-phase marker in the cell cycle, respectively. Sections were then washed three times with PBS followed by incubation for 1.5 h with Alexa red- or green-conjugated secondary antibodies (Alexa-594 labeled anti-mouse IgG and Alexa-488 labeled anti-rabbit IgG; Invitrogen, Oregon, USA) were

used at a 1:2000 dilution. Samples were washed with PBS three times, embedded in fluorescent mounting medium vectashield (Vector Laboratories, Burlingame, CA) and examined with the epi-illumination fluorescence microscope (DP-82, Olympus, Tokyo, Japan) for each fluorescent dye. All images were processed using NIS-elements D software (Nikon, Tokyo, Japan).

Scanning electron microscopy

A 2-cm tissue sample of each intestinal segment was transversely cut, slit longitudinally, opened and washed with 0.1 M phosphate buffered saline (pH 7.4). To prevent curling, the edges of tissue sample were pinned flat to the paraffin-covered bottom of a Petri dish containing a mixture of 3% glutaraldehyde and 4% paraformaldehyde fixative solution (pH 7.4) at room temperature for 2 h. Then, samples were cut into 4x7-mm^2 squares, washed with a 0.1 M cacodylate buffer and postfixed with 1% osmium tetroxide for 2 h. The specimens were washed in distilled deionised water and dehydrated in ethanol of increasing concentration (45, 70, 80, 90 and 100%). These specimens were then freeze-dried in a critical point drying apparatus (Hitachi Freeze Dryer, Hitachi Ltd, Tokyo, Japan), sputter coated with platinum (Hitachi E-1030 Ion Sputter, Hitachi Ltd) and observed with a scanning electron microscope (Hitachi S-4300SE/N, Hitachi Ltd). Morphological alterations of the epithelial cells on the villus tip surface were compared between groups.

Statistical analysis

All data were analyzed as a one-way ANOVA using the general linear models procedure of SAS software (SAS Institute Inc., Cary, NC, USA) and the results were expressed as mean and pooled standard error of mean. Separate analysis was performed employing orthogonal polynomial contrast to determine the effects of graded levels of dietary SPV. A probability less than 0.05 was considered significant and less than 0.1 was considered

Table 3. Effects of dietary silicic acid powder including bamboo vinegar compound liquid (SPV) on chicken performance.

Items	Dietary SPV (%)				SEM	P-value	
	0	0.1	0.2	0.3		Linear	Quadratic
Feed intake (kg/b)	9.731	9.824	9.946	10.051	0.077	0.15	0.97
Initial body weight (kg/b)	0.075	0.075	0.076	0.075	0.001	0.90	0.99
Final body weight (kg/b)	2.491	2.539	2.562	2.620	0.022	0.06	0.91
Body weight gain (kg/b)	2.416	2.464	2.486	2.545	0.023	0.06	0.91
Feed efficiency	0.248	0.251	0.250	0.253	0.001	0.38	0.89

Data were means of 4 replicates.

Table 4. Villus height, villus area, cell area and cell mitosis number of the duodenum, jejunum and ileum in chickens fed 0, 0.1, 0.2 and 0.3% dietary silicic acid powder including bamboo vinegar compound liquid (SPV) diets.

Items	Dietary SPV (%)				SEM	P-value	
	0	0.1	0.2	0.3		Linear	Quadratic
Villus height (mm)							
Duodenum	1.30	1.29	1.31	1.34	0.02	0.34	0.66
Jejunum	1.01	1.01	1.07	1.16	0.02	<0.01	0.22
Ileum	0.62	0.64	0.65	0.66	0.02	0.39	0.75
Villus area (mm^2)							
Duodenum	0.143	0.141	0.148	0.150	0.001	0.03	0.42
Jejunum	0.108	0.107	0.120	0.125	0.002	0.02	0.47
Ileum	0.064	0.065	0.066	0.068	0.001	0.12	0.67
Cell area (μm^2)							
Duodenum	256.54	257.11	260.59	262.14	1.81	0.25	0.89
Jejunum	215.88	215.86	216.16	217.32	1.73	0.53	0.71
Ileum	164.56	165.19	166.57	169.23	0.87	0.11	0.55
Cell mitosis (number)							
Duodenum	805.63	797.25	885.88	897.88	15.53	<0.01	0.93
Jejunum	647.88	641.75	685.50	720.63	12.69	0.03	0.62
Ileum	420.12	421.62	428.37	430.87	3.89	0.16	0.93

Data were means of 4 replicates.

trend.

Results

Animal performance

Compared to the control, feed intake, body weight gain and feed efficiency were not significantly different in the dietary SPV groups (Table 3). Body weight gain tended to increase with increasing levels of SPV ($P<0.1$).

Light microscopic parameters

Villus height and area, epithelial cell area, and cell mitosis numbers of the control and experimental groups are shown in Table 4. Jejunal villus height ($P<0.01$) and duodenal and jejunal villus area ($P<0.05$) were higher in poultry fed the 0.2 and 0.3% SPV diets compared with the control. Epithelial cell areas did not differ among the treatment groups. Duodenal and jejunal cell mitosis increased in poultry fed the 0.2 and 0.3% SPV diets ($P<0.01$ and $P<0.05$, respectively) compared with the control.

Expression patterns of the AP-2α and PCNA protein in the duodenal crypt

Increased levels of activated AP-2α- and PCNA-positive cells were observed in the duodenal crypt of poultry fed with the 0.3% SPV diet compared with the

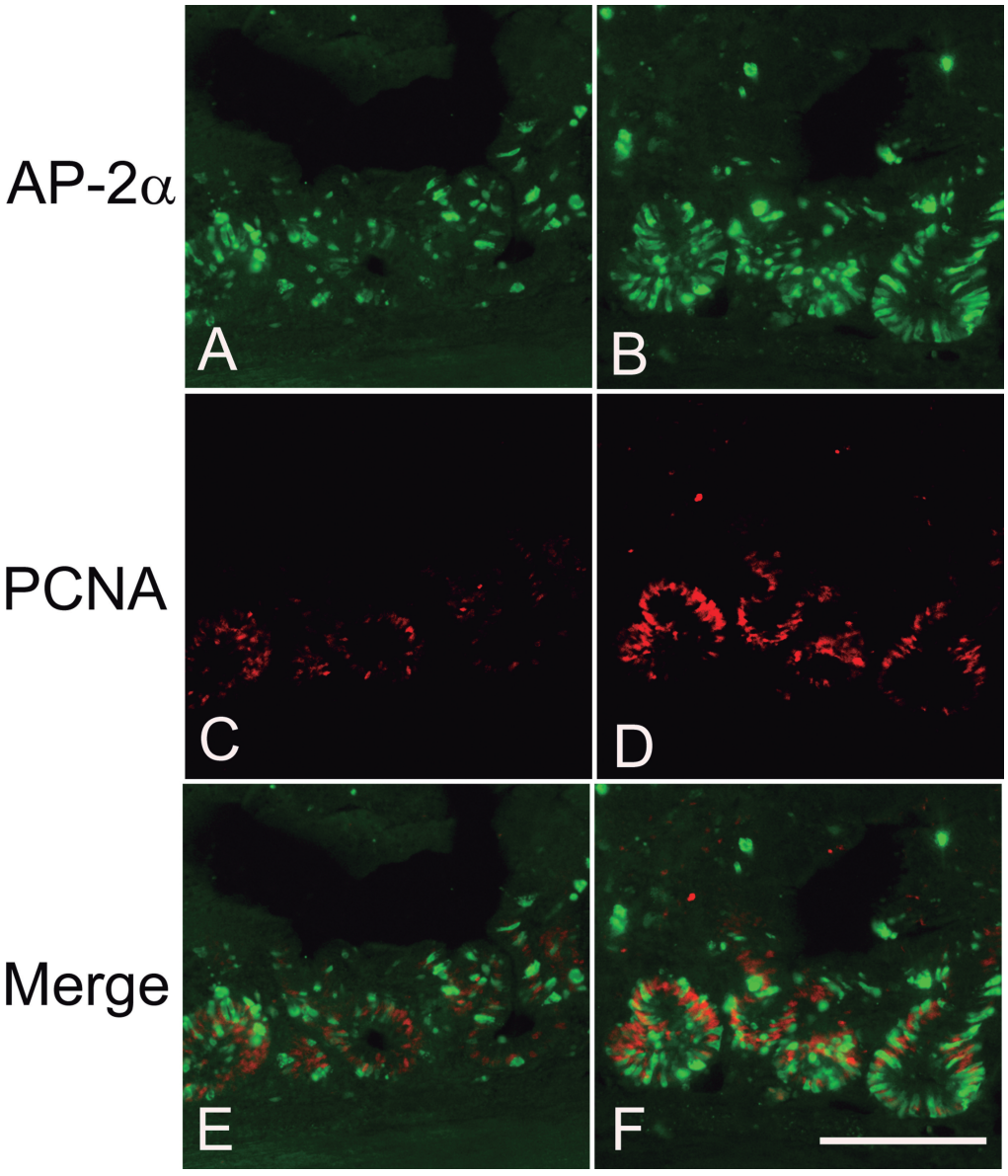

Fig. 1. Examples of mitotic cells in the duodenal crypt in chickens fed the control **(A, C, E)** and 0.3% dietary SPV **(B, D, F)** diets. **(A, B)** Nuclei with green fluorescence are AP-2α-positive (G_1-phase arrest). **(C, D)** Nuclei exhibiting red fluorescene are PCNA-positive (S-phase). **(E, F)** Double staining with anti-AP-2α (Alexa-green) and anti-PCNA (Alexa-red) antibodies are merged into a same panel. The immunofrorecence signals of AP-2α and PCNA are increased in the 0.3% SPV group than the control. Scale bar: 200 μm, x 200

control (Fig. 1A-F). AP-2α-positive cells were highly concentrated in the basal region of the crypt cells in the 0.3% SPV group (Fig. 1B). The lumen regions of the duodenal crypt had increased PCNA-positive cells in poultry fed the 0.3% SPV diet (Fig. 1D) compared with the control (Fig. 1C). Merged fluorescent images indicated that AP-2α- and PCNA-positive cells were differently located in the 0.3% SPV diet (Fig. 1F) group compared with the control (Fig. 1E).

Scanning electron microscopic observations of epithelial cells on the villus tip

The duodenal villus apical surface of the control and 0.1% SPV groups (Fig. 2A and B) had slightly protuberant cells. In the 0.2 and 0.3% SPV groups (Fig. 2C and D), protuberant cells were clearly observed around the central sulcus. Additionally, areas having cells with no microvilli were observed in the 0.3% SPV group.

The jejunal villus apical surfaces of the control and 0.1% SPV groups (Fig. 3A,B) were covered with flat cells, showing a smooth surface. However, in the 0.2 and 0.3% SPV groups (Fig. 3C,D), faintly protuberant cells were present.

The ileal villus apical surface of the control and the experimental groups showed a similar morphology (pictures not shown).

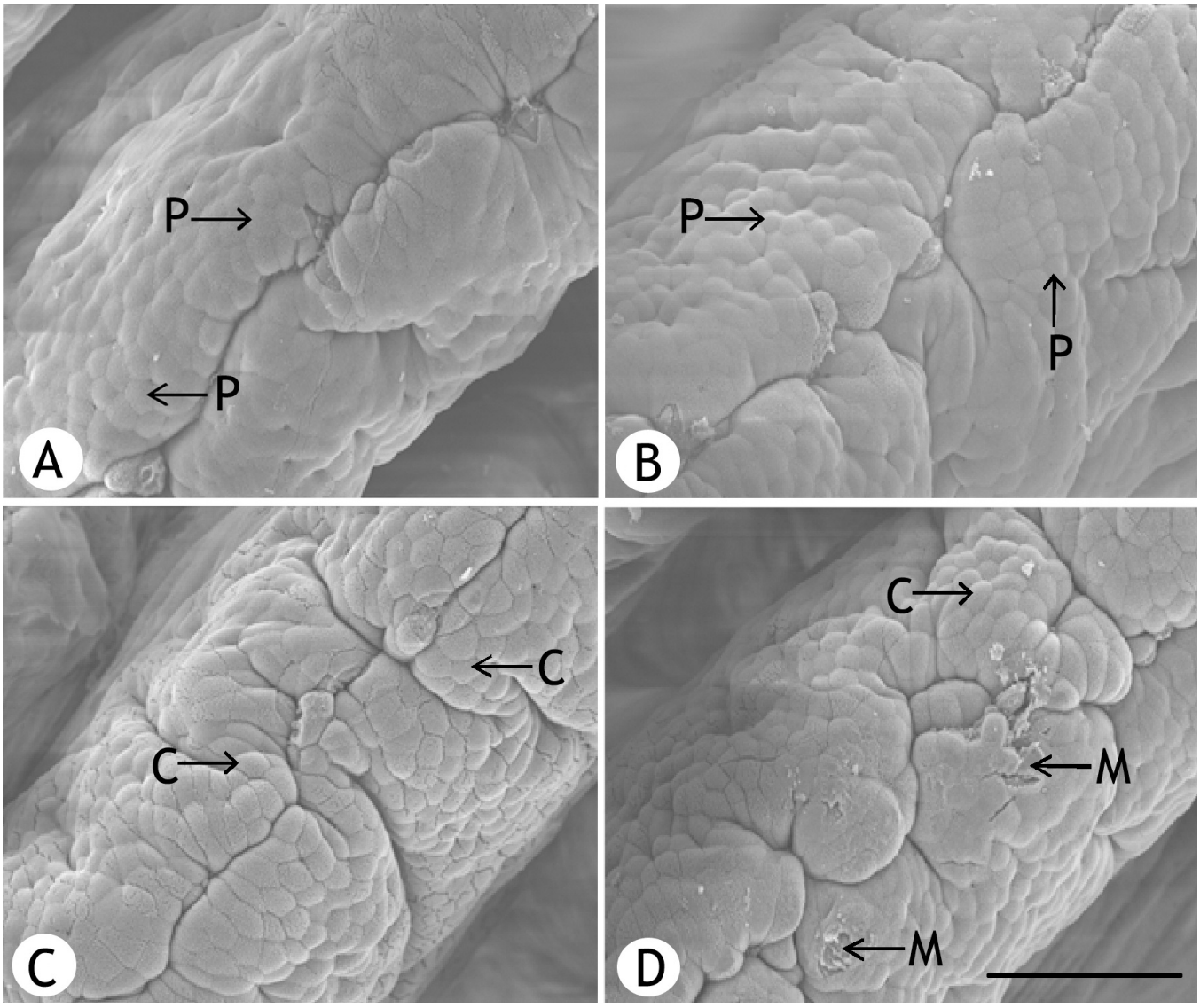

Fig. 2. Duodenal villus apical surface in chickens fed on the control (**A**; arrows with P, protuberant cells), 0.1% (**B**; arrows with P, protuberant cells), 0.2% (**C**; arrows with C, clearly protuberant cells) and 0.3% (**D**; arrow with C, clearly protuberant cells; arrows with M, areas having cells with no microvilli) SPV diets. In the 0.2 and 0.3% SPV groups show clearly protuberant cells, indicating a higher absorptive cell in these groups. Scale bar: 50 μm, x 1000.

Discussion

Many reports indicate that addition of acidifiers to swine diet has a positive influence on growth and performance (Gabert and Sauer, 1994; Roth and Kirchgessner, 1998; Partanen and Mroz, 1999). However, use of acidifiers as a feed additive for poultry has been questioned. One reason for this could be that the results regarding body weight gain and feed efficiency following addition of dietary acidifiers are not as convincing as they are for pigs. The main purpose of this study was to investigate the impact of dietary SPV on the growth and intestinal histological alterations in poultry from 7-112 days of age and to confirm the activation of G1 and S cell cycle phases in the 0.3% SPV group. This study demonstrated a slight increase in body weight gain of the SPV-fed poultry. The seemingly minimal weight gain may be due to the lightweight poultry strain (Sanuki Cochin) used. All treatment groups were numerically higher in body weight gain than the control; the body weight gain of the 0.3% SPV group was 5.3% more than that of the control group. Present results indicate that SPV tends to improve the body weight of lightweight poultry. The nutritional composition of the diets in each group was almost identical; the body weight gain of birds fed SPV can be

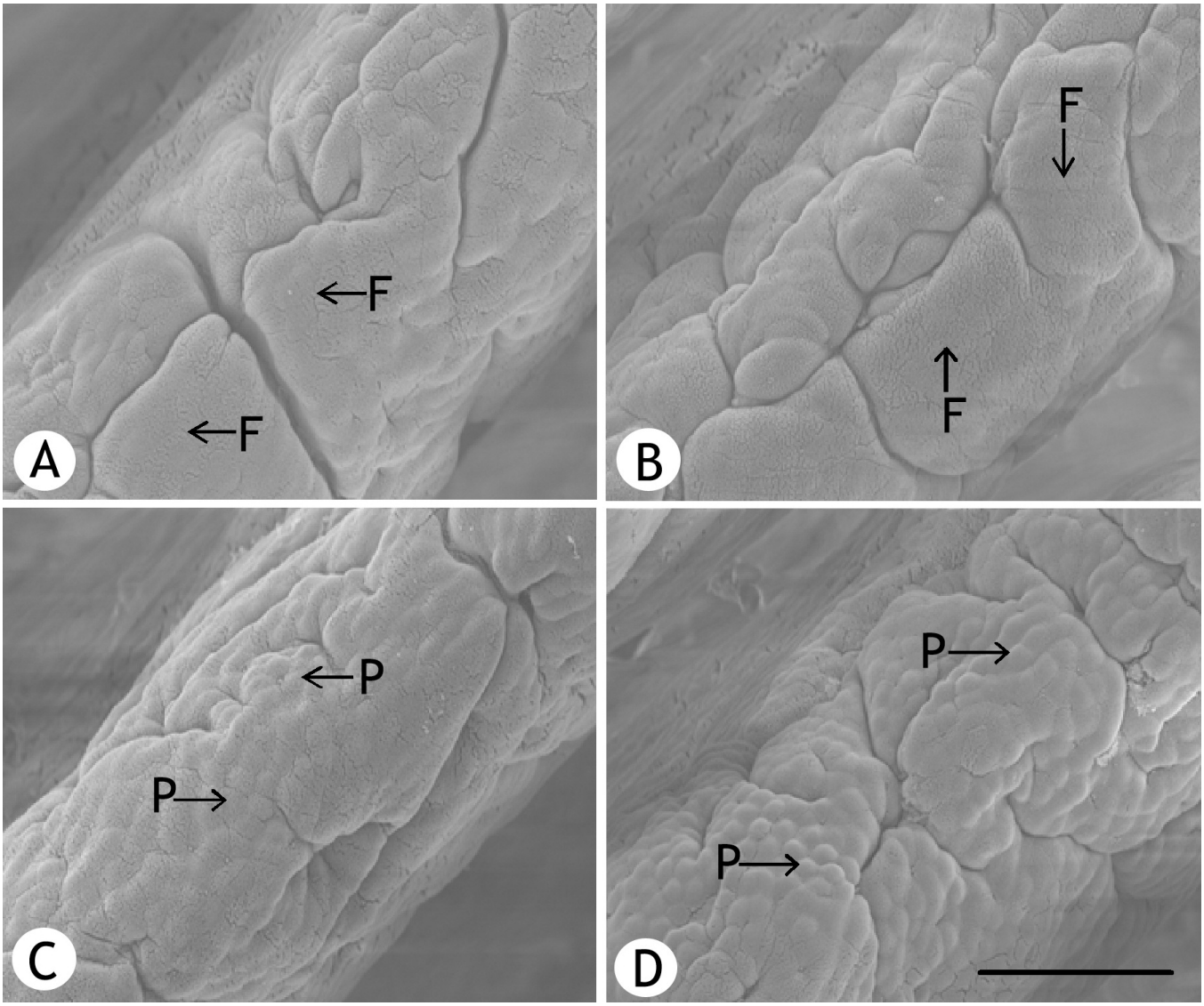

Fig. 3. Jejunal villus apical surface in chickens fed on the control (**A**; arrows with F, flat cells), 0.1% (**B**; arrows with F, flat cells), 0.2% (**C**; arrows with P, protuberant cells) and 0.3% (**D**; arrows with P, protuberant cells) SPV diets. SPV stimulates the function of the epithelial cells in the jejunum especially at 0.2 and 0.3%. Scale bar: 50 μm, x 1000

attributed to the beneficial effects of SPV in promoting intestinal function, as will be described later.

Charcoal powder is generally used with vinegar compound liquid as an additive to animal feed. However, silicic acid powder has been known to adsorb vinegar more strongly than charcoal. Charcoal powder with vinegar is commercially supplemented with 1% of the feed, but silicic acid powder with vinegar can be supplemented with only 0.2% of the feed, resulting in decreased feed cost for the farmer. Hence, silicic acid powders mixed with bamboo vinegar is also more cost effective. Thus, dietary SPV provides the added benefit of more than 200 accessory ingredients, including phenolic compounds, polyphenolic compound, and various organic acids (Kimura et al., 2002). Some of these ingredients, such as guaiacol, cresol and 4-ethyl-2-methoxyphenol, have been reported to have antifungal activity (Ikegami et al., 1998). Moreover, organic acids in bamboo vinegar were reported to increase gastric proteolysis and improve the digestibility of protein and amino acids (Samanta et al., 2010). In addition, these acids have been shown to inhibit the growth of intestinal bacteria which compete with the host animal for available nutrients (Dhawale, 2005). As a result, the growth performance of the host improved. Acetic acid, which is the main component of bamboo vinegar compound liquid, is one of the main short-chain fatty acids produced by intestinal microbes. Acetic acid was reported to have an effect on intestinal functions and metabolism (Lutz and Scharrer, 1991). It was also reported to inhibit the growth of pathogenic bacteria and accelerate the growth of beneficial bacteria (Watarai and Tana, 2005). Beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium* can enhance the metabolism of host birds and improve gut efficiency by increasing nutrient absorption (Gabriel et al., 2006) and accelerating gut development (Furuse et al., 1991), whereas harmful bacteria damage the villus and microvillus of intestinal mucosa and inhibit the secretion of digestive enzymes (Xu et al., 2003). Alternatively, silicic acid included in SPV is a source of silicon element. Silicon was required for maximal enzyme activity of prolyl hydroxylase (Carlisle et al., 1981). However, Elliot and Edwards (1991) found that supplemental silicon (250 ppm) had no effect on growth performance in broiler chickens receiving a basal diet containing silicon from 0.6 to 143 ppm. Thus, it is likely that the higher body weight gain for chickens fed SPV-supplemented diets in the current study was mainly due to the effects of bamboo vinegar compound liquid on improving the circumstances of the gastrointestinal tract. In this study, therefore, SPV may improve body weight by several possible mechanisms, including altering gut pH, selecting for beneficial intestinal organisms, inhibiting the growth of pathogens, and enhancing the digestion and utilization of nutrients.

The digestive function of the small intestine is closely related to mucosal architecture, in particular to the villi density, shape, and size (Thomsen et al., 2006). SPV supplementation appeared to influence several villi parameters in our study. Table 4 shows an increase in jejunal villus height, duodenal and jejunal villus area, and cell mitosis number in the duodenum and jejunum of the 0.2 and 0.3% SVP groups compared with control group. In addition, protuberant cells were also clearly observed in the duodenum and jejunum of the 0.2 and 0.3% SPV groups. This suggests that the ingested feed might be effectively absorbed from the duodenal and jejunal epithelial cells. Intestinal morphology was markedly affected by the diets (Langhout et al., 1999; Yasar and Forbes, 1999). As villi increase surface area and are the first to make contact with nutrients in the lumen, the villi play a crucial role in the digestion and absorption processes of the small intestine. Increased villus height is indicative of a greater absorptive surface area and a better capacity for the absorption of available nutrients (Caspary, 1992). Villus height is increased by the enhanced efficiency of digestion and absorption of the small intestine due to a population of beneficial bacteria that supply nutrients and stimulate vascularisation and development of the intestinal villi (Gilmore and Ferretti, 2003). Choct (2009) reported a shorter villus when the counts of pathogenic bacteria increase in the gastrointestinal tract, which results in fewer absorptive and more secretory cells. Organic acids in bamboo vinegar reduce the growth of many pathogenic bacteria. As a result, it may reduce intestinal colonization and slow the infectious process, thereby decreasing the inflammatory process in the intestinal mucosa, which improves villus height and its functions of secretion, digestion and absorption of nutrients (Iji and Tivey, 1998). As it has been reported in swine that increased intestinal villus height, cell area and cell mitosis numbers are indicators that the function of the intestinal villi is activated (Thomsen et al., 2006), the present significant increase in villus height of jejunum, and villus area of duodenum and jejunum of the 0.2 and 0.3% SPV groups might provide more surface area for nutrient absorption and thus might improve nutrient digestibility.

Also in the present study, protuberant cells were clearly observed on the duodenal and jejunal villus apical surface of the 0.2 and 0.3% SPV groups. Such cells were reported in chickens (Yamauchi et al., 2006), Aigamo ducks (Ruttanavut et al., 2009) and piglets (Mekbungwan et al., 2008) showing heavy body weight gain. Cell protuberances on the villus apical surface have shown an activated absorptive function of the villi (Yamauchi et al., 2006). In addition, the area having cells with no microvilli in the 0.3% SPV group might be induced by activated cell mitosis, resulting in a quicker cell turnover than that of other groups. It is well known that AP-2α and PCNA immunofluorescence can be used as a marker of cell proliferation for G_1-phase (arresting in G_1) cells and S-phase cells of cell cycle, respectively. The present increased duodenal cell mitosis numbers using hematoxylin-eosin method in the 0.3% SPV-fed

birds corresponded with the result that the clearly activated immunofluorescence microscopic observations of increased AP-2α- and PCNA- positive cells in the 0.3% SPV group than the control. This suggests that increased cell mitosis would be induced by activated G_1-phase and S- phases of cell cycle.

The present results showed that 0.3% SPV could effectively stimulate intestinal function in the duodenum and jejunum, but there was no significant effect on the ileum. This may be explained by the fact that under normal circumstances, the major absorption of nutrients occurs in the duodenum and jejunum (Noy and Sklan, 1995). From these studies, the present increased light microscopic parameters and protuberant cells suggest that the function of villi and epithelial cells might be stimulated after feeding dietary SPV.

In conclusion, 0.3% dietary SPV showed hypertrophied intestinal villi and epithelial cells in the duodenum and jejunum due to the activated G_1 - and S - phases of cell cycle, resulting in higher body weight gain. This may suggest that 0.3% SPV can be used as a natural growth promoter in chicken diets.

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