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**Effect of dietary supplementation of silicic acid powder
containing bamboo vinegar on production performance, egg
quality, intestinal microflora and morphology of laying hens**

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Running Head: RATTANAWUT *ET AL.* – LAYING HENS FED SILICIC ACID
POWDER INCLUDING VINEGAR

This study investigated the effects of dietary inclusion of silicic acid powder containing bamboo vinegar liquid (SPV) on production performance, egg quality, intestinal microflora and alterations of intestinal villi in laying hens. Eighty ISA Brown hens were randomly divided into 4 treatment groups, with 5 replicates per treatment and 4 hens per replicate. They were fed *ad libitum* a basal diet supplemented with SPV at 0, 2, 4 or 6 g kg⁻¹ level, for 16 weeks. Egg production significantly increased with the 2 g kg⁻¹ SPV diet for hens 34-37 weeks old, while egg mass was highest for hens fed the 2 g kg⁻¹ SPV diet in 38-41 weeks age range ($P < 0.05$). Internal and external egg quality were not significantly affected by SPV ($P > 0.10$). In the ileal content, populations of *Escherichia coli* and *Salmonella* spp. tended to decrease in the SPV-supplemented groups compared to the control group ($P < 0.10$). Villus height of jejunum and villus area of duodenum and jejunum were highest in the hens fed the 2 g kg⁻¹ SPV diet ($P < 0.05$). The present results indicate that dietary SPV at 2 g kg⁻¹ could stimulate intestinal functions, tended to decrease ileal pathogenic bacteria counts, and resulted in higher egg production performance.

Key words: Silicic acid powder, bamboo vinegar, hen, villus

Abbreviation: SPV, silicic acid powder containing bamboo vinegar liquid

The application of antibiotics to poultry has been banned in many countries because of public concerns about the downsides, such as drug residues in the animal products, imbalances of normal microflora, the development of drug-resistant bacteria, and a reduced ability to cure these bacterial diseases in humans. As a result, efficacious alternatives to replace antibiotics are sought, targeting effective prevention or control of infectious diseases, promotion of growth, and improvement of feed efficiency. In poultry acidifiers are an alternative that promote performance and improve gut health. Acidifier helps to maintain an optimum pH in the stomach, which increases the activity of proteolytic enzymes (Swiatkiewicz et al. 2010). This improves protein digestibility and contributes to weight gain of the host animal. In addition, the growth inhibition of potential pathogenic and zoonotic bacteria, in the feed and in the gastrointestinal tract, benefits the animal health. Positive effects of feeding acidifiers to chickens were reported by many researchers (Alcicek et al. 2004; Aksu et al. 2007; Brzoska and Steck 2007). Recently, Brzoska et al. (2013) reported that broilers fed on 0.3, 0.6 and 0.9% commercial acidifier (butyric acid, ammonium propionate and propionic acid) had better growth performance than the control birds. Bamboo vinegar is a brown-red transparent liquid produced during pyrolysis of bamboo charcoal. It includes more than 200 accessory ingredients, including phenolics, alkanes, alcohols, aldehydes, and various organic acids (Kimura et al. 2002; Lin et al. 2008). The main components of bamboo vinegar are acetic acid and water (Velmurugan et al. 2009). The acid in wood vinegar was reported to inhibit the growth of pathogenic bacteria and to accelerate growth of beneficial bacteria (Watarai and Tana 2005). Silicic acid is a weak acid known to be the active form of silicon in both plants and animals. Silicon is essential for the growth and the skeletal development in chicks (McDowell 1992). A mixture of

silicic acid powder and bamboo vinegar liquid (SPV) has been recently formulated and tested as supplement in animal feeds, with a positive influence on the performance of meat type chickens (Ruttanavut et al. 2012). However, the effects of SPV on production performance of laying hens had not been investigated yet. We hypothesized that the mixture of both substances would be synergistically effective in increasing of laying performance. In this study, the effects of dietary SPV on production performance, egg quality, intestinal microflora, and intestinal morphology, were examined in laying hens.

MATERIALS AND METHODS

Hens and Management

All experiments were performed according to the humane care guidelines for the use of animals in experimentation, as provided by the Prince of Songkla University, Thailand. The ISA Brown hens were obtained at 22 weeks of age, and reared in layer cages in a completely randomized design at ambient temperature (26-31 °C). Light was provided for 16 h (from 6:00 to 22:00) each day. The hens were fed with a conventional layer mash diet for 3 weeks as an adaptation period to the environment, during which the daily egg production and the egg weights were recorded. At 25-wk-old, 80 hens were divided into 4 treatment groups with 5 replicates each, and 4 hens in each replicate. The replicates had initially similar mean body weights ($1,652.9 \pm 54.7$ g) and egg production levels ($95.8 \pm 3.5\%$). The basal diet (Table 1) was in mash form and was formulated to meet or exceed the NRC (1994) requirements for laying hens. The control group was fed a basal diet, and the other groups were fed the basal diet supplemented with SPV at 2, 4 or 6 g kg⁻¹. Commercial SPV with pH=4 (Shikoku Tekuno Co., Ltd, Kagawa,

Japan) had been produced as follows: bamboo vinegar liquid (pH=3.25) (Table 2) was obtained by condensing smoke during the making of bamboo charcoal from moso bamboo (*Phyllostachys pubescens*) by dry distillation at 700 °C in an airless condition, and it was stored for one year. The skimmed solution was distilled to remove harmful substances such as tar. Then the bamboo vinegar (3 L) was allowed to absorb into commercial silicic acid powder (5 kg, 50 mesh particle size), to create SPV. All hens were housed in an environmentally controlled room under a photoperiod of 16 h of light. Each cage was equipped with a feeding trough and a nipple water drinker. Experimental diets and water were provided ad libitum throughout the 16-wk study.

Production Performance, and Egg Quality

Production performance was determined every 4 weeks by monitoring egg production, egg weight, egg mass, feed intake and feed efficiency. Eggs were collected daily and the weight was recorded to calculate mean egg weight. Egg production was expressed on a hen-day basis (%hen-day). Egg mass was calculated by multiplying egg weight by egg production. Daily feed intake was determined by subtracting the remaining feed from the original amount of feed at the end of each day. Feed efficiency was calculated by dividing egg mass by the feed intake. Body weight was measured at the beginning (25 weeks of age) and at the end of the experiment (41 weeks of age) to calculate body weight changes. Egg quality (5 eggs/diet) was assessed from measured egg weight, shell weight, shell thickness, albumen weight, yolk weight, yolk colour, and the Haugh unit of eggs, obtained on the final day of experiment. The weights of the shell, albumen and egg yolk were measured using an electronic digital balance. Shell thickness was estimated as the mean from three measured locations (air cell, equator and sharp end), and was measured by a dial thickness gauge (Peacock, Tokyo, Japan). The albumen

height was measured using a micrometer. Yolk colour was determined using the Roche yolk colour fan scores (RYCF; F. Hoffman-La Roche, Basel, Switzerland), and colours were scored according to 15 sample colours ranging from 1 (the lightest) to 15 (the darkest). The Haugh units were assessed by the following formula: $100 \times \log (H + 7.57 - 1.7 \times W^{0.37})$, where H = albumen height (mm) and W = egg weight (g).

Intestinal Microflora Analyses

In this study, fresh digesta from the ileum were used because the ileum is richer in intestinal microflora population than the duodenum and jejunum (King and Toskes 1979). At the end of feeding period, the hens were killed by decapitation and their intestinal tracts were removed. Samples (5/diet; each pooled from 2 birds) of fresh digesta from the ileum were collected aseptically in sterilized plastic tubes and blended to obtain a homogeneous mass of digesta, and a 1 g sample was transferred to a test tube. Each sample was mixed with 9 mL phosphate-buffered saline and homogenized at 2,500 rpm for 1 min. The homogenized sample was diluted between 10^{-2} and 10^{-6} fold before inoculations onto Petri dishes of sterile agar. *Escherichia coli* was grown on Eosin Methylene Blue (EMB) agar (BBL, Sparks, MD, USA) and *Salmonella* spp. was grown on *Salmonella-Shigella* (SS) agar (Difco, Franklin Lakes, NJ, USA). The colonies on each plate were counted after incubation in an aerobic chamber at 37 °C for 24 h. Colony forming units (cfu) were defined as being distinct colonies measuring at least 1 mm in diameter.

Tissue Sampling and Measurement

Another 5 hens per group were used for morphometrical and histological observations of the villi in each intestinal segment. After decapitation, the entire small intestine was

excised and fixed in 10% neutral-buffered formalin. The tissue samples from the middle of the duodenum, jejunum, and ileum of the small intestine were cut into 3-cm sections. After dehydration in graded alcohol, each intestinal segment was embedded in Paraplast. Transverse 4 μm sections were cut, and stained with haematoxylin and eosin. Villus height and villus area were determined using an automatic image analyzer (Olympus DP73 camera, Olympus Corporation, Tokyo, Japan) connected to a light microscope. Villus height was measured from the villus tip to the bottom. The mean villus height from 5 hens (16 villi from 8 different sections in each segment per hen) is given as the mean villus height for a treatment group. 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 hen. The average of these was assigned to each hen, and the mean of these across 5 hens is given as the mean villus area for one treatment group.

Statistical Analysis

All of the experimental data were statistically analyzed using one-way ANOVA, and significant differences between treatments were determined with Duncan's multiple range test using the SAS program (SAS Institute Inc., Cary, NC, USA). The results are expressed as the mean and the pooled standard error of the mean (SEM). $P < 0.05$ was considered significant, $P < 0.1$ was considered a trend, and $P > 0.1$ was considered not significant.

RESULTS

Production Performance

The production performance is shown in Table 3. Compared to the control group, egg production significantly increased in the hens fed with the 2 g kg⁻¹ SPV diet, during 34-

37 weeks age period ($P < 0.05$), and the overall egg production tended to be higher in the 2 g kg⁻¹ SPV group ($P < 0.1$). Egg mass were higher for the hens fed with 2 and 4 g kg⁻¹ SPV, during 38-41 weeks age period ($P < 0.05$). Body weight changes, egg weight, feed intake, and feed efficiency did not differ significantly between the treatments ($P > 0.10$).

Egg Quality Traits

The SPV supplementation level did not affect mean egg weight, shell weight, shell thickness, albumen weight, yolk weight, yolk colour or Haugh unit ($P > 0.10$) (Table 4).

Ileal Microflora Population

The ileal *E. coli* and *Salmonella* colony counts did not differ significantly between the treatments (Table 5). However, the populations of *E. coli* and *Salmonella* spp. tended to decrease in the SPV-supplemented groups compared to the control group ($P < 0.10$).

Intestinal Morphology

Villus height and villus area of the control and experimental groups are shown in Table 6. Villus height of jejunum and villus areas of duodenum and jejunum were highest in the hens fed with 2 g kg⁻¹ SPV in the feed ($P < 0.05$).

DISCUSSION

In prior studies, the addition of SPV to diets has improved the productive performances of lightweight chickens (Ruttanavut et al. 2012) and fast growing chickens (Rattanawut and Yamauchi 2015), by stimulating intestinal villi and epithelial cells. In the recent

study with older laying hens (from 75 to 80 weeks of age) feeding the acidifier at 2 g kg⁻¹ of the diet beneficially affected broken egg and feed conversion ratio, but had no influence on laying rate and eggshell strength (Park et al. 2009). Brzoska et al. (2013) reported that the amount of acidifier recommended for inclusion in poultry diets depends on several factors, mainly on alkalizing effects of feed ingredients and mineral supplement such as calcium content. In this study, adding 2 g kg⁻¹ SPV to the feed was effective in improving the egg production and egg mass of laying hens, during the age periods 34-37 and 38-41 weeks, respectively. A higher egg performance with the 2 g kg⁻¹ SPV diet might be due to the beneficial effects of silicic acid and bamboo vinegar liquid. The organic acids in bamboo vinegar reportedly increase gastric proteolysis and improve the digestibility of proteins and amino acids (Samanta et al. 2010). Acetic acid is the main organic acid component in bamboo vinegar. It can control the balance of intestinal microflora and pathogens (Sorrells and Speck 1970). Acetic acid and phenolic compounds contained in wood vinegar have been reported as anti-germination agents (Yatagai et al. 2002) and termiticides (Mu et al. 2004). Pigs fed a diet supplemented with 4 g kg⁻¹ bamboo vinegar grew faster than the controls (Wang et al. 2012). Alternatively, silicic acid included in the SPV is a source of silicon element. Silicon is required for the maximal enzyme activity of prolyl hydroxylase, a determinant of the rate of collagen biosynthesis (Carlisle et al. 1981). Thus, it is likely that the higher egg production performance for hens fed the 2 g kg⁻¹ SPV diet in the current study was induced by both bamboo vinegar and silicic acid, through improving the gastrointestinal tract environment. In our results the hens fed the 6 g kg⁻¹ SPV diet had similar performance to the control hens. An excessive amount of acidifiers in the diet may disturb the acid-base balance of hens. In addition, a higher level of SPV may affect the

balance of gut microflora. Wang et al. (2012) found that the richness of intestinal bacteria tended to decrease with the increase of bamboo vinegar inclusion in the diet.

In the hens fed the 2 g kg⁻¹ SPV diet, intestinal villus height of the duodenum and villus area of the duodenum and the jejunum were the highest across the treatments. An increased size of the intestinal villi implies a greater surface area for nutrient absorption and thus improved nutrient digestibility. Gilmore and Ferretti (2003) found that villus height is increased with an enhanced efficiency of digestion and absorption of the small intestine, due to a population of beneficial bacteria that supplies nutrients and stimulates vascularisation and development of the intestinal villi; whereas Choct (2009) found shorter villi with increased counts of pathogenic bacteria in the gastrointestinal tract. Bamboo vinegar diluted eight-fold killed fecal *E. coli*, *Staphylococcus aureus* and *Candida albicans* (Jiang 2005), while a 100-fold dilution has antimicrobial activity against *E. coli* (Wang et al. 2012). Chu et al. (2013) reported that population of lactic acid bacteria in the feces was higher, while the population of coliform bacteria and *Salmonella* were lower in pigs fed with bamboo vinegar in the feed. These results indicate that bamboo vinegar can reduce harmful intestinal bacteria, but increases beneficial bacteria. In the current study, supplementing the diet with SPV tended to decrease ileal *E. coli* and *Salmonella* spp. of the laying hens. This may be due to the active ingredients in bamboo vinegar reducing the growth of pathogenic bacteria. As a result, the SPV supplementation may reduce intestinal colonization and slow infectious processes, thereby decreasing the inflammatory processes in the intestinal mucosa, thus improves villus size in duodenum and jejunum. Yamauchi (2007) reported that increased villus height and villus area in the intestine indicate that the intestinal villi are activated. The present results showed that 2 g kg⁻¹ SPV could effectively stimulate

intestinal function in the duodenum and jejunum, indicating that most nutrients are absorbed in the upper segment of the intestine.

In conclusion, 2 g kg⁻¹ dietary SPV supplementation increased the size of intestinal villi in the duodenum and jejunum, tended to decrease ileal pathogenic bacteria counts, resulting in higher egg performance. The results suggest that 2 g kg⁻¹ SPV could be used as a beneficial feed additive in layer production.

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Table 1. Feed formulation and chemical composition of basal diet (as-fed basis)

Item	Amount (g kg ⁻¹)
Ingredient	
Corn	595.0
Soybean meal	200.0
Rice bran	42.5
Fish meal	65.8
Oyster shell	56.3
Dicalcium phosphate	20.4
Plant oil	10.0
DL-Methionine	2.0
Salt	3.0
Premix ^a	5.0
Calculated analysis	
Crude protein	180.0
Metabolisable energy (MJ kg ⁻¹)	11.8
Crude fiber	36.1
Crude fat	49.3
Calcium	33.0
Available phosphorus	5.5
Lysine	9.7
Methionine	5.3

^a premix: 2.0 MIU vitamin A, 0.32 MIU vitamin D₃, 2,000 mg vitamin E, 330 mg vitamin K₃, 220 mg vit B₁, 450 mg vitamin B₂, 4.5 mg vitamin B₁₂, 600 mg niacin, 100 mg copper, 150 mg iodine, 130 mg cobalt, 10 g iron, 8.8 g manganese, 8.8 g zinc, 25 g preservative, up to 1 kg filter.

Table 2. Chemical properties of bamboo vinegar compound liquid

Item	Composition (g kg ⁻¹)
Total organic content	113.7
Acetic acid	28.7
Methanol	0.7
Formaldehyde	0.03
Phenol	1.77
Cresol	0.43
Tar	7.3

Table 3. Effects of dietary silicic acid powder containing bamboo vinegar liquid on performance of laying hens during 25-41 weeks of age (n = 5)

Parameters	Dietary SPV (g kg ⁻¹)				SEM	P value
	0	2	4	6		
Body weight changes (g)	145.32	142.15	135.71	122.52	11.46	0.814
Egg production (%)						
26-29 week	97.14	96.78	97.14	96.07	0.69	0.950
30-33 week	97.85	98.92	97.85	96.07	0.65	0.508
34-37 week	94.33 ^{bc}	98.57 ^a	97.49 ^{ab}	93.10 ^c	0.73	0.012
38-41 week	96.03	96.92	96.17	94.11	0.39	0.055
26-41 week	96.34	97.80	97.16	94.84	0.44	0.091
Egg weight (g)						
26-29 week	55.53	55.08	54.70	55.43	0.43	0.920
30-33 week	57.12	58.03	56.46	54.87	0.59	0.301
34-37 week	58.64	58.53	58.05	57.26	0.49	0.775
38-41 week	59.05	60.79	60.72	59.13	0.44	0.336
26-41 week	57.58	58.11	57.48	56.67	0.45	0.701
Egg mass (g/hen/day)						
26-29 week	53.93	53.35	53.18	53.27	0.66	0.982
30-33 week	55.91	57.42	55.31	52.77	0.85	0.293
34-37 week	55.33	57.70	56.63	53.26	0.70	0.092
38-41 week	56.71 ^{ab}	58.94 ^a	58.40 ^a	55.61 ^b	0.49	0.046
26-41 week	55.48	56.85	55.85	53.74	0.60	0.309
Feed intake (g/d)						
26-29 week	109.00	108.10	108.29	107.50	0.87	0.953

30-33 week	116.25	117.21	115.71	111.10	1.01	0.115
34-37 week	113.61	114.14	113.25	108.10	1.21	0.261
38-41 week	114.14	113.10	113.81	112.32	1.26	0.963
26-41 week	113.25	113.14	112.76	109.75	0.67	0.213
Feed efficiency						
26-29 week	0.495	0.494	0.491	0.496	0.007	0.990
30-33 week	0.481	0.490	0.473	0.475	0.006	0.816
34-37 week	0.486	0.506	0.500	0.494	0.005	0.562
38-41 week	0.498	0.522	0.514	0.495	0.007	0.598
26-41 week	0.490	0.502	0.495	0.490	0.005	0.795

Note: Means within a row not sharing a lowercased italic letter differ significantly at the $P < 0.05$ level.

Table 4. Effects of dietary silicic acid powder containing bamboo vinegar liquid on egg quality traits of laying hens from 26 to 41 weeks of age (n = 5)

Parameters	Dietary SPV (g kg ⁻¹)				SEM	P value
	0	2	4	6		
Egg weight (g)	58.73	59.16	58.53	57.59	0.35	0.472
Shell weight (g)	6.46	6.50	6.58	6.26	0.04	0.190
Shell thickness (mm)	0.37	0.36	0.37	0.36	0.01	0.531
Albumen weight (g)	38.19	38.48	37.70	37.52	0.31	0.729
Yolk weight (g)	14.09	14.17	14.26	13.80	0.11	0.515
Yolk color	7.23	7.15	7.37	7.35	0.05	0.372
Haugh unit	84.18	83.85	84.16	84.29	0.29	0.950

Table 5. Ileal microflora counts of hens fed 0, 2, 4 and 6 g kg⁻¹ dietary silicic acid powder containing bamboo vinegar liquid diets (log₁₀ cfu/g of wet digesta) (n = 5)

Parameters	Dietary SPV (g kg ⁻¹)				SEM	P value
	0	2	4	6		
<i>Escherichia coli</i>	3.91	3.86	3.87	3.88	0.01	0.064
<i>Salmonella</i> spp.	3.38	3.32	3.29	3.27	0.01	0.057

Table 6. Villus height and villus area of the duodenum, jejunum and ileum in hens fed 0, 2, 4 and 6 g kg⁻¹ dietary silicic acid powder containing bamboo vinegar liquid diets (n = 5)

Parameters	Dietary SPV (g kg ⁻¹)				SEM	P value
	0	2	4	6		
Villus height (mm)						
Duodenum	1.32	1.38	1.34	1.28	0.01	0.059
Jejunum	0.89 ^{ab}	0.94 ^a	0.92 ^{ab}	0.86 ^b	0.01	0.013
Ileum	0.48	0.50	0.47	0.46	0.01	0.335
Villus area (mm ²)						
Duodenum	0.118 ^{ab}	0.122 ^a	0.119 ^{ab}	0.115 ^b	0.008	0.034
Jejunum	0.074 ^b	0.083 ^a	0.080 ^a	0.073 ^b	0.002	0.002
Ileum	0.034	0.037	0.035	0.032	0.001	0.229

Note: Means within a row not sharing a lowercased italic letter differ significantly at the $P < 0.05$ level.