Effects of bamboo charcoal powder including vinegar supplementation on performance, eggshell quality, alterations of intestinal villi and intestinal pathogenic bacteria populations of aged laying hens

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ABSTRACT
This study examined the supplementation level effects of bamboo charcoal powder including vinegar (BCV) on performance, eggshell quality, alterations of intestinal villi and intestinal pathogenic bacteria populations, in aged laying hens. A total of 200 laying hens (60 weeks of age) were randomly allotted into four treatment groups, with five replicates per treatment and 10 hens per replicate. Hens were fed a basal diet, supplemented with BCV at 0, 0.5, 1.0 or 1.5% level, ad libitum for 12 weeks. Egg production, egg weight, egg mass, feed intake and feed efficiency were not affected by the dietary treatment (p > .05). Damaged egg rate decreased in the hens fed the 1.0 and 1.5% BCV diets during the late feeding stage (69 to 72 weeks of age), and eggshell thickness increased in the 1.5% BCV group (p < .05). In the ileal content, population of Escherichia coli decreased with BCV in the diet, while colony counts of Salmonella spp. were comparatively low with 1.0 and 1.5% BCV (p < .01). Villus height and villus area of duodenum were higher in hens fed with 1.0 and 1.5% diets (p < .01), while villus size of jejunum was higher in hens fed with BCV diets (p < .05). The results of this study demonstrate that a level of 1.0% BCV in a layer’s diet is sufficient for decreasing pathogenic bacteria and stimulating intestinal functions; and that dietary supplementation of BCV at 1.5% can improve eggshell thickness in aged laying hens.

ARTICLE HISTORY
Received 28 September 2016
Revised 20 November 2016
Accepted 5 December 2016

KEYWORDS
Aged hen; bamboo charcoal; bamboo vinegar; intestine; pathogenic bacteria

Introduction

The laying cycle of a chicken flock usually covers a span of about 12 months. When hens get older, the eggs get larger, but the shell gland still deposits the same amount of calcium (Ca) on the shells, making them thinner. Also, aged hens are reportedly less efficient in absorbing Ca than younger ones (Al-Batshan et al. 1994). For these reasons, the incidence of cracked eggs could increase in the late-phase production cycle. This problem has raised the interest of many researchers for improving eggshell quality using various approaches. Most of the studies on nutrition effects on eggshell quality in laying hens have focussed on dietary Ca manipulation as the primary means to improve eggshell quality. However, increasing dietary Ca alone reportedly impairs the absorption of other minerals, such as phosphorus, magnesium, manganese, and zinc, causing secondary deficiencies. In addition, excess calcium significantly reduces egg production, egg weight and feed consumption (Harms & Waldroup 1971). In a recent study, Jiang et al. (2013) reported that laying hens fed diets with high Ca concentrations (4.4%) experienced decreased eggshell quality (shell thickness) in comparison with a control group (3.7% Ca). Therefore, increasing Ca level in the diet to improve eggshell quality might not be a practical solution, but enhancing Ca availability and absorption in the gut might be useful.

The gut microflora plays a major protective function, maintaining integrity of the intestinal mucosa. Intestinal barrier dysfunction leads to a progressive increase of mucosal permeability, which facilitates pathogen infections (Lambert 2009). As a hen gets older, its gut microflora changes and the gut mucosal system becomes more susceptible to lose its integrity (Burel & Valat 2009). Damaging the intestinal integrity
encourages pathogenic gut bacteria at the expense of beneficial bacteria. Adding beneficial feed additives to the diet can recover the intestinal integrity, improve gut health, and thus increase nutrient availability and absorption (Awad et al. 2009; Abdelqader et al. 2013). Bamboo charcoal is known as a universal adsorbent, because it contains a complex network of pores of various shapes and sizes (Zhao et al. 2008). It has been used in powder form as an oral antidote to reduce the absorption of poison from the gastrointestinal tract (Anjaneyulu et al. 1993). When activated charcoal was added to diets, the body weight gain and feed efficiency of chickens tended to increase (Samanya & Yamauchi 2002). Bamboo vinegar is an acidic by-product of bamboo charcoal production. 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, which contribute 3.5–4% and 80–90%, respectively (Velmurugan et al. 2009). The acid in wood vinegar enhances the growth of *Bifidobacterium* and *Enterococcus* but inhibits the growth of *Salmonella* species (Watarai & Tana 2005). In addition, dietary vinegar enhances intestinal Ca absorption in rats by improving Ca solubility and by the trophic effect of the acetic acid contained in vinegar (Kishi et al. 1999). A mixture of bamboo charcoal powder and bamboo vinegar (BCV) has been recently formulated and tested as animal feed supplement. BCV has been shown to induce a significant increase in egg production by stimulating intestinal functions of laying hens in the early phase of production (Yamauchi et al. 2010). However, effects of BCV on production performance and eggshell quality of aged laying hens have not yet been investigated. In this study, the effects of BCV treatment on performance, eggshell quality, alterations of intestinal villi and intestinal pathogenic bacteria populations, were examined in aged laying hens.

### Materials and methods

#### Birds 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. A total of two-hundred 55-week-old ISA Brown hens, obtained from a commercial source, were placed in a poultry house in cages (two birds per cage) on a wire-mesh floor at ambient temperature. The cage dimensions were 40 cm × 45 cm × 35 cm, equating to 1800 cm² total floor space per cage. During the pre-experimental period (55 to 60 weeks of age), the hens were fed *ad libitum* with a conventional layer mash diet, and the daily egg production and the egg weights were recorded.

At 60-week-old, the hens were randomly assigned to one of four treatments, each comprising 5 replicates, and 10 birds in each replicate. The replicates had initially similar mean body weights and egg production levels. During the experiment, the hens had free access to mash feed and water, and were exposed to a 16 L:8 D lighting schedule. The composition of the basal diet is given in Table 1. The control group was fed a basal diet, and the other groups were fed the basal diet supplemented with BCV at 0.5, 1.0 or 1.5%. Commercial BCV was produced by the company (Super BOB®, Shikoku Tekuno Co., Ltd, Kagawa, Japan) as follows: bamboo vinegar was obtained as condensate by cooling 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. Then, the skimmed solution was distilled to remove harmful substances such as tar. This bamboo vinegar compound was absorbed into bamboo charcoal powder, in the ratio of 3 L/8 kg. Chemical properties of bamboo vinegar and composition of bamboo charcoal powder are presented in Tables 2 and 3, respectively (as provided by the

| Item         | Amount, g/100 g |
|--------------|-----------------|
| Ingredient   |                 |
| Corn         | 53.63           |
| Soybean meal | 24.35           |
| Rice bran    | 5.35            |
| Fish meal    | 3.0             |
| Oyster shell | 8.44            |
| Dicalcium phosphate | 1.57      |
| Plant oil    | 2.86            |
| DL-Methionine| 0.2             |
| Salt         | 0.3             |
| Premix a     | 0.3             |
| Calculated analysis |          |
| Crude protein| 17.5            |
| Metabolizable energy, kcal/kg | 2800 |
| Crude fibre  | 3.61            |
| Crude fat    | 5.9             |
| Calcium      | 4.0             |
| Available phosphorus | 0.4      |
| Lysine       | 0.93            |
| Methionine   | 0.5             |

Premix: 2.0 MU vitamin A, 0.32 MU vitamin D₃, 2,000 mg vitamin E, 330 mg vitamin K₃, 220 mg vit B₃, 450 mg vitamin B₉, 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.
manufacturer). The hens were fed an experimental diet until 72 weeks of age.

**Performance and egg quality**

Body weight was measured at the commencement (60 weeks of age) and the end (72 weeks of age) of the trial. Laying performance was determined every 4 weeks by monitoring egg production, egg weight, egg mass, damaged egg rate, 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). The collected eggs were classified as either normal or damaged for calculating the damaged egg rate; the latter included misshapen eggs, broken eggs, cracked eggs and shell-less eggs. Egg mass was calculated by multiplying egg weight by egg production. Feed intake was determined by subtracting the remaining feed from the original amount of feed at the end of each week. Feed efficiency was calculated by dividing egg mass by the feed intake. Egg quality was assessed from measured egg weight, eggshell strength, eggshell weight, eggshell percent, eggshell thickness, albumen weight, yolk weight, yolk colour, and the Haugh unit of each egg, obtained on the final day of the experiment. The weight of the collected eggs from each group was recorded. The eggshell breaking strength was measured using an eggshell strength tester (DET6000, NABEL Co., Ltd, Kyoto, Japan), and the maximum force required to crack the shell surface was recorded. After the eggs were broken on a plate, the weights of the shell, albumen and egg yolk were measured using an electronic digital balance. Eggshell percent was calculated by dividing dry shell weight by egg weight and multiplying by 100. Eggshell thickness was estimated as the mean from three measured locations (air cell, equator and sharp end), and was measured by a digital micrometer (Mitutoyo Corporation, Kanagawa, Japan). Yolk colour and Haugh units were automatically measured using the digital egg tester (DET6000, NABEL Co., Ltd, Kyoto, Japan).

**Intestinal pathogenic bacteria analyses**

Ileal digesta contents (5 samples/diet) were collected at the end of the experiment and then aseptically placed in sterile bags, later blended to obtain a homogeneous mass of digesta, and a 1 g sample was transferred to a test tube. Samples were taken to assays within 1 h after collection. Each sample was mixed with 9 mL sterile 0.9% NaCl, and homogenised at 2500 rpm for 1 min. Homogenates of digesta samples were then serially diluted with phosphate-buffered saline from $10^{-2}$ to $10^{-6}$ mL. One-tenth millilitre of each diluted sample was coated on the appropriate agar media, in duplicate for enumeration of the selected microbial populations. Bacterial counts were performed for the appropriate dilutions. *Escherichia coli* and *Salmonella* spp. were assessed using Eosin Methylene Blue agar (BBL, Sparks, MD) and *Salmonella-Shigella* agar (Difco, Franklin Lakes, NJ), respectively. The colonies on each plate were counted after incubation in an aerobic chamber at 37°C for 24 h. Colony-forming units (CFUs) were defined as being distinct colonies measuring at least 1 mm in diameter. Results are expressed as log 10 CFU/g of fresh sample.

**Tissue sampling and measurement**

At the end of the feeding period (72 weeks of age), 5 birds per group were used for morphometrical and histological observations of the villi in each intestinal segment. After decapitation, their intestine and caecum were removed. The midpoint of the duodenum, the midpoint between the bile duct entry and Meckel’s diverticulum (jejunum), and the midpoint between Meckel’s diverticulum and the ileo-caecal junction (ileum) were fixed in 10% neutral-buffered formalin. After dehydration in graded alcohol, each intestinal segment was embedded in Paraplast Plus (Sigma-Aldrich Co, St Louis, MO). Transverse 4 μm sections were cut, and stained with haematoxylin and eosin. Villus height and villus area were determined at a $10 \times$ magnification using a light microscope. Villus height was measured from the villus tip to the bottom. The mean villus height from 5 birds (16 villi from

| Table 2. Chemical properties of bamboo vinegar compound liquid. |
|---------------------------------------------------------------|
| **Item** | **Composition, g/100 g** |
| 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 |

| Table 3. Composition of bamboo charcoal powder. |
|------------------------------------------------|
| **Item** | **Composition, g/100 g** |
| Ash | 6.35 |
| Nitrogen | 0.57 |
| Phosphate | 1.06 |
| Potassium | 2.10 |
| Silicon dioxide | 1.20 |
8 different sections in each segment per bird) is given as the mean villus height for one 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 bird. The average of these was recorded as the mean for each bird. Finally, the mean across 5 birds was calculated and considered the mean villus area for one group (Rattanawut 2014).

**Statistical analysis**

Production performance data was subjected to repeated-measures ANOVA using the GLM procedure of SAS (Institute Inc., Cary, NC). The egg quality variables, intestinal pathogenic bacteria populations and intestinal villi alterations were analysed using one-way ANOVA, and significant differences between treatments were determined with Duncan’s multiple range test. The results are expressed as the mean and the pooled standard error of the mean (SEM). As the performance data obtained and compared between the weeks within the group were not significant, it was removed. In the current study, \( p < .05 \) was considered significant, and \( p < .10 \) was considered a tendency.

**Results**

**Laying performance**

Laying performance is summarised in Table 4. There were no significant differences in body weight changes, egg production, egg weight, egg mass, feed intake and feed efficiency between the hens fed BCV-supplemented diets and those fed the control diet, either during the experimental feeding phases or across the whole experiment (\( p > .05 \)). During the late feeding stage (weeks 69–72), the rate of damaged eggs decreased significantly (\( p < .05 \)) in the 1.0% and 1.5% BCV groups compared with controls, and the damaged egg rate tended to be lower (\( p < .10 \)) in the 1.0% and 1.5% BCV groups for the whole experiment.

**Egg quality traits**

The effects of dietary BCV supplementation on egg quality in laying hens are presented in Table 5. No significant differences were found between the groups with respect to eggshell weight, eggshell percent, albumen weight, yolk weight, yolk colour, and egg albumen quality expressed in Haugh units. Eggshell

![Table 4](image-url)
thickness was significantly (p < .05) improved in hens fed with 1.5% BCV diet, whereas the 1.0% BCV group was intermediate. Eggshell strength tended to be higher in the 1.0 and 1.5% BCV groups than in the controls (p < .10).

**Ileal pathogenic bacteria population**

The population of ileal *E. coli* decreased (p < .01) with BCV supplementation and the *E. coli* numbers were strongly reduced in 1.0% and 1.5% BCV groups (Table 6). The number of ileal *Salmonella* spp. in hens fed 1.0% and 1.5% BCV diets was lower (p < .01) than in hens fed the control or 0.5% BCV diets.

**Intestinal villi morphology**

Data on intestinal villi morphology are presented in Table 7. Villus height and villus area of duodenum were higher in hens fed with 1.0% and 1.5% BCV diets (p < .01), while villus height and area of jejunum were higher in hens fed the BCV diets (p < .05). There were no significant differences in ileal villus height and area between hens fed BCV-supplemented diets and hens fed the control diet.

**Discussion**

In our previous study, supplementing the diet with 0.5% and 1.0% BCV tended to improve egg production of laying hens in the early phase of production (weeks 22–39) (Yamauchi et al. 2010). Rattanawut (2014) also reported that the addition of 1.0% BCV to diets improved the body weight gain in Betong chickens. Such effects were attributed to the beneficial effects of BCV in stimulating intestinal functions. In the present study, although egg production did not improve with feeding BCV, the rate of damaged eggs was significantly reduced in hens fed 1.0 and 1.5% BCV diets during the late laying period, from 69 to 72 weeks of age, and tended (p = .073) to be lower in these groups for the whole experiment. Decrease in the damaged egg rate with BCV supplementation, as observed in the present study, primarily reflected improved eggshell quality, including eggshell thickness and eggshell strength. Eggshell thickness increased in the hens fed with 1.5% BCV diet, and eggshell strength tended (p = .069) to be higher in the hens fed with 1.0% and 1.5% BCV diets. The positive effects of BCV in feed on eggshell quality could be attributed to the beneficial effects of bamboo charcoal and bamboo vinegar in promoting intestinal function, which may help to assimilate more nutrients. Bamboo charcoal reportedly has higher adsorption capacity than wood charcoal, because of the special micro-pore structure of bamboo.
stems (ChungPin et al. 2004); bamboo charcoal is known to have about 4 times more cavities, 3 times more mineral content and 4 times better absorption rate (Zhao et al. 2008). Watarai and Tana (2005) reported that activated charcoal from the bark given orally could reduce intestinal Salmonella enterica serovar Enteritidis carriage and minimise the removal of normal bacterial flora (Enterococcus faecium) in the intestinal tract. They also reported that wood vinegar liquid inhibited S. Enteritidis growth, whereas growth of E. faecium and Bifidobacterium thermophilum was enhanced by it. Acetic acid is the main organic acid component in bamboo vinegar. It can control the balance of intestinal microflora and pathogens (Sorrells & Speck 1970) and affects intestinal functions and metabolism (Lutz & Scharrer 1991). The results of some experiments with layers and old breeder hens have demonstrated that organic acids may improve the utilisation of minerals and can positively affect eggshell quality (Park et al. 2002; Sengor et al. 2007). One contributing mechanism is the reduction of gastrointestinal pH, which increases the activity of digestive enzymes and the solubility of minerals (Świątkiewicz et al. 2010). Dietary inclusion of organic acids is also reported to decrease intestinal pH and increase Ca solubility, which increases Ca level in blood and improves eggshell quality (Abdel-Fattah et al. 2008; Soltan 2008). In this study, therefore, BCV may have improved eggshell quality through several possible mechanisms, including decreasing gut pH, selecting for beneficial intestinal organisms, inhibiting the growth of pathogens and enhancing the digestion and utilisation of minerals.

In the present study, intestinal villus height and area in the duodenum and the jejunum of aged laying hens increased when feeding BCV at 1.0% and 1.5% levels. An increased villus size provides greater absorptive surface area and a better capacity for absorbing available nutrients. The increased villus absorptive area could also increase Ca absorption. Gilmore and Ferretti (2003) reported that villus height is increased by the enhanced efficiency of digestion and absorption in the small intestine, when a population of beneficial bacteria supplies nutrients and stimulates vascularisation and development of the intestinal villi. 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). In contrast, harmful bacteria damage the villi and microvilli in intestinal mucosa and inhibit the secretion of digestive enzymes (Xu et al. 2003). Choc (2009) found shorter villi when the counts of pathogenic bacteria increased in the gastrointestinal tract, resulting in fewer absorptive and more secretory cells. In the current study, the ileal E. coli and Salmonella spp. populations decreased on feeding aged laying hens with 1.0 and 1.5% BCV. This may be due to the organic acids in bamboo vinegar reducing the growth of pathogenic bacteria. This might reduce intestinal colonisation and slow down infectious processes, thereby decreasing the inflammations in the intestinal mucosa, which improves villus height and its functions of secretion, digestion and absorption of nutrients. Increased villus size in the duodenum and jejunum are indicators that the function of the intestinal villi is activated by BCV in the feed. The present results show that 1.0% and 1.5% BCV could effectively stimulate intestinal function in the duodenum and jejunum, but there were no observed significant effects 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 & Sklan 1995), while the ileum appears to have relatively less intestinal absorptive function (Yamauchi et al. 1996).

Conclusions

In conclusion, 1.0% and 1.5% dietary BCV increased the size of intestinal villi in the duodenum and jejunum, decreased ileal pathogenic bacteria counts and improved eggshell quality. The results of this study demonstrate that a level of 1.0% BCV in a layer’s diet is sufficient for reducing the rate of damaged eggs during the late laying period (69–72 weeks of age), and dietary supplementation of BCV at 1.5% can improve eggshell thickness in aged laying hens.

Acknowledgements

The authors would like to thank Associate Professor Seppo Karrila, Faculty of Science and Industrial Technology, Prince of Songkla University, Surat Thani Campus, Thailand, for critical review and adding valuable comments to the manuscript prior submission.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Funding

This research was financially supported by Research and Development Office from Prince of Songkla University (Grant No. SIT570776S-0) and also from Prince of Songkla University, Surat Thani Campus, 2016.
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