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The Dietary Effects of Fermented *Chlorella vulgaris* (CBT®) on Production Performance, Liver Lipids and Intestinal Microflora in Laying Hens

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ABSTRACT : Fermented *Chlorella vulgaris* CBT® was evaluated for its effects on egg production, egg quality, liver lipids and intestinal microflora in laying hens. One hundred and eight Hy-line Brown layers (n = 108), 80 wk of age, were fed a basal diet supplemented with CBT® at the level of 0, 1,000 or 2,000 mg/kg, respectively for 42 d. Egg production was measured daily and egg quality was measured every two weeks. Five eggs from each replicate were collected randomly to determine egg quality. Egg production increased linearly with increasing levels of CBT® supplementation ($p<0.05$), although there was no significant effect of treatment on feed intake. Egg yolk color ($p<0.001$) and Haugh unit ($p<0.01$) improved linearly with increasing dietary CBT®. Hepatic triacylglycerol level was linearly decreased with increasing dietary CBT® ($p<0.05$). The supplemental CBT® resulted in linear ($p<0.001$) and quadratic ($p<0.01$) response in population of cecal lactic acid bacteria. In conclusion, fermented *Chlorella vulgaris* supplemented to laying hen diets improved egg production, egg yolk color, Haugh unit and positively affected the contents of hepatic triacylglycerol and the profiles of cecal microflora. (**Key Words :** Fermented *Chlorella vulgaris*, Laying Hen, Liver Lipid, Intestinal Microflora, Egg Quality)

INTRODUCTION

Chlorella vulgaris (*C. vulgaris*), a genus of unicellular green algae containing abundant chlorophyll is known as functional food world wide. It is a good source of protein, lipid, carotenoids, vitamins, minerals, pigment (Kay, 1991), and contains essential amino acids in excellent ratios (Borowitzka, 1988; Schubert, 1988). It has been shown to have beneficial effects in poultry, such as broilers' skin pigmentation (Lipstein and Hurwitz, 1980), immuno-modulatory activities on broilers (Kotrbácek et al., 1994), egg yolk pigmentation (Lipstein et al., 1980), and laying hens' intestinal Lactobacillius diversity (Janczyk et al., 2009). Additionally, in experimental animals, it affects growth rate (Konishi et al., 1996), and lipid contents in the liver and serum (Shibata et al., 2001). Furthermore the extracts of *Chlorella pyrenoidosa* had antibacterial activities against *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli*. The antimicrobial activity of microalgae was explained by the presence of cyclic peptides, alkaloids and lipopolysaccharides (Rania and Hala, 2008).

Several cultivation technologies of microalgal production have been introduced by researchers and commercial producers for efficient use of *Chlorella*: Open tanks, open ponds, photobioreactors and fermentation reactors (Apt and Brehrens, 1999; Tredici, 1999). Due to the fact that *Chlorella* has a peculiar algal ordor, it may have negative effect on the intake. Fermentation process can improve its flavor and make an active ingredient of *Chlorella* into a readily absorbable state. Keijiro (2011) found that dietary fermented *Chlorella* significantly improved protein digestibility compared to non-fermented *Chlorella* and remarkably increased oral intake of β-glucan and γ-aminobutyric acid in rats. It was suggested that fermenting *Chlorella* with baker's yeast and lactic acid bacteria improved the flavor and absorption of *Chlorella*.

However, information on the effects of fermented *Chlorella* on laying hens is very limited. Therefore, the objective of this study was to evaluate the dietary effects of

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a commercial product of fermented *Chlorella vulgaris* (CBT®) on egg production, egg quality, liver lipids and cecal microflora in laying hens.

MATERIALS AND METHODS

Fermented *C. vulgaris*

A commercial product, *C. vulgaris* microalgae produced by fermentation (CBT®) was obtained from Celltech, Co. Ltd, Korea. *C. vulgaris* was inoculated with baker's yeast and lactic acid bacterium. After 72 h of dry fermentation with cereal broth, fermented *C. vulgaris* then dried at low temperature and ground into powder. The chemical content of CBT® analyzed by Korea Feed Ingredients Association of corporation was as following; crude protein, 24.85%; crude fiber, 5.36%; ether extract, 2.42%; Ca, 0.20%; and P, 0.65%.

Animals and experimental design

A total of one hundred and eight 80-wk-old Hy-Line Brown laying hens were used in this study. Four replicate groups of 9 hens each (3 adjacent cages containing 3 hens/ 45×62×66 cm cage) were allotted to 3 dietary treatments in a completely randomized design. Prior to the experimental period, the birds had a 1-wk adaptation period. A corn-soybean meal-based diet (Table 1) was formulated to meet or exceed the nutrient recommendations of the NRC (1994). The treatment diets were made by adding CBT® at the level of 0, 1,000, or 2,000 mg/kg, respectively to the basal diet. Feed and water were provided *ad libitum*. A room temperature of 25±3°C and a photoperiod of 16 L:8 D were maintained throughout the experimental period. The feeding period lasted for 6 wks. All animal care procedures were approved by the Institutional Animal Care and Use Committee at Konkuk University.

Sampling and measurements

Feed intake (g/hen/d) was recorded weekly by replicate. Eggs were collected daily, and egg production and egg mass (grams of egg produced per day) were determined weekly. The mean egg weight was measured by the weekly basis, excluding cracked and soft-shell eggs. Interior and exterior qualities of eggs were measured biweekly. Eggshell thickness (without shell membrane) of the eggs was measured by micrometer (Digimatic micrometer, Series 293-330, Mitutoyo, Japan). Breaking strength of uncracked eggs was measured with an eggshell strength tester (FHK, Fujihira Ltd., Tokyo, Japan). Eggshell color, albumen height and yolk color were measured by using egg multi tester provided by TSS (Technical services and supplies Ltd, York, England), and Haugh unit was determined using the Haugh unit formula (Haugh, 1937).

At the end of experiment, 10 birds per treatment were slaughtered. The blood serum was centrifuged at 1,500 rpm for 10 min. At necropsy, the liver, spleen and abdominal fat were immediately removed and weighed. Relative weights of organ (liver, spleen) and tissue (abdominal fat) weights per 100 g body weight (BW) were calculated. Data were expressed as grams of organ per 100 g of BW. The serum, liver and cecal samples were preserved at -20°C until further analysis. The activities of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) in the serum were measured according to the colorimetric method using a GOT-GPT assay kit (GOT-GPT assay kit, Asan Pharmaceutical, Hwaseong-si, Korea).

For microbial tests, after mixing contents of ceca from the sacrificed birds, one gram of the content was adjustably weighed and transferred into a test tube containing 9 ml of salt medium. The samples were serially diluted in 10-fold steps using pre-reduced salt medium according to the technique of Miller and Wolin (1974). Bacterial total microbes were enumerated on nutrient agar (Difco, BD Science, USA), presumptive lactic acid bacteria were enumerated on MRS agar (Difco, BD Science, USA) and

Table 1. Formula and chemical composition of experimental diet

Ingredients	%
Yellow corn	59.55
Soybean meal	17.76
Wheat bran	3.00
Lupine	2.00
DDGS[1]	3.00
Rapeseed meal	2.00
Tallow	1.68
Limestone	9.13
Mono dicalcium phosphate	1.20
DL-methionine (98%)	0.13
Choline chloride (50%)	0.05
Salt	0.10
$NaHCO_3$	0.20
Vit. mixture[2]	0.10
Min. mixture[3]	0.10
Total	100.00
Calculated values	
ME (kcal/kg)	2,713
Crude protein (%)	15.50
Crude fat (%)	4.39
Ca (%)	3.70
Available P (%)	0.35
Lysine (%)	0.75
Met+cys (%)	0.65

[1] DDGS = Dried distillers grains with solubles.
[2] Vitamin mixture provided the following nutrients per kg: vitamin A, 40,000 IU; vitamin D_3, 8,000 IU; vitamin E, 10 IU; vitamin K_3, 4 mg; vitamin B_1, 4 mg; vitamin B_2, 12 mg; vitamin B_6, 6 mg; vitamin B_{12}, 20 μg; pantothenic acid, 20 mg; folic acid, 2 mg; nicotinic acid, 60 mg.
[3] Mineral mixture provided the following nutrients per kg: Fe, 30 mg; Zn, 25 mg; Mn, 20 mg; Co, 0.15 mg; Cu, 5 mg; Se, 0.1 mg.

Table 2. Effects of dietary CBT® supplementation on feed intake and production performance in laying hens[1]

Items	CBT® (mg/kg)			SEM[2]	p-value	
	0	1,000	2,000		Linear	Quadratic
Feed intake (g/d/bird)	122.4	122.9	123.0	4.11	0.915	0.960
Egg production (%)	55.4	57.7	59.0	1.20	0.031	0.722
Egg weight (g/egg)	68.9	69.0	69.0	0.61	0.920	0.981
Egg mass (g/d/bird)	39.1	39.4	39.9	0.75	0.412	0.954

[1] Values are presented as the least square of means of 4 groups (each group was composed of 3 cages, 3 hens/cage).
[2] SEM = Standard error of the means.

presumptive coliform bacteria were enumerated on MacConkey agar (Difco, BD Science, USA). Those plates incubated aerobically at 37°C for 24 h. The results obtained were presented as base-10 logarithm colony-forming units per gram of cecal content.

The contents of the cholesterol were determined by Iatroscan (MK-6 TLC/FID analyzer, Iatron Laboratories, Inc., Japan) united of couples of silica coated thin layer chromatography and a flame ionization detector. The lipids of liver and serum were dissolved and extracted by the method of Folch et al. (1957) and the contents of liver and serum lipids were detected by the method of An et al. (1997).

Statistical analysis

The experimental data were analyzed using Generalized Linear Model procedures of SAS (SAS Institute, 2002). Orthogonal polynomials contrasts were used to determine the linear and quadratic effects of the dietary CBT® supplementation according to the following general model: $Y = \mu+\alpha+\varepsilon$, where Y was the observed response variables; μ was the overall mean; α was the effect of diet and ε was the random error. For all the data, the cage lot (3 adjacent cages) was considered as an experimental unit. The alpha level used for determination of statistical significance was 0.05.

RESULTS AND DISCUSSION

Feed intake and egg production

The effects of dietary fermented *C. vulgaris* (CBT®) on feed intake and egg production are shown in Table 2. The results indicated that egg production was linearly improved as the dietary CBT® supplementation increased ($p<0.05$). Feed intake, egg weight and egg mass were not affected by the dietary treatments. Kim (2011) found that dietary supplementation of *C. vulgaris* at the level of 0.1% or above increased egg production rate and daily egg mass in laying hens. Abril et al. (2000) reported that fermented *Shizochytrium sp*. significantly increased egg production at the level of 4.8% of the diet. On the other hand, Halle et al. (2009) reported that supplementation of spray-dried *C. vulgaris* at the level of 2.5 g/kg significantly decreased feed intake without significant difference in egg production among *C. vulgaris* and control groups. Discrepant results among the experiments seemed to be due to types of algae, dosage at feeding and processing techniques. Obviously, fermentation of *C. vulgaris* seemed to have advantages in improving egg production of aged laying hens. A part of the incremental increase in egg production might due to improvement of *C. vulgaris* availability by fermenting process. The dosages of fermented *C. vulgaris*, age and the strain of hens might be considered in further work.

Egg quality

In order to compare the egg quality among the control and experimental groups, the measurements on eggs were executed every two weeks and the results are presented in Table 3. Egg yolk color was linearly improved with increasing dietary CBT® ($p<0.001$). Haugh unit increased linearly as the increasing supplemental CBT® ($p<0.01$). No significant differences were found in eggshell color,

Table 3. Effects of dietary CBT® supplementation on egg quality in laying hens[1]

Items	CBT® (mg/kg)			SEM[2]	p-value	
	0	1,000	2,000		Linear	Quadratic
Eggshell color, unit	29.2	28.7	31.2	0.82	0.132	0.167
Yolk color, Roche yolk color fan	4.18	4.70	5.00	0.11	<0.001	0.410
Eggshell strength (kg/cm^2)	2.30	2.28	2.30	0.12	0.884	0.933
Eggshell thickness (0.01 mm)	33.4	33.5	33.7	0.41	0.561	0.885
Haugh unit	74.5	83.1	84.8	2.15	0.008	0.220

[1] Values are presented as the least square of means of 4 groups (each group was composed of 3 cages, 3 hens/cage).
[2] SEM = Standard error of the means.

Table 4. Effects of dietary CBT® supplementation on relative weight of organs and blood parameters in laying hens[1,2]

Items	CBT® (mg/kg)			SEM[3]	p-value	
	0	1,000	2,000		Linear	Quadratic
Liver (g/100 g BW)	1.98	1.94	1.70	0.15	0.171	0.618
Spleen (g/100 g BW)	0.11	0.12	0.09	0.02	0.404	0.389
Abdominal fat (g/100 g BW)	4.11	4.57	3.31	0.53	0.234	0.252
GOT (IU/L)	119.5	115.9	113.1	8.13	0.577	0.968
GPT (IU/L)	8.76	7.34	8.26	0.82	0.665	0.273

[1] Values are presented as the least square of means of 4 groups (each group was composed of 3 cages, 3 hens/cage).
[2] GOT = Glutamic-oxaloacetic transaminase; GPT = Glutamic-pyruvic transaminase.
[3] SEM = Standard error of the means.

eggshell strength and eggshell thickness among the treatments. The results were similar to those observed by Kim (2011) with dietary supplementation of 0.2% or more *C. vulgaris* in laying hens. Lipstein et al. (1980) supplemented algae meal (mainly *Chlorella*) to feed and noticed more intensive yellow color of the yolk in the experimental group. Grigorova (2005) indicated that feeding diets supplemented with *Chlorella* genus improved eggs morphological characteristics and yolk pigmentation, which are consistent with the present study. The beneficial effects on egg yolk color might be attributed to carotenoids contents in *Chlorella*. The fermentation of this substance seemed not to destroy the pigments. The single most important factor affecting the albumen quality of the freshly laid egg is the age of the bird. With advancing flock age, Haugh unit scores decrease (Williams, 1992). Although the age of the hens used in the present study was relatively old, the supplemental CBT® resulted in linear response in Haugh unit ($p<0.01$). No in-depth studies have been made on how the dietary algae improved Huaugh unit of eggs. However, since several investigators reported that growth factor in *Chlorella* (CGF) contained growth promoting substances such as S-nucleotide adenosyl peptide complex (Kanno et al., 1996; Han et al., 2002), CGF might affect protein synthesis and secretion in oviduct of laying hens.

Relative weights of various organs and blood biochemical parameters

The dietary effects of CBT® on relative weights of liver, spleen and abdominal fat and enzyme of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) are shown in Table 4. Liver, spleen and abdominal fat were not affected by the level of dietary CBT®. Kim (2011) also had not found any effects of dietary dried *C. vulgaris* on sizes of liver and spleen in broiler and laying chickens, which were similar to the results observed in this experiment. In a rat experiment, Lee et al. (2008) found liver weight was significantly less in the *Chlorella*-fed group than in the standard diet group, but no significant difference in spleen weight or epididymal and perirenal fat pad weights. These reports were in agreement with the present observation. It was revealed that vital organs did not change with the administration of fermented *C. vulgaris* and had no enlargement in liver, spleen and abdominal fat. The supplementation of dietary CBT® had no effects on activities of serum GOT and GPT among the treatment groups. The effects of dietary *C. vulgaris* on GOT and GPT have been investigated by Kim (2011) as well in which the treated birds with 0.1% and more of powdered *C. vulgaris* lowered blood GOT. It was reported that level of GOT and GPT activities were the indicative of liver damage in laying hens (Goswami and Robblee, 1958; Lumeiji, 1997). Commercial flocks with diseases caused higher activities of GOT in serum. Large amount of transaminase is released into blood mostly on liver cell damage (So et al., 2009). Based on these findings, CBT® administered at the levels evaluated in this study may not exert adverse effects on laying hens.

Lipid fractions of serum and liver

The effects of CBT® on the contents of various lipid fractions in the serum and liver of laying hens are shown in Table 5. No differences in cholesterol ester, free cholesterol, triacylglycerol and phospholipid levels appeared in the serum of CBT® fed hens compared to the control. The hepatic triacylglycerol level was linearly decreased as dietary CBT® supplementation increased ($p<0.05$). The hepatic cholesterol, free cholesterol and phospholipid were not affected by the treatments.

A previous study with laying hens fed *C. vulgaris* at the age of 71 wks old resulted in no differences in either serum or hepatic lipid fractions (Kim, 2011). The discrepancy among experiments might result from the fermentation process used and the age of laying hens. Studies on rats have shown that *Chlorella* has the ability to reduce lipid concentration in serum and liver when supplemented in a high-fat diet. Marie and Vladimir (2001) showed 1% *Chlorella* powder added in standard diet did not significantly affect lipid metabolism. However, a high-fat diet containing 1% *Chlorella* powder significantly inhibited

Table 5. Effects of dietary CBT® supplementation on lipid fractions of serum and liver in laying hens[1]

Items	CBT® (mg/kg)			SEM[2]	p-value	
	0	1,000	2,000		Linear	Quadratic
Serum lipid fractions	---------- mg/dl ----------					
Cholesterol ester	99.88	95.67	100.70	3.05	0.847	0.237
Free cholesterol	104.88	118.50	110.99	19.89	0.811	0.694
Triacylglycerol	1,667.66	1,621.45	1,603.14	264.81	0.845	0.970
Phospholipid	939.31	978.42	1,011.44	225.36	0.803	0.992
Hepatic lipid fractions	---------- mg/g ----------					
Cholesterol ester	3.92	3.90	3.84	3.89	0.105	0.645
Free cholesterol	1.49	1.42	1.33	1.42	0.051	0.895
Triacylglycerol	7.12	4.91	5.10	0.43	0.045	0.126
Phospholipid	8.95	9.30	9.41	0.81	0.702	0.901

[1] Values are presented as the least square of means of 4 groups (each group was composed of 3 cages, 3 hens/cage).
[2] SEM = Standard error of the means.

the increment of total chloresterol and triglyceride in the serum and liver than the control group. It has been referred to decreased hepatic lipogenesis and influx of fatty acyl-CoA from adipose tissue and increased VLDL secretion or both as possible mechanisms with respect to reduction in hepatic triacylglycerol. The lowering effect of fermented *C. vulgaris* on triglyceride levels, which was observed in this study, could be associated with the inhibition of hepatic fatty acid synthesis and triglyceride production, thus limiting the output of VLDL.

Cecal microflora

The dietary effects of CBT® on cecal microflora are presented in Table 6. The level of dietary CBT® exerted linear ($p<0.001$) and quadratic ($p<0.01$) effects on population of cecal lactic acid bacteria.

It is known that large numbers of bacteria are capable of digesting many algal components. The ability of a gut flora to degrade algal polysaccharides or other complex plant polymers in an animal's diet can increase its host's digestive efficiency. Among polysaccharides, the major polymers of the ingested epiphytic algae include starch, found in the green algae, and cellulose in the green, brown and red algae (Lasker and Giese, 1954; Prim and Lawrence, 1975). Lin (1969) reported that addition of *Chlorella* growth factor (CGF) to a standard growth medium increased the growth Lactobacillus by up to 400%. Janczyk et al. (2009) indicated that feeding *C. vulgaris* resulted in increased Lactobacilli diversity in crop and ceca in laying hens, which was confirmed in the present results. The beneficial effect of dietary CBT® on egg production presented in Table 2 might be related to the positive effect of dietary CBT® on intestinal microflora. Cecal lactic acid bacteria might affect pathogenic microflora such as *S. enteritidis* (Surachon et al., 2011), thus improve host's health status and productivity.

In conclusion, the present study showed that the fermented *Chlorella vulgaris* supplemented to laying hens diets improved egg producton, egg yolk color, Haugh unit, reduced contents of hepatic triacylglycerol and positively affected the profiles of cecal microflora. Longer term supplementation studies using hens of different strains with various production stages are suggested in order to clarify effects on performance.

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Table 6. Effects of dietary CBT® supplementation on cecal microflora in laying hens[1]

Items	CBT® (mg/kg)			SEM[2]	p-value	
	0	1,000	2,000		Linear	Quadratic
Total microbes, log10 (cfu/g)	7.24	6.89	7.41	0.31	0.699	0.276
Lactic acid bacteria, log10 (cfu/g)	7.13	8.21	8.27	0.13	<0.001	0.009
Coliforms, log10 (cfu/g)	5.07	4.91	5.73	0.41	0.331	0.375

[1] Values are presented as the least square of means of 4 groups (each group was composed of 3 cages, 3 hens/cage).
[2] SEM = Standard error of the means.

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