

**Feed supplementation with red seaweeds, *Chondrus crispus* and *Sarcodiotheca gaudichaudii*, affects performance, egg quality, and gut microbiota of layer hens**

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**ABSTRACT** The aim of this study was to evaluate the effect of the inclusion of red seaweed supplementation to standard poultry diets on production performance, egg quality, intestinal histology, and cecal short-chain fatty acids in Lohmann Brown Classic laying hens. A total of 160 birds were randomly assigned to 8 treatment groups. Control hens were fed a basal layer diet; positive control hens were fed a diet containing 2% inulin; and 6 treatment groups were fed a diet containing one of the following; 0.5, 1, or 2% *Chondrus crispus* (CC0.5, CC1, and CC2, respectively) and one of the same 3 levels of *Sarcodiotheca gaudichaudii* (SG0.5, SG1, and SG2, respectively). Dietary supplementation had no significant effect on the feed intake, BW, egg production, fecal moisture content, and blood serum profile of the birds. The feed conversion ratio per gram of egg was significantly more efficient ( $P = 0.001$ ) for CC2 and SG2 treatments. Moreover, SG1 supplementa-

tion increased egg yolk weight ( $P = 0.0035$ ) and birds with CC1 supplementation had higher egg weight ( $P = 0.0006$ ). The SG2 and CC2 groups had greater ( $P < 0.05$ ) villus height and villus surface area compared with the control birds. Seaweed supplementation increased the abundance of beneficial bacteria [e.g., *Bifidobacterium longum* (4- to 14-fold), *Streptococcus salivarius* (4- to 15-fold)] and importantly reduced the prevalence of *Clostridium perfringens* in the gut of the chicken. Additionally, the concentrations of short-chain fatty acids, including acetic acid, propionic acid, *n*-butyric acid, and *i*-butyric acid, were significantly higher ( $P < 0.05$ ) in CC and SG treatments than in the control. In conclusion, dietary supplementation using red seaweed inclusions can act as a potential prebiotic to improve performance, egg quality, and overall gut health in layer hens.

**Key words:** *Chondrus crispus*, *Sarcodiotheca gaudichaudii*, prebiotics, beneficial bacteria, short-chain fatty acid

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**INTRODUCTION**

Antibiotics have been used in poultry feed to control disease-causing pathogens in layer chickens and as a growth promoter in the broiler industry (Baurhoo et al., 2007; Li et al., 2007; Awad et al., 2009). The prolonged and indiscriminate use of antibiotics in livestock led to concerns such as development of antibiotic-resistant strains of pathogens, high concentrations of antibiotic residues in meat and meat products, and un-

desirable changes in the microbial communities of animal gastrointestinal tracts (Filazi et al., 2005; Shargh et al., 2012; Tellez et al., 2012). As a consequence, since January 1, 2006, the European Union banned the use of antibiotics as a growth promoter in broiler chicken production (Castanon, 2007). In North America, there is an increased public awareness of the negative effect of antibiotics in livestock production. Therefore, there is strong interest in the development of alternatives to antibiotics (Yan et al., 2011). Control measures such as competitive exclusion cultures and vaccination have contributed significantly to reduce pathogen (e.g., *Salmonella*) infections in layer hens (Penha Filho et al., 2009). Additionally, ingredients with antimicrobial properties such as enzymes, peptides, bacteriophages, organic acids, plant extracts, probiotics, and prebiot-

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ics have been investigated for use in poultry industry (Hinton and Mead, 1991; Joerger, 2003).

Prebiotics are defined as a “non-digestible food ingredient that affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995). In other words, prebiotics improve gastrointestinal health by providing a substrate for the growth and establishment of beneficial bacteria within the gut (Cummings and Macfarlane, 2002). Prebiotics influence gastrointestinal health by a variety of mechanisms such as production of metabolites, namely, lactic acid, inhibiting or reducing the growth and establishment of pathogenic microbes, modifying metabolism of intestinal microbes, and stimulation of gut-microbe-mediated host immunity against microbial and metabolic diseases (Reddy, 1999). Prebiotics also improve epithelial cell integrity by stimulating epithelial stem cell differentiation and angiogenesis. Some examples of beneficial probiotic bacteria found in the gut microflora of chickens include *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, and *Streptococcus*. These bacteria, which are present in the small intestine, use nondigestible polysaccharides and dietary fiber for energy (Mussatto and Mancilha, 2007). Prebiotics improve the activity of beneficial microbiota that produces metabolites, which in turn enhance the ability of animals to absorb minerals and vitamins in both the small and large intestine (Sako et al., 1999).

The prebiotic effects of nondigestible oligosaccharides such as inulin, mannan-oligosaccharides, fructo-oligosaccharides, and galacto-oligosaccharides as feed supplements have been reported in studies involving layer hens (Li et al., 2007; Shang et al., 2010). In addition, health-promoting oligosaccharides, derived from seaweeds, have been evaluated for their use in poultry (Dibaji et al., 2014). Results of a recent study with broilers suggested that supplementing seaweed into the diet enhanced health and productivity, largely by increasing the growth of beneficial gut-microbiota in the lower gastrointestinal tract and by alleviating inflammation resulting from overstimulation of the innate immune system (Abudabos et al., 2013; Evans and Critchley, 2014). Algal biomass and algal products are widely used as animal feed supplements in several countries. It is estimated that about 30% of the algal biomass produced is used as animal feed (Richmond, 2004).

Gudiel-Urbano and Goñi (2002) reported that red and brown seaweeds have prebiotic properties that alter metabolic activity of beneficial microbiota and reduce the prevalence of pathogenic bacteria in rats. Seaweeds such as *Porphyra yezoensis*, *Laminaria japonica*, and *Hizikia fusiformis* have been shown to alter nutrient digestibility by binding to bile salts and inhibiting uptake of fats resulting in lower levels of blood cholesterol in humans (Wang et al., 2001). Additionally, supplementation of layer hen diets with the seaweed *Macrocystis pyrifera* resulted in an elevated levels of n-3 fatty acids in the egg (Carrillo et al., 2008). Moreover, feeding

seaweeds has resulted in an increase in the growth rate and nutrient uptake in chickens and ducks (El-Deek and Mervat Brikaa, 2009).

Edible red macroalgae (i.e., *Chondrus crispus*, *Palmaria palmata*, *Porphyra* sp., and *Mastocarpus stellatus*) are commercially harvested along Pacific and Atlantic coast. Also, certain selected strains of red seaweed, such as *C. crispus*, are grown on land (Hafting et al., 2012). Red seaweeds are rich in phycocolloids (these can be carrageenans, agar) and phycobiliproteins. Red seaweeds are good source of dietary fiber, minerals, vitamins, phlorotannins, carotenoids, amino acids, and several health-promoting compounds, providing a source of raw material for the nutraceutical and pharmaceutical industries (Holdt and Kraan, 2011).

Seaweed polysaccharides can be considered as an ideal prebiotic due to properties such as a) selectivity by beneficial bacteria but not pathogenic strains, b) non-digestibility (i.e., resistant to the digestive enzymes), and c) fermentability as a substrate for intestinal microbiota. In vitro studies showed that glycerol galactoside of the red alga *Pyropia yezoensis* was resistant to action of saliva, pancreatic and digestive enzymes (Muraoka et al., 2008). Several algal polysaccharides are fermented by specific subsets of gut microbes; for example, agarose-derived neoagar-oligosaccharides were fermented by beneficial gut bacteria *Lactobacillus* sp. and *Bifidobacterium* sp. and not by pathogenic strains such as *Escherichia coli* and *Enterococcus* sp. (Hu et al., 2006). Similarly, fecal microbiota have been reported to selectively ferment alginate-derived oligosaccharides (Michel et al., 1999).

The potential activity of a prebiotics can be analyzed by determining the fermentation end products of the gut microbiome [i.e., short-chain fatty acids (SCFA)]. In the colon, SCFA are formed by fermentation of undigested carbohydrates and proteins. They play an important role in the control of colonic diseases, proliferation of colonocytes, and health of the intestinal mucosa in chickens. Short-chain fatty acids such as butyric, propionic, and acetic acids also aid in mineral uptake and provide extra energy to the birds (Lan et al., 2005). Michel et al. (1999) studied the effect of alginate and laminarin-derived oligosaccharides on SCFA in human fecal flora. There was no difference in the total SCFA; however, the concentration of propionate increased significantly in the treatments as compared with fructo-oligosaccharide control. This study indicated that seaweeds could be fermented by intestinal microbiota to produce beneficial by-products such as SCFA.

The aim of the present study was to investigate the effect of dietary inclusion of 2 red seaweed species (i.e., *C. crispus* or *Sarcodiotheca gaudichaudii*) on layer productivity, histomorphology of integrity of intestinal mucosa, as well as composition of microbiota, small intestine, and ceca of layer hens. A basal diet and a ration containing inulin (an established prebiotic) served as negative and positive controls.

## MATERIALS AND METHODS

### Birds and Housing

A total of 160 commercial, laying hens (Lohmann Brown Classic) at 67 wk of age were used in a 30-d trial. All birds were randomly assigned to 1 of 32 wire cages in the upper tiers, of a 2-sided, 3-tier battery cage system, 5 birds per cage. Feed and water were available ad libitum throughout the trial. A controlled environment was established with 16 h of light per day, and the temperature was set at 25°C. All experimental procedures were carried out in accordance with the Canadian Council of Animal Care (1993) guidelines.

### Preparation of Seaweed-Supplemented Feed and Experimental Design

Cultivated samples of the red seaweeds *C. crispus* (CC) and *S. gaudichaudii* (SG) were provided by Acadian Seaplants Limited, Nova Scotia, Canada. The seaweeds were grown in an on-land cultivation facility. Freshly harvested biomass was dried at 50°C for 24 h and ground to a powder (mesh size, 0.4 mm) using a micro Wiley mill, standard model 3 (Arthur H. Thomas Co., Philadelphia, PA). Oligo-Fiber DS2 inulin (Cargill Inc., Wayzata, MN), a commercial prebiotic, was used

as one of the controls in this experiment. The dried seaweeds (SG and CC) were incorporated into chicken feed at concentrations of 0.5, 1, and 2% (wt/wt). Experimental diets were made to be isocaloric by varying the percentage of carbohydrate and protein sources. Two control treatments were included in the experiment; a basal layer diet served as the negative control, whereas the basal diet containing 2% (wt/wt) inulin served as the positive control. The compositions of diets used in the experiment are given in Table 1. Each treatment was randomly assigned to 4 cages; therefore, each cage of 5 birds was considered an experimental unit.

### Layer Performance and Egg Quality

Eggs were collected daily and the numbers recorded throughout the experiment. Feed intake and BW (by cage) were determined on d 0, 14, and 28 of the experiment. Feed was weighed each day before adding into the feeders, and feed consumption was calculated by weighing feeders at the end of each observation period. Additionally, 4 eggs per cage were collected on d 0, 14, and 28. Weight, specific gravity, and other quality parameters were determined for collected eggs. From these data, the feed conversion ratio (FCR) for each cage was calculated using the following formula:

**Table 1.** Dietary composition of experimental diets

Item	Seaweed meal <sup>1</sup> (%)							
	Control	IN2	CC0.5	CC1	CC2	SG0.5	SG1	SG2
Ingredient (% as fed)								
Corn	53.53	55.54	58.91	58.34	57.21	58.94	58.38	57.29
Soybean meal	19.53	20.09	19.28	19.16	18.90	19.25	19.10	18.78
Wheat	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
SG meal	—	—	—	—	—	0.50	1.00	2.00
CC meal	—	—	0.50	1.00	2.00	—	—	—
Ground limestone	4.86	4.91	4.91	4.91	4.91	4.91	4.91	4.91
Barley	5.00	—	—	—	—	—	—	—
Mono-dicalcium phosphorus	0.19	—	—	—	—	—	—	—
Oyster shell	2.43	2.45	2.45	2.45	2.45	2.45	2.45	2.45
Shell mix	2.43	2.45	2.45	2.45	2.45	2.45	2.45	2.45
Poultry fat	1.0	1.58	0.56	0.80	1.30	0.56	0.81	1.30
Inulin	—	2.00	—	—	—	—	—	—
MCL4 <sup>2</sup>	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Biophytase	—	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Methionine premix <sup>3</sup>	0.135	0.14	0.15	0.15	0.17	0.15	0.15	0.17
Iodized salt	0.30	0.30	0.24	0.19	0.07	0.25	0.20	0.10
Total	100	100	100	100	100	100	100	100
Nutrient content, calculated								
ME (kcal·kg <sup>-1</sup> )	2,820.56	2,820.56	2,820.56	2,820.56	2,820.56	2,820.56	2,820.56	2,820.56
Protein (%)	15.22	15.22	15.22	15.22	15.22	15.22	15.22	15.22
Crude fiber (%)	2.28	2.28	2.28	2.28	2.28	2.28	2.28	2.28
Calcium (%)	3.91	3.91	3.91	3.91	3.91	3.91	3.91	3.91
Nonphytate phosphorus (%)	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.26
Lysine (%)	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Methionine + cysteine (%)	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58

<sup>1</sup>Treatment group (IN2: contained 2% of inulin; SG0.5: contained 0.5% of *Sarcodiotheca gaudichaudii*; SG1: contained 1% of *S. gaudichaudii*; SG2: contained 2% of *S. gaudichaudii*; CC0.5: contained 0.5% of *Chondrus crispus*; CC1: contained 1% of *C. crispus*; CC2: contained 2% of *C. crispus*).

<sup>2</sup>Providing per kilogram of diet: retinol, 7,800 IU/kg; cholecalciferol, 2,500 IU/kg; DL- $\alpha$ -tocopherol acetate, 20 IU/kg; thiamine, 1.94 mg/kg; riboflavin, 7.6 mg/kg; pantothenic acid, 7.2 mg/kg; niacin, 30.7 mg/kg; pyridoxine, 3.96 mg/kg; choline chloride, 641 mg/kg; vitamin K, 2.97 mg/kg; biotin, 0.16 mg/kg; cyanocobalamin, 12 mg/kg; antioxidant, 1 mg/kg; manganese, 70.2 mg/kg; zinc, 66 mg/kg; iron, 33 mg/kg; copper sulfate, 25 mg/kg; iodine, 0.9 mg/kg; selenium, 0.15 mg/kg; ethoxyquin, 50 mg/kg; and folic acid, 0.66 mg/kg.

<sup>3</sup>Methionine premix is composed of 50% wheat middlings and 50% DL-methionine.

$$\text{FCR} = \frac{\text{feed consumed (kg)}}{\text{average egg weight (kg)} \times \text{total number of eggs}}$$

The eggs were subsequently broken to determine albumen height (using a QCH albumen height gauge, Technical Services and Supplies, York, UK), yolk weight, eggshell weight, and eggshell breaking strength (using TA.xt Plus, Texture Technologies Corp., Scarsdale, NY). The albumen was separated from the yolk and shell; thereafter, albumen weight was calculated by subtracting the yolk weight and eggshell weight from the weight of the egg. Shells with their membrane intact were rinsed and dried for 48 h, and dry shell weights were measured.

### **Excreta Moisture Content**

On d 15 and 27 of the trial, 3 independent samples of excreta were collected with a sterile spatula from 16 randomly selected cages (2 cages per treatment). Care was taken to avoid feed spillage and bird feathers from inclusion with the excreta samples. Each collection was weighed and placed in a resealable plastic bag. Subsequently, each sample was dried in a forced-air oven at 70°C for 24 h and weighed. Percent moisture was calculated using the following formula (Miles et al., 2011; van der Hoeven-Hangoor et al., 2013):

$$\text{Mn} = \left[ \frac{(\text{Ww} - \text{Wd})}{\text{Ww}} \right] \times 100,$$

where Mn is moisture percentage of material n, Ww is the wet weight of the sample, and Wd is the weight of the sample after drying.

### **Collection of Blood Samples and Chemical Analysis**

Blood samples were taken from the brachial vein of birds on d 28 in Vacutainer tubes containing sodium heparin and were used for determination of the plasma component (Lan et al., 2005). Approximately 8 mL of blood was collected from 32 randomly selected birds (4 birds/treatment) using a 21-gauge needle. Blood plasma was isolated by centrifuging the tubes at 3,000 × g for 10 min at 4°C. The separated supernatant, obtained as plasma, was transferred into individual vials and stored at -20°C for chemical analysis. The blood plasma analysis was carried out at Diagnostic Services, Atlantic Veterinary College, University of Prince Edward Island.

### **Histomorphological Analysis and Sample Collection**

One bird per cage was randomly chosen (d 28, n = 4) and euthanized by cervical dislocation. Each bird was

weighed and the ileum, ceca, spleen, liver, and heart were removed. The contents of ceca were expelled by finger pressure and the empty segments were weighed. A section of 0.5 to 1.0 cm of the distal ileum, immediately before the ileo-cecal junction, was collected (d 0, 14, 28) and fixed in buffered, neutral formalin [10% (vol/vol)] for histological studies (Awad et al., 2009).

The ileal samples were placed in formalin and processed for microtomy. Briefly, each sample was sliced into 3 sections and dehydrated in a series of alcohol with increasing concentration (70 to 100%). The tissue slices were permeated with xylene and fixed in paraffin wax. A section (0.5 μm thick) was cut with a microtome and mounted on to a glass slide. Each slide was stained by the procedure described by Drury and Wallington (1980) and was used for histological measurements. Images were scanned using a Nikon Super Cool Scan 400ED (Nikon Inc., Tokyo, Japan) and Sigma Scan Pro 5 (SPSS Inc., Chicago, IL) was used for measuring villi height, width and area, crypt depth, and mucosal depth of the scanned images. Eight to 10 measurements of each component were made per slide. Villi heights were measured from the base of the intestinal mucosa to the tip of the villus, crypt depth was measured between the start of mucosa to the bottom end of villi, and the mucosal depth was measured from one end of the crypt to the end of the serosa (Samanya and Yamauchi, 2002).

### **Analysis of Intestinal Microbiota**

The microbial population within the gut was determined by quantitative, real-time PCR based on a method as described by Middelbos et al. (2007) with minor modifications. Briefly, genomic DNA was isolated from the ileum contents (stored at -80°C) using a DNA extraction kit (QIAamp DNA Stool Mini Kit, Qiagen, Valencia, CA; Faber et al., 2012) according to the manufacturer's instructions. The DNA extracted from the samples was quantified spectrophotometrically using Nano-Drop ND-2000 (Thermo Scientific, Wilmington, DE). The transcript levels of species-specific 16S rRNA gene for *Bifidobacterium longum* (Matsuki et al., 2003), *Lactobacillus acidophilus* (Haarman and Knol, 2006), *Streptococcus salivarius* (Furet et al., 2009), and *Clostridium perfringens* (Wu et al., 2011) were quantified using StepOnePlus Real-Time PCR system (Applied Biosystems, ON, Canada). The reaction mix contained 2 ng of DNA, 5 μL of SYBR Green I master mix (Applied Biosystems) and 300 nM of each gene-specific primer. Additionally, the total populations of ileal contents were quantified using a universal primer set (Haarman and Knol, 2006).

### **Gas Chromatographic Analysis of Cecal Contents**

The SCFA in the cecal contents were analyzed, as described by Martin et al. (2007). Briefly, 300 mg of ce-

cal content ( $n = 4$ ) was homogenized in 2 mL of buffer, containing 0.1% (wt/vol)  $\text{HgCl}_2$ , 1% (vol/vol)  $\text{H}_3\text{PO}_4$ , and 0.045 mg/mL 2,2 dimethyl butyric acid as an internal standard. The diluted slurry was centrifuged at  $500 \times g$  for 30 min at  $4^\circ\text{C}$  and the supernatant containing SCFA was collected for analysis (Martin et al., 2007). The sample (0.5  $\mu\text{L}$ ) was analyzed using a gas chromatograph (Bruker 430), equipped with a flame ionization detector. A DB-FFAP (Diphenyl-Free Fatty Acid phase) column (Agilent Technology, Santa Clara, CA; length 30 m, internal diameter 530  $\mu\text{m}$  with 1- $\mu\text{m}$  film thickness) was used in the analysis. A cleaning injection of 1.2% formic acid wash was run following each sample run. The initial temperatures for each sample run for the oven, injector, and detector were 80, 180, and  $220^\circ\text{C}$ , respectively. The system used helium as the carrier gas with a constant pressure of 68.9 kPa. The SCFA in the samples were identified using an external standard containing acetate, propionate, iso-butyrate, *n*-butyrate, iso-valerate, and *n*-valerate and quantified using the internal standard. The concentration of SCFA in the samples was determined as described by Zhao et al. (2006).

### Statistical Analysis

A completely randomized design, with 8 dietary treatments as the main factors, was used. Each cage of 5 birds was considered as an experimental unit, which was replicated 4 times for each treatment group. The main effects of diets, bird age, and the interaction between these effects were analyzed using ANOVA, with a  $P$  value  $<0.05$  using the PROC MIXED procedure (software version 9.3, SAS Institute Inc., Cary, NC). When significant effects of treatment were found, means were separated using Tukey analysis for the pair-wise comparison to differentiate treatment means.

## RESULTS

### Feed Efficiency and Layer Performance

The effects of red seaweed dietary supplementation on feed intake, BW, and FCR are summarized in Table 2. No interactions between the dietary inclusions of the seaweeds and bird age, on response variables were detected; hence, only cumulative data are presented in Table 2. Dietary supplementation with CC and SG did not affect feed intake ( $P > 0.05$ ) and average BW of hens, during the period of the experiment. Initially, the data from eggs/cage were collected on d 0 and the production percentage was calculated; this served as a baseline from which to compare the effects of treatments over the experimental period. The baseline egg production was not significantly different for birds assigned to each treatment. The hen-day egg production was significantly higher ( $P < 0.05$ ) in the birds fed with 2% SG (94.6) and 1% CC (90.1), compared with inulin and control birds (85.3 and 88.2 respectively). Consequently, 2% SG and 1% CC improved the FCR ( $P = 0.001$ ) compared with other treatments (1.97, 1.69, 1.72 for control, SG, and CC, respectively), whereas 1% SG and 2% CC were intermediate (1.91 and 1.79 for SG and CC, respectively) and not different from the control. Moreover, lower inclusion levels for dietary seaweed (0.5% SG and CC) reduced egg production (79 and 72, respectively) and increased FCR (2.04 and 2.13, respectively) of the birds. Additionally, inulin, an established prebiotic, used as positive control had no significant effect ( $P > 0.05$ ) on layer hen performance and egg quality (Table 2).

### Egg Quality

The effect of dietary seaweed supplementation on egg quality is presented in Table 2. Significant treat-

**Table 2.** Effect of selected cultivated red seaweed dietary supplementation on layer hen performance and egg quality

Diet <sup>1</sup>	Feed intake (g/d)	Egg production (%)	FCR <sup>2</sup> (g/g)	BW (kg)	Egg weight (g)	Yolk weight (g)
Control	109.98 <sup>a</sup>	88.21 <sup>abc</sup>	1.97 <sup>abc</sup>	2.06 <sup>a</sup>	63.43 <sup>b</sup>	17.27 <sup>b</sup>
IN2	107.79 <sup>a</sup>	85.35 <sup>abc</sup>	1.98 <sup>abc</sup>	2.02 <sup>a</sup>	63.44 <sup>b</sup>	17.38 <sup>b</sup>
SG0.5	106.48 <sup>a</sup>	79.64 <sup>cd</sup>	2.04 <sup>ab</sup>	2.12 <sup>a</sup>	65.44 <sup>ab</sup>	17.91 <sup>ab</sup>
SG1	103.37 <sup>a</sup>	81.96 <sup>bcd</sup>	1.91 <sup>abcd</sup>	2.04 <sup>a</sup>	65.63 <sup>ab</sup>	18.72 <sup>a</sup>
SG2	105.16 <sup>a</sup>	94.64 <sup>d</sup>	1.69 <sup>d</sup>	2.12 <sup>a</sup>	65.11 <sup>ab</sup>	17.66 <sup>ab</sup>
CC0.5	104.32 <sup>a</sup>	72.85 <sup>d</sup>	2.17 <sup>a</sup>	2.13 <sup>a</sup>	65.20 <sup>ab</sup>	17.63 <sup>ab</sup>
CC1	105.16 <sup>a</sup>	90.17 <sup>ab</sup>	1.72 <sup>cd</sup>	2.16 <sup>a</sup>	66.82 <sup>a</sup>	18.48 <sup>ab</sup>
CC2	103.55 <sup>a</sup>	86.07 <sup>abc</sup>	1.79 <sup>d</sup>	2.21 <sup>a</sup>	65.21 <sup>ab</sup>	17.80 <sup>ab</sup>
SEM <sup>3</sup>	1.68	2.13	0.06	0.66	0.63	0.27
Interaction, $P$ -value						
Diet	0.11	$<0.001$	$<0.001$	0.43	0.006	0.0035
Week	$<0.0001$	0.79	0.0004	0.67	$<0.0001$	0.0116
Week $\times$ diet	0.99	0.66	0.5763	0.59	0.0860	0.2488

<sup>a-d</sup>Within a column, values with the same letter grouping are not significantly different ( $P < 0.05$ ).

<sup>1</sup>Values are means for each treatment group (IN2: contained 2% of inulin; SG0.5: contained 0.5% of *Sarcoditheca gaudichaudii*; SG1: contained 1% of *S. gaudichaudii*; SG2: contained 2% of *S. gaudichaudii*; CC0.5: contained 0.5% of *Chondrus crispus*; CC1: contained 1% of *C. crispus*; CC2: contained 2% of *C. crispus*).

<sup>2</sup>FCR = feed conversion ratio.

<sup>3</sup>Four replicates of 5 hens each per treatment.

**Table 3.** Effect of selected cultivated red seaweed dietary supplements on histomorphological parameters of the distal ileum section in layer hens

Diet <sup>1</sup>	Villus height ( $\mu\text{m}$ )	Villus width ( $\mu\text{m}$ )	Crypt depth ( $\mu\text{m}$ )	Mucosal depth ( $\mu\text{m}$ )	VSA <sup>2</sup> ( $\text{mm}^2$ )
Control	658.82 <sup>b</sup>	149.16 <sup>b</sup>	159.86 <sup>b</sup>	490.75 <sup>b</sup>	1.02 <sup>b</sup>
IN2	756.63 <sup>ab</sup>	157.42 <sup>ab</sup>	185.85 <sup>ab</sup>	517.34 <sup>ab</sup>	1.47 <sup>ab</sup>
SG0.5	735.83 <sup>ab</sup>	179.50 <sup>ab</sup>	241.20 <sup>ab</sup>	476.21 <sup>b</sup>	1.56 <sup>ab</sup>
SG1	848.72 <sup>ab</sup>	181.56 <sup>ab</sup>	245.29 <sup>ab</sup>	590.04 <sup>ab</sup>	1.72 <sup>a</sup>
SG2	896.82 <sup>a</sup>	190.67 <sup>ab</sup>	277.63 <sup>ab</sup>	593.94 <sup>ab</sup>	1.76 <sup>a</sup>
CC0.5	801.22 <sup>ab</sup>	174.68 <sup>ab</sup>	293.35 <sup>ab</sup>	706.50 <sup>a</sup>	1.69 <sup>ab</sup>
CC1	744.92 <sup>ab</sup>	221.03 <sup>a</sup>	264.59 <sup>ab</sup>	663.07 <sup>ab</sup>	1.41 <sup>a</sup>
CC2	909.47 <sup>a</sup>	204.40 <sup>ab</sup>	328.80 <sup>a</sup>	636.24 <sup>ab</sup>	2.00 <sup>a</sup>
SEM <sup>3</sup>	35.58	13.08	26.57	38.10	0.13
<i>P</i> -value	0.0044	0.0317	0.0468	0.0143	0.0023

<sup>a,b</sup>Within a column, values with the same letter grouping are not significantly different ( $P < 0.05$ ).

<sup>1</sup>Values are means for each treatment group (IN2: contained 2% of inulin; SG0.5: contained 0.5% of *Sarcodiotheca gaudichaudii*; SG1: contained 1% of *S. gaudichaudii*; SG2: contained 2% of *S. gaudichaudii*; CC0.5: contained 0.5% of *Chondrus crispus*; CC1: contained 1% of *C. crispus*; CC2: contained 2% of *C. crispus*).

<sup>2</sup>VSA = villus surface area.

<sup>3</sup>Four replicates per treatment.

ment effects were observed for the weights of total egg, yolk, and eggshell. Supplementation of diets with 1% SG increased egg-yolk weight ( $P = 0.0035$ ) and total egg weight ( $P = 0.0006$ ), compared with control diets; however, 0.5 and 2% SG supplementation was not different from the control. Additionally, total egg ( $P = 0.006$ ) and eggshell weights ( $P = 0.0006$ ) were higher in birds fed with 1% CC than in 0.5 and 2% CC treatments. Egg albumen height, yolk color, and shell thickness were not affected by dietary supplementation with either seaweed. There were no interactions between the dietary inclusion of seaweeds and bird age on yolk weight, eggshell thickness, and weight.

### Moisture Content

Dietary supplementation with the selected red seaweeds did not affect ( $P = 0.36$ ) the moisture content of excreta on d 15 and 25 (data not shown). A 2-way interaction between excreta collection days and dietary treatments was not significantly different ( $P = 0.59$ ). However, the total moisture content of the excreta collected on d 25 was higher (78.43,  $P = 0.003$ ), as compared with d 15 (75.27). This difference could be attributed to the age of bird and abiotic factors such as temperature and humidity (data not shown).

### Histomorphological Analysis

The mean values of villus height, width, area, and depth of crypt and mucosa from distal ileal samples are shown in Table 3. The average villus height increased ( $P = 0.004$ ) in birds fed 2% SG and CC seaweed. Crypt depths were deeper for 2% CC supplementation than other treatments ( $P = 0.046$ ). Additionally, dietary inclusions of 2% CC and SG also increased the villus width ( $P = 0.03$ ) and area ( $P = 0.002$ ) compared with the control birds. Positive control inulin showed higher values for villus height, width, area, and depth of crypt

and mucosa than control but was not significantly different ( $P > 0.05$ ).

### Blood Serum Profile

Serum sodium levels were reduced ( $P = 0.03$ ) in laying hens fed 2% dietary SG and CC (149 and 148.7 mEq/L, respectively) compared with the control group (156 mEq/L) fed the basal layer diet. There was no significant effect due to feed supplementation of seaweed meal on blood serum concentrations of phosphorus, chlorine, calcium, total protein, potassium, glucose, creatine kinase, aspartate amino transferase, and uric acid (data not shown).

### Organ Weight

Dietary supplementation of feed with selected red seaweeds increased ( $P < 0.0001$ ) the weight of ceca, as compared with controls (Table 4). The cecal weights increased ( $P < 0.001$ ) in birds fed with 2% CC (13.10 g) and 2% SG (12.48 g), as compared with the control groups (7.15 g). There were no significant effects on liver, spleen, ileum, or heart weights (data not shown).

### Ileal Microbial Analysis

Red seaweed supplementation altered the relative abundance of beneficial bacteria (*Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Streptococcus salivarius*) and pathogenic bacteria (*Clostridium perfringens*) in the ileal contents of chicken (Figure 1). The abundance of *Bifidobacterium longum* increased ( $P < 0.001$ ) by 14- and 9-fold in 1 and 2% SG treatments, respectively, and 4-fold in the 1% CC supplementation. Additionally, the relative abundance of *Streptococcus salivarius* was 15-fold higher ( $P < 0.001$ ) in 1% CC and 4-fold higher in the 2% CC and SG dietary supplementations. Interestingly, all treatments including positive

**Table 4.** Effect of selected cultivated red seaweed dietary treatments on cecum weight relative to BW per kilogram in layer hens (g)<sup>1</sup>

Item	Control	IN2	SG0.5	SG1	SG2	CC0.5	CC1	CC2	SEM	P-value
Cecum <sup>2</sup>	7.15 <sup>bc</sup>	7.63 <sup>bc</sup>	6.15 <sup>c</sup>	7.64 <sup>bc</sup>	12.48 <sup>a</sup>	9.31 <sup>abc</sup>	10.43 <sup>ab</sup>	13.10 <sup>a</sup>	0.73	<0.0001

<sup>a-c</sup>Within a row, values with the same letter grouping are not significantly different ( $P < 0.05$ ).

<sup>1</sup>Values are means for each treatment group (IN2: contained 2% of inulin; SG0.5: contained 0.5% of *Sarcodiotheca gaudichaudii*; SG1: contained 1% of *S. gaudichaudii*; SG2: contained 2% of *S. gaudichaudii*; CC0.5: contained 0.5% of *Chondrus crispus*; CC1: contained 1% of *C. crispus*; CC2: contained 2% of *C. crispus*).

<sup>2</sup>The values are reported as least squares means (n = number of birds, n = 4/treatment).

control inulin decreased the prevalence of *Clostridium perfringens* compared with the negative control ( $P < 0.001$ ). The abundance of *Lactobacillus acidophilus* increased ( $P < 0.001$ ) by 4-fold in the inulin treatment and decreased in the CC treatments.

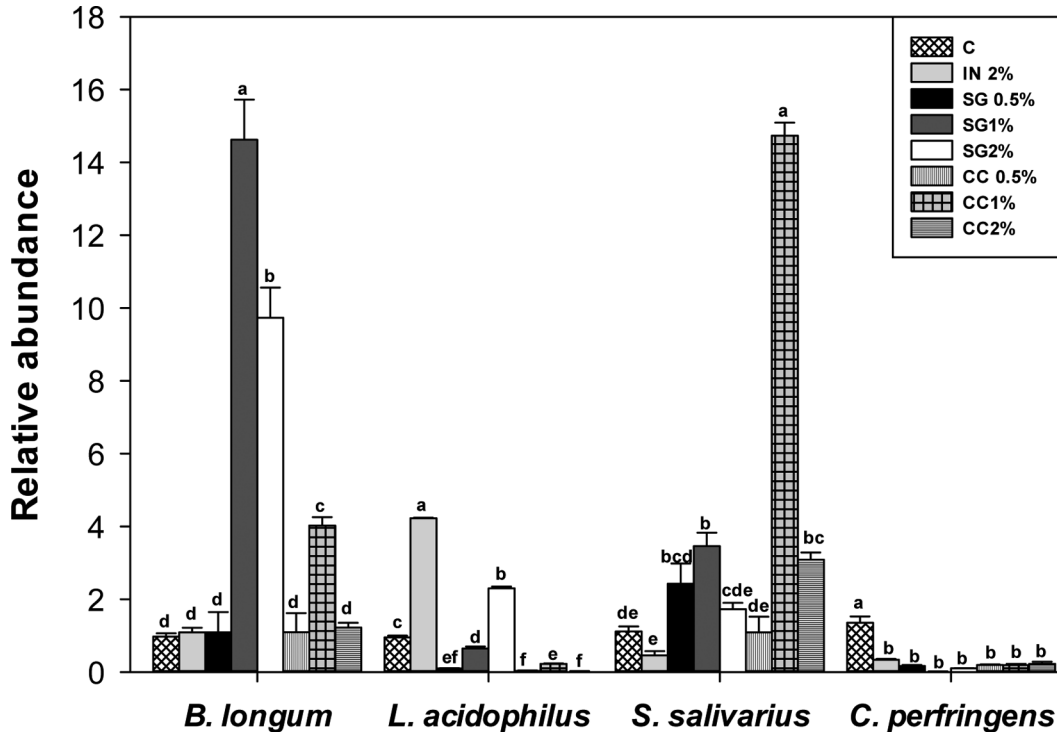
**SCFA Analysis**

Dietary supplementation with selected cultivated red seaweeds affected concentration of some SCFA digesta contents (Figure 2). The concentration of acetic acid was greater ( $P = 0.02$ ) in the cecal digesta of those birds fed 1% SG and CC (52.21 and 51.53 mmol/kg, respectively), compared with the basal diets (29.94 mmol/kg) or inulin (36.11 mmol/kg). Also, the concentrations of propionic acid, *n*-butyric acid, and *i*-butyric acid increased by 2- to 3-fold in SG and CC treatments ( $P = 0.01$ ,  $P = 0.01$ , and  $P = 0.04$ , respectively). No significant differences in the concentration of *n*-valeric and *i*-valeric acids were observed in any of the treatments (Figure 2).

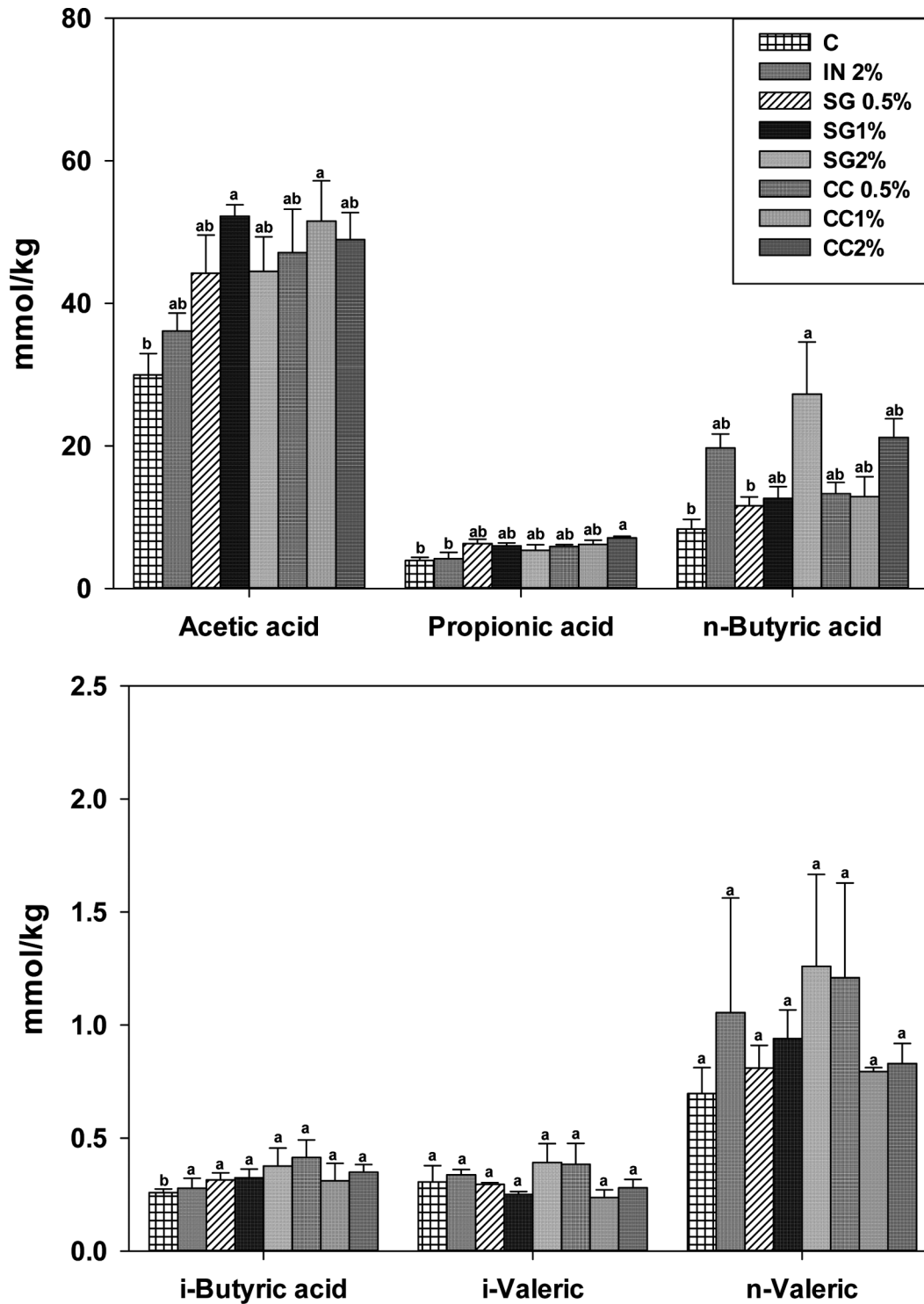
**DISCUSSION**

Supplementation of layer feed with 2 red seaweeds, *Chondrus crispus* and *Sarcodiotheca gaudichaudii*, resulted in a significant improvement in layer performance and an increase in the population of beneficial bacteria with a decrease in the abundance of pathogenic *Clostridium perfringens* in the gut (Table 2 and Figure 1). The role of gut microbiota in animal health is well documented. The inclusion of an oligosaccharide-rich diet, like seaweeds, in animal feed improved growth performance, immune status, and gut microbiota (Strand et al., 1998). The change in gut microbiota are largely caused by complex polysaccharides in the feed that are resistant to acid hydrolysis in the upper gastrointestinal tract of higher animals (O’Sullivan et al., 2010); many of these polysaccharides are then fermented in the lower gastrointestinal tract by beneficial bacteria.

In the present study, addition of red seaweeds in the feed did not affect feed intake in layer hens, and similar results were observed in other studies in which the feed



**Figure 1.** Real-time PCR quantification of microbial population in the ileal contents of layer hens. Relative abundance of *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Streptococcus salivarius*, and *Clostridium perfringens* in the chicken ileum. Error bars indicate SE, and different letters (a-f) indicate significantly different mean values ( $P < 0.001$ ). C = control; IN = inulin; SG = *Sarcodiotheca gaudichaudii*; CC = *Chondrus crispus*.



**Figure 2.** Concentration of short-chain fatty acids (mmol/kg) in the cecal digesta of layer hens. Data are representative of mean measurements from 4 individual chicken cecal digesta. Error bars indicate SE, and different letters (a,b) indicate significantly different mean values. C = control; IN = inulin; SG = *Sarcodiotheca gaudichaudii*; CC = *Chondrus crispus*.

was supplemented with brown seaweeds, *Macrocystis pyrifera*, *Sargassum sinicola*, and a green seaweed *Enteromorpha* sp. (Carrillo et al., 2008). In our study, although the feed intake and the egg production were not significantly different in the treatments, close examination of the data revealed that red seaweed treatments (2% CC and SG) significantly improved FCR (Table

2) and an increase in egg and yolk weight in CC1 and SG1 treatments. Previous work with mannan oligosaccharide or the red microalga *Porphyridium* sp. did not result in an increase in FCR (Ginzberg et al., 2000; Bozkurt et al., 2012). This variation may be attributed to the age or strain (or both) of birds used in the experiment and also the type of seaweed used and inclu-



sion rates, as well as the differences in the experimental conditions such as temperature and humidity (Geier et al., 2009; Zhang et al., 2012). The increase in egg and yolk weight with 1% level of seaweeds (1% CC and SG) might be attributed to higher yolk protein synthesis and an increase in water and mineral availability (Novak et al., 2004) and greater retention of feed in the gastrointestinal tract and thus a better ileal digestibility of the feed (Piray et al., 2007). Several reports indicated that dietary inclusion of seaweed improved egg albumen height and egg yolk color (Herber-McNeill and Van Elswyk, 1998; Ginzberg et al., 2000; Carrillo et al., 2008; Zahroojian et al., 2011; Carrillo et al., 2012).

*Chondrus crispus*- or *S. gaudichaudii*-supplemented feed had no effect on the weight of the spleen, heart, ileum, or liver of birds. However, a significant increase in the weight of ceca was observed. An increase in the weight of the ceca correlated with an increase in the population of beneficial bacteria in the ceca. Cecal weight is an indicator of fermentation activity in the ceca (Oyarzabal and Conner, 1996). A significant increase in cecal weight was observed in broilers fed with direct-fed microbial and fructo-oligosaccharide, and a subsequent reduction in *Salmonella* infection was observed (Oyarzabal and Conner, 1996).

Seaweed-supplemented feed induced changes in the histomorphology of the small intestine; there was a significant increase in villi height and crypt depth (Table 3). The intestine is the major site of enzymatic digestion, and absorption of nutrients, efficiency of absorption, and hence FCR largely depends on the histomorphology of the intestine (Awad et al., 2008). Previous studies have shown that the intestinal histomorphology, specifically structures such as villi, crypts, and the thickness of mucosa, were altered by the composition of diet (Samanya and Yamauchi, 2002; Nain et al., 2012). The increase in villi height and crypt depth is associated with healthy turnover of epithelial cell and active cell mitosis (Fan et al., 1997; Samanya and Yamauchi, 2002). There is evidence suggesting a healthy gut microbiota aid healthy turnover of intestinal epithelial cells and reduced inflammation caused by pathogens and toxins (Baurhoo et al., 2007).

In this study, the relative abundance of probiotic bacteria *Bifidobacterium longum* and *Streptococcus salivarius* were higher in birds fed with 1% CC, 1% SG, and 2% SG. This result was similar to a previous report on the effect of dietary mannan oligosaccharides, galactoglucomannan oligosaccharide-arabinoxylan, and dried whey in layer and broiler chickens (Baurhoo et al., 2007; Donalson et al., 2008; Faber et al., 2012). Beneficial microorganisms suppress the growth of pathogens by mechanisms such as competitive exclusion, secretion of SCFA, and antimicrobial peptides as well by priming host immune system (Torok et al., 2008). Interestingly, a negative correlation was observed between *Bifidobacterium* sp. and *Clostridium perfringens*, suggesting *Bifidobacterium* sp. as a suppressor of *C. perfringens* (Gibson et al., 2005). Taken together, the dietary red

seaweeds *C. crispus* and *S. gaudichaudii* increased epithelial cell turnover by altering the gut microbiota in favor of the beneficial bacteria. These results suggested that dietary supplementation with seaweeds may increase the population of probiotic bacteria and at the same time reduced the abundance of harmful enteric bacteria.

A change in the gut microbiota affects microbial SCFA. Short-chain fatty acids are major end products of microbial fermentation, which stimulate gut fluid absorption and also have proliferative effects on colonocytes (Mountzouris et al., 2007). The concentration of SCFA in the intestine largely depends on the fermentative substrate and microbial diversity in the gut (Cummings and MacFarlane, 1991). Seaweed in the feed significantly increased the concentration of acetate and propionate in the cecum (Gomez-Ordóñez et al., 2012). Similarly, Deville et al., (2007) reported that laminarin, a brown seaweed polysaccharide, increased mucosal absorption and butyrate production. Our results on the concentration of SCFA are in agreement with these published studies. Acetic acid is primarily produced by cellulose fermentation by *Bacteriodes* and *Bifidobacterium* (Apajalahti et al., 2001). Thus, the higher abundance of *Bifidobacterium longum* in birds fed with seaweed might have contributed to the increased concentration of acetic acid. The higher concentration of acetate, butyrate, and propionate observed in our study were also consistent with previous findings in layer and broiler chickens (Apajalahti et al., 2001; Donalson et al., 2008; Rehman et al., 2008; Faber et al., 2012). Butyric acid acts as an energy substrate for epithelial growth and regulates cellular differentiation, growth, permeability, and gene expression. Moreover, SCFA lower pH of ceca resulting in reduction in the growth of pathogens such as *Salmonella* (Hinton et al., 1990). Faber et al. (2012) correlated the concentration of cecal SCFA to the increase in cecal weight. These authors suggested that SCFA acted as the main energy source for intestinal epithelial cells and stimulated cell growth, resulting in an increase in intestinal weight (Frankel et al., 1994; Faber et al., 2012). Thus, in our study, it is possible that the higher concentration of SCFA could have resulted in higher cecal weight.

In conclusion, supplementation of layer feed with the red seaweeds *Chondrus crispus* and *Sarcoditheca gaudichaudii* improved the performance of layers possibly as a result of an increase in the population of beneficial bacteria and a reduction of pathogenic bacteria in the gut, improvement in villi height, crypt depth, and an increase in the concentration of SCFA. Thus, the cultivated strain of *C. crispus* and *S. gaudichaudii* could be used as a prebiotic for layer hens.

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