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Original Article

Investigation of Egg Productivity, Egg Quality, Egg Storability, Organ Characteristics, Gut Microbiota, and Health Parameters with Different Addition Levels of Fermented Allium Tuberosum

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ABSTRACT

Allium tuberosum (Chinese chives; CC) contains large amounts of metabolites that have antibacterial, antioxidant, and anti-inflammatory properties. In our previous study, fermented CC was shown to improve physiological functions and have positive results in broilers. In this study, we investigated egg productivity, egg quality, blood parameters, organ and intestinal characteristics, and gut microbiota by adding solid-state fermented CC (FCC) to laying hen diets. A total of 120 laying hens (40-weeks-old) were randomly assigned to one of the three dietary treatment groups: (1) basal diet + carrier mixture (CM) 1.5% (CC juice concentration 0%; CON); (2) basal diet + 0.5% FCC + 1.0% CM (CC juice concentration 0.1%; FCC 0.1%); (3) basal diet + 1.5% FCC (CC juice concentration 0.3%; FCC 0.3%). Each treatment was designed with 10 replicates, and 4 hens were assigned to each replicate. The experiment lasted for 5 weeks. As a result, the FCC 0.1% group had a heavier egg weight, and the FCC 0.3% group had higher feed intake ($p<0.05$). However, feed conversion ratio (FCR) was similar in all treatments ($p>0.05$). Egg quality was also not observed to be affected by the supplementation of FCC ($p>0.05$). However, interestingly, when eggs were stored for 4 weeks, FCC treatment hens showed lower malondialdehyde (MDA) concentrations than CON ($p<0.05$). Although the content of blood components was similar ($p>0.05$), FCC 0.1% affected ileum length and potential pathogen growth ($p<0.05$). This study suggests that FCC as a feed additive for laying hens improves egg productivity, and storage period, and helps maintain health.

INTRODUCTION

Antibiotic feed additives have been extensively used in the livestock industry to meet increased demand for animal-derived products due to the growing population since the 1960s (Mingmongkolchai & Panbangred, 2018). The original purpose of antibiotics was the management of clinical infectious diseases as chemotherapeutic agents. However, antibiotics were revealed to be effective in improving industrial growth performance, feed efficiency, mortality, and quality of animal products in livestock animals at a sub-therapeutic dosage, and began to be used as feed additives worldwide (Moore *et al*., 1946; Chattopadhyay, 2014). Despite providing these beneficial effects for both animals and producers from an economical perspective, the ban on the use of antibiotic feed additives is expanding worldwide, with growing concerns about the emergence of antimicrobial resistance, which is one of the biggest threats to global health and food security nowadays (Vanaerschot *et al*., 2020; Moon *et al*., 2021). Therefore, research for feed resources, additives, feeding management, and welfare has been focused on improving or maintaining productivity without conventional anti-microbial substances.

Phytogenic feed additives (PFAs) are one of the economically ideal products with confirmed positive effects on productivity performance, intestinal health, and antioxidant and anti-inflammatory status of poultry through numerous studies over the past few years (Kothari *et al*., 2019; Singh *et al*., 2020; Abdelli *et al*., 2021). The PFA itself could exert antioxidant, antimicrobial, anti-viral, and anti-inflammatory activity, and has this has been shown to improve feed intake, digestive enzyme secretions, enhance immunity, and regulate gut microbiota in poultry (Abou-Elkhair *et al*., 2018; Kothari *et al*., 2019; Sharma *et al*., 2020; Abdelli *et al*., 2021). In this context, the genus *Allium*, consisting of approximately 915 species, holds exorbitant potential due to the presence of a variety of bioactive compounds, including polyphenols, saponins, organosulfur compounds (OSCs), fructans, and fructo-oligosaccharides (FOS) (Teshika *et al*., 2019; Kothari *et al*., 2020). Several studies have reported the benefits of using *Allium* sp. (in particular garlic and onion) in poultry diets, with growth-improving, antioxidant activity, and immune-modulating properties (Lee *et al*., 2016; Omer *et al*., 2019; Ismail *et al*., 2021). Furthermore, natural herbal plants such as garlic, onion, moringa, and several seeds have been reported to have positive effects on livestock due to their flavonoids and other phenolic compounds (Dehshahri *et al*., 2012; Abdelrahman *et al*., 2015; Lee *et al*., 2020).

Allium tuberosum (Chinense chive, CC) is also a plant belonging to the *Allium* plant genus, and is a natural product used worldwide as food, spice, and herbal medicine, similarly to garlic, onion, chives, and rakkyo (Lee *et al*., 2022). This plant contains various major bioactive components such as organosulfur compounds (OSCs) and polyphenols, and is widely used in folk medicine due to its excellent antioxidant and antibacterial properties (Ni *et al*., 2019; Putnik *et al*., 2019). In particular, fermentation using *Lactobacillus plantarum* is known to improve the content of metabolites such as kaempferol, isorhamnetin, and quercetin in CC, enhancing antibacterial and antioxidant activity (Kothari *et al*., 2020). For this reason, various studies have been conducted on functional feed additives using CC (Moon *et al*., 2021; Niu *et al*., 2020; Lee *et al*., 2022). However, there are few research cases that analyze changes in productivity, livestock product quality, and differences in body condition when fermented CC are applied used in livestock feed.

Therefore, in this study, the effect of adding solid-state fermented CC (FCC) in the diet of laying hens was investigated. Egg productivity, egg quality, malondialdehyde (MDA) difference between stored eggs, blood parameters, organ and intestinal characteristics, and gut microbiota were investigated according to the addition level. These results are expected to prove that FCC is an effective feed additive for maintaining productivity, and improving egg quality and health status in laying hens.

MATERIALS AND METHODS

Ethical statement

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (Approval number: KU21192) of Konkuk University (Seoul, Republic of Korea).

Feed additives and feed composition

The CC were purchased from a local market (Gwangjin-gu, Seoul, Republic of Korea) and CC juice was prepared by squeezing it using a juicer (Angel-7700, Angel Juicer, Busan, Republic of Korea). In addition, the fermentation strain used in this study was *L. plantarum* SK4719, a microorganism that was isolated from CC juice during our previous research, and used to manufacture fermented feed additives for broilers (Kothari *et al*., 2020; Lee *et al*., 2022). Briefly, *L. plantarum* SK4719 was initially inoculated into de Man Rogosa Sharpe (MRS, Difco, USA) culture medium and cultured at 30°C and 100 rpm for 18 h. After washing three times with sterilized distilled water, the same volume of sterilized distilled water as the MRS culture medium used during culture was dispensed. Afterwards, feed additives for each treatment group were prepared by mixing each raw material at the ratio shown in Table 1, which also contains ingredient analysis results. FCC was mixed and packaged in a rigid plastic container and incubated at 30°C for 24 h (number of *L. plantarum* SK4719 in final product: over 10^7 colony forming units (CFU)/mL).

The basal diet for all treatments was based on NRC (1994), and a corn-soybean meal-based diet was formulated to meet or exceed the nutritional requirements of laying hens (Table 2).

Table 1 – Ingredients and chemical composition of the experimental additive[1].

Items	CM	FCC
Material composition (%)		
Solid carrier[2]	50	50
Distilled water	45	20
Molasses	5	5
CC juice	-	20
Lactobacillus plantarum SK 4719 bacterial culture	-	5
Total	**100**	**100**
Crude protein, %	8.48	8.81
Crude fat, %	1.98	2.25
Crude fiber, %	2.15	1.94
Ash, %	5.21	2.55
Ca, %	0.12	0.18
Total phosphorus, %	0.34	0.34
Sulfur, %	0.20	0.20
Met+Cys[3], %	0.03	0.30
Gross energy, kcal/g	2,018	2,144

[1]Abbreviations: CM = carrier mixture; FCC = solid-state fermented Chinese chives
[2]Solid carrier (%): corn (53.8) + soybean meal (10.2) + corn gluten meal (15.4) + distiller's dried grains with solubles (12.8) + rice bran (7.7) + dry yeast (0.1).
[3]Met: Methionine; Cys: Cysteine.

Table 2 – Ingredients and chemical composition of the basal diet.

Ingredients, %	Amount, %
Corn	61.44
Soybean meal	16.10
Canola meal	6.500
Corn gluten meal	3.150
Tallow	1.050
Salt	0.280
Syn-Lysine, 25% (liquid)	0.264
Mono-dicalcium phosphate (MDCP)	0.667
L-Methionine, 100%	0.066
Limestone	10.11
Choline-Cl 50%	0.009
Phytase	0.050
$NaHCO_3$	0.051
Vitamin mix[1]	0.105
Mineral mix[2]	0.160
Total	**100.00**
Calculated nutrient values	
Crude protein, %	16.00
Ether extract, %	3.70
Crude fiber, %	2.80
Ca, %	4.00
Phosphorus, %	0.49
Available phosphorus, %	0.22
Cys+Met[3], %	0.67
Ash, %	12.40
TMEn[4], %	2,780

[1]Vitamin mixtures provided the following nutrients per/diet: vitamin A, 9,450 IU; vitamin D3, 2,310 IU; vitamin E, 15.75 IU; vitamin K3, 2.1 mg; vitamin B1, 1.575 IU; vitamin B2, 5.25 mg; vitamin B6, 3.15 mg; vitamin B12, 0.021 mg; pantothenic acid, 10.5 mg; niacin, 31.5 mg; biotin, 0.16 mg; folic acid, 1.05 mg.
[2]Mineral mixture provided the following nutrients per/diet: Fe, 42 mg; Co, 0.05 mg; Cu, 42 mg; Mn, 63 mg; Zn, 52.5 mg; I, 1.05 mg; Se, 0.11 mg.
[3]Cys: Cysteine; Met: Methionine.
[4]TMEn: nitrogen corrected true metabolizable energy.

Experimental animal and design

A total of 120 ISA Brown laying hens (40-weeks-old) were randomly assigned to three treatment groups of 4 birds, in 10 replicates. The experimental facility was a 1-tier battery-cage with a length of 43 cm, a depth of 45 cm, and a height of 42 cm, and two birds were raised per cage (two cages per replicate). Three treatments were designed based on the final CC juice concentration in the feed for each treatment, as follows: (1) basal diet + 1.5% CM (CC juice concentration 0%; CON); (2) basal diet + 0.5% FCC + 1.0% CM (CC juice concentration 0.1%; FCC 0.1%); (3) basal diet + 1.5% FCC (CC juice concentration 0.3%; FCC 0.3%). Each experimental diet was mixed using a feed mixer (DKM-350SU, DAE KWANG CO., LTD., Hwaseong-si, Korea) for 10 min.

All hens were allocated into cages randomly and housed for a 2-weeks-long adaptation period during which they were fed the basal diet without any experimental additive and the egg production rate of hens was measured in each cage. After the adaptation period, all hens were weighed and assigned to one of the three dietary treatment groups to equalize the initial egg production rate (89.44 ± 0.07%) and BW (1982.50 ± 5.02 g) between the treatment groups under a completely randomized design (CRD). The experimental period lasted for 5 weeks (43 to 47 weeks of age). During the entire experimental period, feed in mash form and water were provided *ad libitum*. The environmental conditions were maintained at controlled temperature (23 ± 2°C), humidity (25 ± 1%), and light (16L:8D) settings throughout the entire experimental period.

Egg productivity

The number of eggs laid by birds in each replicate was noted every day at 10 am and expressed as the percentage of egg production. The egg production rate was calculated from the total number of eggs laid during each week divided by the total number of hen days in that week on a replicate basis. All collected eggs were weighed on a daily basis to obtain the average weight of the eggs. Egg mass was calculated as a multiplication of the egg production rate by the average egg weight. Feed intake was calculated as the difference between the amount of supplied feed and remaining feed at a weekly basis. The feed conversion ratio (FCR) was calculated by dividing feed intake by egg mass.

Egg quality

30 eggs per treatment group (3 eggs per replicate) were randomly collected after each week and used to measure egg quality. Haugh unit, eggshell breaking strength, and egg yolk color were measured using a digital egg tester (DET6000, NABEL Co., Ltd., Kyoto, Japan). Haugh unit is calculated using the following equation: $100 \times \log(H + 7.57 - 1.7 \times W^{0.37})$, where H = albumen height (mm) and W = egg weight (g). The eggshell thickness was measured using a digital egg caliper (Digital Caliper, NABEL Co., Ltd., Mitutoyo, Japan) after removing the eggshell membrane.

Determination of malondialdehyde concentration in egg yolk

In the last week of the experiment, a total of 90 eggs (30 eggs per treatment group) were collected and stored in an incubator at 25°C for 4 weeks. Lipid peroxidation assays of the egg yolk according to egg storage were compared and analyzed by measuring thiobarbituric acid reactive substances (TBARS), following the method described by Botsoglou *et al*. (1994). Briefly, two egg yolk samples stored for 4 weeks were pooled together, homogenized, and considered as one replicate. Then, 1.5 g of pooled egg yolk was mixed with 5% aqueous trichloroacetic acid solution containing 0.8% butylated hydroxytoluene, and homogenized at 4,000 rpm for 5 min. Next, it was reacted with thiobarbituric acid reagent and incubated at 70°C for 30 mins. The mixture was cooled to room temperature and then the absorbance of the mixture was measured at 532 nm relative to the blank reaction mixture. The TBARS concentrations were calculated using MDA as the reference standard.

Sample collection

At the end of the experiment, 30 hens (10 hens per treatment group) were randomly selected from each treatment. Selected laying hens were euthanized using CO_2 gas, and then individual samples were collected to analyze blood parameters, organ and intestinal characteristics, and intestinal microbial flora.

Biochemical properties in blood plasma

After euthanasia, blood samples were collected through cardiac puncture, immediately dispensed into vacutainer serum tubes (BD, New Jersey, USA), and stored at 4°C. The collected blood was then centrifuged at 1,500 rpm for 10 min to collect serum and stored at -20°C until analysis. As analysis indicators, total cholesterol (TCHO), high-density lipoprotein-cholesterol (HDL-C), triglyceride (TG), and lactate dehydrogenase (LDH) were analyzed. These were analyzed using an automated clinical chemistry analyzer (FUJI DRI-CHEM 7000i, FUJIFILM Corporation, Japan), and the concentration of low-density lipoprotein + very low-density lipoprotein (LDL + VLDL) was calculated considering the concentration of TCHO and HDL-C.

Organ and intestinal characteristics

The organs collected were the liver and spleen, which are related to immune function. Each organ sample was weighed and expressed as relative organ mass (g/100 g body weight). The samples of intestinal sections were obtained from the pancreatic loop (duodenum), between the pancreatic loop and Meckel's diverticulum (jejunum), between Meckel's diverticulum and the ileocecal junction (ileum), and cecum on both sides (cecum) of the intestine. The lengths of the duodenum, jejunum, ileum, and cecum were expressed as relative length (cm/100 g of body weight).

Viable microbial cell counts in cecal microbiota

The number of viable bacteria in the cecum was measured with the standard agar plating method using MacConkey (MAC), *Salmonella shigella* (SS), MRS, *Bifidobacteria* selective agar (BSA) and nutrient agar (NA) (Difco, Franklin Lakes, NJ, USA). This method was performed by following procedure described by Lee *et al*. (2022). Briefly, samples taken from the cecum were immediately kept on ice, transported to the laboratory, and analyzed within 24 h of collection. The mixture was suspended by mixing 1 g of sample with 9 mL of distilled water and serially diluted by repeating the same procedure. Then, 10 uL was spotted on each plate, and the dispensed medium was cultured in an incubator at 37°C for 24 or 48 h. The number of bacterial colonies was then counted and expressed as $\log_{10}$CFU/g.

Statistical analysis

The experimental data were analyzed using SAS software version 9.4 (SAS Institute, Cary, NC, USA), through the generalized linear model (GLM) procedure. Orthogonal polynomial contrasts were used to determine the linear and quadratic effects of dietary dosage of FCC considering significance difference at *p*<0.05 between treatment groups. The data of laying performance were analyzed by considering replicates as the experimental units. The mean of collected eggs for each replicate was considered the experimental unit. For the analysis of blood biochemical properties, organ and intestine characteristics, and intestinal microbiota, the individual bird was considered the experimental unit. Data were presented as the mean and standard error of means (SEM).

RESULTS

Egg productivity

Table 3 shows the supplemental effect of the FCC on the egg productivity of laying hens in our study. There was no significant difference in the egg laying rate and amount according to the addition of FCC between treatment groups (*p*>0.05). However, the average egg weight was highest in the group supplemented with FCC 0.1% (Quadratic, *p*<0.05), and feed intake increased linearly with the addition level (Linear, *p*<0.05). FCR was similar for all treatments.

Table 3 – Effect of dietary supplementation of solid-state fermented Chinese chives on the laying performance in laying hens.

Items	Treatments[1]			SEM	*p*-value	
	CON	FCC 0.1%	FCC 0.3%		Linear	Quadratic
Egg production rate, %	90.97	91.22	94.79	1.451	0.262	0.749
Average egg weight, g	58.75[b]	61.18[a]	59.13[b]	0.372	0.888	0.003
Egg mass, g/hen/d	53.45	55.74	56.03	0.882	0.295	0.463
Feed intake, g/hen/d	111.93[b]	116.66[ab]	118.07[a]	1.102	0.032	0.234
FCR, g feed/g egg	2.102	2.112	2.116	0.030	0.863	0.936

[1]CON: basal diet with 1.5% CM; FCC 0.1%: basal diet with 0.5% FCC + 1.0% CM; FCC 0.3%: basal diet with 1.5% FCC.
[a-b]Means within a same row with different letters differ significantly (***p***<0.05).

Egg quality

The egg quality results of laying hens according to FCC addition levels are summarized in Table 4. The analyzed indicators (Haugh unit, egg yolk color, eggshell breaking strength, and eggshell thickness) were all similar regardless of the amount of FCC added (*p*>0.05).

Table 4 – Effect of dietary supplementation of solid-state fermented Chinese chives on egg quality in laying hens.

Items	Treatments[1]			SEM	*p*-value	
	CON	FCC 0.1%	FCC 0.3%		Linear	Quadratic
Haugh unit	85.28	84.71	84.92	0.501	0.834	0.691
Egg yolk color	8.05	7.93	7.92	0.040	0.253	0.396
Eggshell breaking strength, kg/cm^2	4.59	4.50	4.57	0.066	0.977	0.577
Eggshell thickness, mm	0.42	0.43	0.42	0.003	0.873	0.409

[1]CON: basal diet with 1.5% CM; FCC 0.1%: basal diet with 0.5% FCC + 1.0% CM; FCC 0.3%: basal diet with 1.5% FCC.

Malondialdehyde content of stored eggs

Table 5 shows the difference in MDA levels after the storage of eggs in each treatment group at room temperature for 4 weeks. Interestingly, the FCC groups had lower MDA levels than the CON group (Linear, *p*<0.05).

Table 5 – Differences in MDA content in eggs stored for 4 weeks according to the addition level of solid-state fermented Chinese chives.

Items	Treatments[1]			SEM	*p*-value	
	CON	FCC 0.1%	FCC 0.3%		Linear	Quadratic
MDA, ug/g yolk	0.527[a]	0.491[b]	0.473[b]	0.008	0.004	0.220

[1]CON: basal diet with 1.5% CM; FCC 0.1%: basal diet with 0.5% FCC + 1.0% CM; FCC 0.3%: basal diet with 1.5% FCC.
[a-b]Means within a same row with different letters differ significantly (*p*<0.05).

Blood biochemical properties

The biochemical properties of the blood plasma of laying hens are shown in Table 6. Total cholesterol content, HDL, LDL+VLDL, triglyceride, and LDH contents were not affected by FCC supplementation (*p*>0.05).

Table 6 – Effect of dietary supplementation of solid-state fermented Chinese chives on the blood biochemical properties of laying hens.

Items	Treatments[1]			SEM	*p*-value	
	CON	FCC 0.1%	FCC 0.3%		Linear	Quadratic
TCHO, mg/dL	132.3	119.2	134.8	5.39	0.681	0.253
HDL-C, mg/dL	56.17	46.83	54.17	2.49	0.981	0.120
LDL+VLDL, mg/dL	76.17	72.33	80.67	4.32	0.611	0.593
TG, mg/dL	2483.3	2136.7	2306.7	136.0	0.738	0.357
LDH, mg/dL	6860.0	7086.7	7340.0	256.4	0.481	0.910

[1]CON: basal diet with 1.5% CM; FCC 0.1%: basal diet with 0.5% FCC + 1.0% CM; FCC 0.3%: basal diet with 1.5% FCC.

Organ and intestinal characteristics

Results for the organ characteristics are shown in Table 7. The weight of liver and spleen was similar in all treatments. No significant differences were observed in the duodenum, jejunum, and cecum (*p*>0.05), but the length of the ileum was shortest in the FCC treatment group (Quadratic, *p*<0.05).

Table 7 – Effect of dietary supplementation of solid-state fermented Chinese chives on the organ and small intestinal characteristics of laying hens.

Items	Treatments[1]			SEM	*p*-value	
	CON	FCC 0.1%	FCC 0.3%		Linear	Quadratic
Liver, g/100 g of BW	2.48	2.20	2.18	0.061	0.066	0.163
Spleen, g/100 g of BW	0.08	0.09	0.09	0.004	0.690	0.416
Duodenum, cm/100 g of BW	1.48	1.38	1.37	0.040	0.332	0.508
Jejunum, cm/100 g of BW	3.06	2.90	2.76	0.069	0.541	0.101
Ileum, cm/100 g of BW	3.47[a]	3.03[b]	3.13[b]	0.072	0.098	0.030
Cecum, cm/100 g of BW	1.62	1.47	1.55	0.046	0.682	0.198

[1]CON: basal diet with 1.5% CM; FCC 0.1%: basal diet with 0.5% FCC + 1.0% CM; FCC 0.3%: basal diet with 1.5% FCC.
[a-b]Means within a same row with different letters differ significantly (*p*<0.05).

Viable microbial cell counts in cecal microbiota

Table 8 shows the distribution of microbes in the cecum for each treatment group. In the case of MAC, *Escherichia coli* selective media, the FCC 0.1% treatment group showed the lowest number of viable bacteria (Quadratic, $p<0.05$), and the number of microorganisms observed in MRS and NA was also lowest in the FCC 0.1% treatment group (Quadratic, $p<0.05$). However, the number of microorganisms observed in SS and BSA media was similar for all treatments ($p>0.05$).

Table 8 – Effect of dietary supplementation of solid-state fermented Chinese chives on cecal microbiota in laying hens.

Items	Treatments[1]			SEM	*p*-value	
	CON	FCC 0.1%	FCC 0.3%		Linear	Quadratic
	log CFU/g					
MAC	6.57^{a}	5.63^{b}	5.79^{ab}	0.157	0.139	0.043
SS	6.17	5.81	5.77	0.155	0.505	0.636
MRS	9.00^{a}	8.39^{b}	8.68^{ab}	0.080	0.189	0.002
BSA	7.10	7.19	7.19	0.103	0.775	0.777
NA	8.97^{a}	8.39^{b}	8.46^{b}	0.085	0.024	0.014

[1]CON: basal diet with 1.5% CM; FCC 0.1%: basal diet with 0.5% FCC + 1.0% CM; FCC 0.3%: basal diet with 1.5% FCC.
[a-b]Means within a same row with different letters differ significantly ($p<0.05$).

DISCUSSION

Generally, *Allium*-based phytobiotics have been recognized to improve production performances in hens by many studies, including laying performance (Damaziak *et al*., 2017; Omer *et al*., 2019; Abad *et al*., 2020; Kothari *et al*., 2021; Moon *et al*., 2021). Abad *et al*. (2020) reported that supplementation of commercial *Allium* extracts increased the egg weight and egg productivity of laying hens. In the studies by Damaziaki *et al*. (2017) and Omer *et al*. (2019), *A. sativum*, *A. cepa*, and their mixture improved the general health parameters, egg weight, and feed efficiency. Indeed, the genus *Allium* stores selenium compounds that can be absorbed into egg yolk (Kothari *et al*., 2019). These studies may support the finding that egg weight was higher in the FCC 0.1% supplemented diet in our study, which may be due to the absorption of compounds present in FCC into the egg yolk and egg white, leading to increased egg weight. Furthermore, the improvement of feed palatability in poultry fed with phytogenic additives consisting of *Allium* spp. has been reported by numerous studies (Goodarzi *et al*., 2014; Kothari *et al*., 2021; Moon *et al*., 2021). Our previous study reported that supplementation of a phytogenic blend containing CC juice at 0.3% of dosage improved feed intake without affecting other performance parameters in laying hens (Moon *et al*., 2021). A study by Kothari *et al*. (2020) reported that microbial fermentation could increase the palatability of livestock by reducing the tannin content of CC. In our study, feed intake increased as the added level of FCC increased. This is believed to be due to the increased feed palatability of laying hens due to microbial fermentation and the addition of PFA.

One of the beneficial effects of photobiotic antioxidants, which have been proposed as natural food preservatives, is their ability to inhibit oxidation and enhance food freshness more effectively than synthetic antioxidants (Al-Harthi, 2014). Several researchers reported that egg quality, including Haugh units, improved when natural products such as brown algae, papaya, pine needles, and CCs were added to laying hen feed (Al-Harthi, 2014; Moon *et al*., 2021; Dissa *et al*., 2023). However, interestingly, in some studies, the addition of natural products containing functional substances in the feed of laying hens had no effect on improving egg quality or, on the contrary, resulted in its decrease (Saki *et al*., 2014; Park *et al*., 2018; Kothari *et al*., 2021). Additionally, egg quality has shown different results depending on the level of addition of natural products (Abou-Elkhair *et al*., 2018; Dilawar *et al*., 2021). Moon *et al*. (2021) noted that even if the same PFA is supplemented, the quality improvement effect varies depending on livestock breed, age, rearing environment, and amount of additives used. In our study, despite varying the level of use and addition of natural products, there was no positive effect on egg quality. Considering this, it seems necessary to conduct research on the appropriate addition levels to improve egg quality.

During food storage, lipid oxidation is a particularly important factor affecting quality (Radwan Nadia *et al*., 2008). As polyunsaturated lipids are oxidized, hydrogen peroxide is formed (Radwan Nadia *et al*., 2008). This has a negative impact on the overall quality of the food, including flavor and taste, and can cause a decrease in nutritional value and the production of toxins (Radwan Nadia *et al*., 2008). Meanwhile, natural products with abundant antioxidant effects can be a potential means to increase the storage capacity of livestock products by reducing lipid peroxidation (Kara *et al*., 2016). MDA, a lipid peroxidation product, is an important biomarker that can evaluate oxidation status (Xie *et al*., 2019). A decrease in MDA and an increase in the activity and mRNA expression of antioxidant enzymes mean that the antioxidant capacity of laying hens is improved (Xie *et al*., 2019). In our study, when comparing MDA levels in egg yolk of eggs stored for 4 weeks, the FCC-treated group showed lower MDA content in egg yolk. Carotenoids, xanthophylls, and carotenes in egg yolk are associated with egg yolk color and act as antioxidants that prevent lipid oxidation (Saleh *et al*., 2021). These are influenced by the diet consumed by the laying hens, and plants of the genus *Allium* are known to contain large amounts of xanthophylls (Khoo *et al*., 2011; Saleh *et al*., 2021). In this context, our study also shows that the antioxidant components in FCC consumed by laying hens reduced lipid peroxidation in egg yolk.

Supplementing poultry feed with aromatic plants is known to stimulate the digestive system by improving digestive enzyme production and strengthening liver function (Abou-Elkhair *et al*., 2014). These natural products are a cheap and easily available way to alleviate lipid deposition and liver damage in laying hens (Zhu *et al*., 2022). The liver-damage-alleviating effects of natural products can be evaluated through the accumulation of fat in the liver, and the state of inflammatory response suppression (Zhu *et al*., 2022). In addition, the liver and spleen are important immune organs of poultry, and many researchers have investigated the effects of using natural products with strong antioxidant activity on poultry health (Lee *et al*., 2022). In our previous study (Lee *et al*., 2022), when FCC was added to broiler diets, it had a positive effect on immune parameters by lowering spleen weight compared to CON. Additionally, in a study using garlic, another plant of the *Allium* genus, a decrease in the weight of the liver and bursa of Fabricius was reported (Lee *et al*., 2016; Ismail *et al*., 2021). However, in this study, there was no difference in the weight of the liver and spleen of laying hens despite the addition of FCC. In the future, detailed studies on liver function and immune status are needed, including liver fat content, immunoglobulin, and antibody titers,.

In our study, the length of the ileum in the FCC treatment group was shorter than in the CON group. The structure of the small intestine plays an important role in the digestion and absorption of nutrients, and it is known that the digestibility of nutrients varies depending on its length and weight (Lee *et al*., 2022). Ege *et al*. (2019) reported that feed conversion efficiency improves as small intestine length decreases. This suggests that the short intestine allocates more energy to productivity than maintenance, and absorbs and uses nutrients more efficiently. These properties can be influenced by the composition of feed ingredients or the addition of feed additives (Lee *et al*., 2022). The same fermented CCs product had no effect on the length of the small intestine in broiler chickens, but showed a heavy weight compared to body weight (Lee *et al*., 2022). From this, it is believed that the use of fermented chives has a positive effect on the intestinal and digestive characteristics of poultry.

Infection with pathogenic microorganisms causes intestinal damage and diarrhea, as well as economic losses in the global poultry industry (Huang *et al*., 2022). Pathogenic microorganisms that infect poultry cause impaired nutrient absorption, decreased poultry productivity, and pose a serious threat to human health through the livestock products (Huang *et al*., 2022). Meanwhile, many studies have reported that plant-based additives inhibit the growth of bacteria such as *Salmonella* and *E. coli*, showing a similar effect to antibiotics (Bajagai *et al*., 2022). It is known that this antibacterial activity varies depending on the species of plant used, whether multiple plants are mixed, and the manufacturing process, including fermentation (Kothari *et al*., 2020; Bajagai *et al*., 2022). Kothari *et al*. (2022) reported that fermented CCs showed stronger antibacterial activity than unfermented CCs, and revealed that the content of substances with antibacterial activity was improved through fermentation (Kothari *et al*., 2020). This can also be explained by the results of the previous study on the ability of feed additives containing fermented chives and non-fermented chives to inhibit intestinal pathogens in broiler chickens (Lee *et al*., 2022). A study by Park *et al*. (2016) also revealed that when fermented vegetable additives were offered, the intestinal *E. coli* population decreased as the addition level increased. When looking at our results from this perspective, it appears that the functional substances exerted anti-pathogen action and improved the health of laying hens that consumed FCC.

Blood characteristics are important indicators of the stability of feed additives for livestock (Lee *et al*., 2022). Among them, LDH, along with AST and ALT, are indicators of abnormal liver function when feed additives are applied (Lee *et al*., 2022). Meanwhile, plants of the *Allium* genus have lipid- and cholesterol-lowering properties in poultry (Kothari *et al*., 2019). Due to these functions, these plants are used as lipid-lowering and hypocholesterolemic agents around the world (Kothari *et al*., 2019). Among them, garlic is known to lower cholesterol in the body by inhibiting major enzymes such as malic enzyme, fatty acid synthase, and glucose-6-phosphate dehydrogenase (Kothari *et al*., 2019). Additionally, one study found that onions, another *Allium* genus plant, increased HDL levels and lowered triglyceride levels in broiler chickens (Goodarzi *et al*., 2013). This was reported to be due to onion-derived sulfur components that oxidize the thiol compounds that exist in free form or are bound to proteins and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) required for lipid synthesis (Goodarzi *et al*., 2013). However, in our study, LDH levels were similar between experimental groups and the CON, were stable upon intake, and additive supplementation did not show a positive effect on lipid metabolism in the body. Therefore, it seems necessary to set the optimal addition amount and to explore specific mechanisms for lipid metabolism in laying hens using FCCs.

CONCLUSION

FCC supplementation positively influenced the health and nutrient digestibility of laying hens, as evidenced by a reduction in potential intestinal pathogens and a shorter ileum length. Additionally, the improvement in the shelf life of eggs, demonstrated by lower MDA content after 4 weeks of storage, suggests that FCC can be an effective and safe feed additive for enhancing egg quality and promoting hen health.

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AUTHOR CONTRIBUTIONS

Conceptualization, Kim SK; methodology, Lee WD, Moon SG, Lee AR, Kim KI and Kim SK; software, Lee WD, Moon SG, Lee AR and Kim KI; validation, Lee WD, Moon SG, Lee AR, Kim KI, Kim JI, Ga GW, Kim YG, On JY, Jeon SW, Kim SH, Choi MG and Kim SK; formal analysis, Lee WD, Moon SG, Lee AR and Kim KI; investigation, Lee WD, Moon SG, Lee AR, Kim KI, Kim JI, Ga GW, Kim YG, On JY, Jeon SW, Kim SH, Choi MG and Kim SK; resources, Lee WD, Moon SG, Lee AR, Kim KI and Kim SK; data curation, Lee WD, Moon SG, Lee AR and Kim KI; writing—original draft preparation, Lee WD, Moon SG and Lee AR; writing—review and editing, Lee WD, Moon SG, Lee AR, Kim KI, Kim JI, Ga GW, Kim YG, On JY, Jeon SW, Kim SH, Choi MG and Kim SK; visualization, Lee WD, Moon SG, Lee AR, Kim KI, Kim JI, Ga GW, Kim YG, On JY, Jeon SW and Kim SK; supervision, Kim SK; project administration, Kim SK; funding acquisition, Kim SK. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

The data are available upon request from the corresponding author.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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