Thesis for

the Degree of Master of Science in Agriculture

The effect of fermented buckwheat on producing L-carnitine and Gamma-aminobutyric acid (GABA) enriched designer eggs

L-카르니틴 및 감마-아미노뷰티릭산이 증가된 디자이너 달걀 생산에 대한 발효 메밀의 영향

February 2017

By

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Abstract

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Gamma-aminobutyric acid (GABA) enriched designer eggs

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The potential of fermented buckwheat as feed additives was studied to increase L-carnitine and gamma-aminobutyric acid (GABA) in designer eggs. Buckwheat contains high levels of lysine, methionine, and glutamate, which are precursors for the synthesis of L-carnitine and GABA. *Rhizopus oligosporus* was used for the fermentation of buckwheat to produce L-carnitine and GABA that exert positive effects like enhanced metabolism, antioxidant activities, immunity, and blood pressure control.

A novel analytical method for simultaneously detecting L-carnitine and GABA was developed using LC/MS and LC/MS/MS. The fermented buckwheat extract contained 4 and

34 fold-increased L-carnitine and GABA, respectively, than normal buckwheat. Compared to control, the fermented buckwheat extract-fed group showed enriched L-carnitine (13.6%) and GABA (8.4%) in the yolk. However, only L-carnitine was significantly different ($p<0.05$). Egg production (9.4%), albumen weight (2.1%), and shell weight (5.8%) were significantly increased ($p<0.05$). There was no significant difference in yolk weight, total cholesterol (1.9%) and triglyceride (4.9%) in the yolk was lowered ($p<0.05$).

Fermented buckwheat as feed additives has the potential to produce L-carnitine and GABA enriched designer eggs with enhanced nutrition and homeostasis. The L-carnitine and GABA enriched designer eggs pose significance to be utilized in superfood production and supplement industries.

keywords: Buckwheat, Fermentation, *Rhizopus oligosporus*, L-carnitine, Gamma-aminobutyric acid, Designer Egg

Student Number **:** 2015 - 20006

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I. INTRODUCTION

Buckwheat (*Fagopyrum esculentum*) is a pseudocereal plant in the *Polygonaceae* family mainly cultivated for its nutrient-dense, gluten-free seeds. Besides its high concentration of flavonoids,[1] dietary fiber,[2] and micronutrients (zinc, copper and manganese),[3] these grain-like seeds also contain high levels of amino acids such as lysine, methionine and glutamate,[4] L-carnitine, which is synthesized from lysine and methionine,[5] is a quaternary ammonium compound that plays a major role in the mitochondrial oxidation of fatty acids.[6] In addition, it is an effective antioxidant and improves male fertility.[7,8] L-carnitine is commonly found in meat, fish, and mushrooms, but in low amounts in fruits, crops, and vegetables.[9,10] Lonza, the world's largest manufacturer of L-carnitine, developed a biotransformation process for the biological resolution of racemic mixtures of L-carnitine. [11-13] This process used the chemical precursor, γ-butyrobetaine, and a mutant microorganism whose L-carnitine dehydrogenase was deleted to transform into L-carnitine.

Glutamate is used for the synthesis of GABA,[14] which is a non-protein amino acid and major inhibitory neurotransmitter in the central nervous system. GABA exerts multiple positive effect on mammali an physiology, some of which include reduced anxiety blood pressure regulation, enhanced immunity,[15] and antihypertensive effects.[16] This metabolic byproduct of plants and microorganisms is abundant in lactobacillus-fermented foods like yogurt, kefir, and kimchi.[17] In the simple, one-step biosynthesis of GABA, glutamic acid decarboxylase (GAD) enzyme catalyzes the decarboxylation of glutamate to GABA.[18] This reaction holds high catalytic efficiency with environmental compatibility, and requires mild reaction conditions.[19]

L-carnitine and GABA as potential feed additives have recently gained increasing interest, particularly for the improvement of livestock production by L-carnitine and GABA metabolic functions.[20,21] Especially, there have been several reports about

transferring L-carnitine[22,23] and GABA through the poultry diet into eggs.[24,25] These are related to the concept of designer food that hold benefits other than traditional nutritional value.[26] However, previous research used either L-carnitine[22,23] or GABA[27] as the feed additive, focusing on a single effect for eggs.

In addition, the analytic methods that had been reported were not efficient for the detection of L-carnitine or GABA in foods. For the detection of GABA, pre-column ninhydrin-based derivatization for a RP-HPLC was utilized for the detection of GABA.[24] However, the Pre-column derivatization comprises a series of time-consuming steps and the ninhydrin reagent derivatizes primary and secondary amines,[28] which means the reagent cannot be used for L-carnitine, a quaternary amine compound. For the detection of L-carnitine, enzymatic methods have been applied.[22,29] These methods required additional steps such as neutralization[22] and

lyophilization,[30] followed by deproteinization. Therefore, there are limitations when applying pre-existing methods for analyzing both L-carnitine and GABA in foods such as eggs.

Rhizopus spp is one of the genera in *Mucoromycotina* that is a dominant fungus in special fermented soybean products like Indonesian tempeh. *Rhizopus* spp has been reported to synthesize L-carnitine and GABA.[5,31]

In this study, buckwheat was fermented using *Rhizopus oligosporus* (*R. oligsporus*) isolated from tempeh. After fermentation, the buckwheat extract contained increased amounts of L-carnitine and GABA and was used as a complex additive in poultry feed to determine whether L-carnitine and GABA can be transferred into the eggs. For this purpose, we developed a novel, simplified detection method for both L- carnitine and

GABA in eggs using LC/MS/MS with common solvents, simple aqueous extractions, and acetonitrile deproteinization.

II. MATERIALS AND METHODS

1. Microbial strain and culture condition

R. oligosporus, isolated from tempeh, whose microbial strain was identified using Biolog (Biolog Inc., CA, USA) and 16s rRNA in previous study[32] was used for fermentation and maintained on Potato Dextrose Agar (PDA, Difco, Detroit, MI, USA) plates at 28℃ for 2 days.

2. Preparation of fermented buckwheat

R. oligosporus was grown on PDA medium at 28℃ for spore preparation. Buckwheat (1 kg) was suspended in 650 mL of distilled water in a plastic bag with a cotton cap for aeration and autoclaved at 121℃ for 20 min. Then, 1 mL of *R. oligosporus* spore (1.6 x 10^6 mL^{-1}) was inoculated. The mixture was incubated at 28℃ for 5-7 days. Fermented buckwheat was extracted with 2 L of distilled water for 30 min and centrifuged at 6,760 x g for 20 min. The supernatant was lyophilized at -10 ~ 0℃

under 10 Pa. (Eyela FD-550, Tokyo Rikakikai Co., Japan) and stored at -20℃ for further study.

3. Analysis of the fermented buckwheat

Two of the pre-existing methods[33,34] and the novel methodology developed in this study were used for the determination of L-carnitine and GABA. First, L-carnitine was analyzed by using the L-carnitine colorimetric & fluorometric assay commercial kit and the standard protocol (Biovision, CA, USA) with a mass spectrophotometer.[33] Second, HPLC-UV was performed using pre-column derivatization with p-bromophenacyl bromide.[34] A modified method with LC/MS or LC/MS/MS was used as the following: Fermented buckwheat sample was prepared at 100 mg mL^{-1} with dilution by acetonitrile and filtered with a 0.2 μm membrane syringe filter (Sartorius AG, Germany), then 1 μL of the sample was injected into the LC/MS (Waters, MA, USA). The system used was Waters Acquity H-class with Waters QDa detector (Waters, MA.

USA) and Waters Acquity UPLC Beh Hilic 1.7 µm, 2.1 x 100 mm column. Solvent A was 15 mM ammonium formate with 0.1% (v/v) formic acid and solvent B was acetonitrile with 0.1% (v/v) formic acid. The sample and column temperatures were 10°C and 40°C, respectively. The following elution gradient was applied: 0–3min, 10% A; 3.1–5min, 10–30% A; 5.1–6min, 30–60% A; then a 4 min equilibrium step. Electrospray ionization (ESI) positive and selective ion recording (SIR) mode (162 m/z for L-carnitine and 104 m/z for GABA) were used. The capillary energy was 1.5 kV and the cone voltage was 10 V for L-carnitine and 5 V for GABA. 90% acetonitrile (v/v) was used as blank. Calibration curves were prepared with external standard method with L-carnitine concentration from 0.01 to 1 µg mL^{-1} and GABA concentration from 0.1 to 10 µg mL^{-1}, and the linearity between concentration of standards and area was evaluated ($r^2 > 0.99$). Then, recovery was confirmed by standard addition technique using three addition levels in order to determine the matrix effect of buckwheat or

fermented one on quantification. Each recovery was analyzed by t-test in SPSS (V.23.0 for Windows, SPSS, Chicago, IL) to evaluate significant difference from 100% ($p < 0.05$) (Table S1).

Table S1 Recovery of the L-carnitine and GABA in each samples by standard addition technique

System	Type	Sample	Recovery (%)[ab]
LC/MS	L-carnitine	Buckwheat	101.1 ± 9.3
	GABA		107.3 ± 8.0
	L-carnitine	Fermented buckwheat	100.6 ± 4.5
	GABA		97.3 ± 9.6
LC/MS/MS	L-carnitine	Egg	106.7 ± 4.5
	GABA		100.7 ± 5.7

[a] Recovery was calculated by three addition level in each type of samples and indicated by mean ± standard deviation (n = 3)

[b] Recovery was analyzed by t-test ($p<0.05$) and no significant difference was detected from 100%.

4. The feeding experiment with Hy-Line brown hens

The feeding experiment with Hy-Line brown hens was conducted at the animal facility in Seoul National University (Pyeongchang, Korea) under the institutional guidelines with prior approval from the Institutional Animal Care and Use Committee (IACUC number: SNU-140930-3). A total of forty, 20-week old Hy-Line brown hens were treated for 4 weeks. These laying hens were divided into 2 groups of control and treatment, each constituting 20 hens and individually housed into 0.05 x m^2 space with ambient temperature (20°C) and 50~55% relative humidity. Light was provided for 16 h (from 05:00 to 21:00). The fermented buckwheat extract was fed with 16 g kg^{-1} feed (1.6%, w/w) during 4 weeks for treatment group. The general nutritional content of common forage was analyzed by Cargill Agri Purina, Inc (Pyeongchang, Korea) (Table S2). Prior to the sampling period, the laying hens were fed as the above procedure for 1 week. After this period, the eggs were collected by hand-picking every 2 days for 3 weeks.

Table S2 Chemical composition of poultry forage

Test Name	Analyte	Result (w/w)
Ash 600℃ 4 h	Ash	7.49 %
Calcium	Ca	2.00 %
Crude protein rapidN	CP	17.13 %
Dry matter #1 60℃ overnight	DM1	100 %
Dry matter #2 135℃ 2 h	DM2	87.57 %
Ether extract fat by ankorn	Fat	2.55 %
Crude fiber	Fiber	3.61 %
Phosphorus	P	0.59 %
LC/MS	L-carnitine	2.5 mg kg^{-1}
LC//MS	GABA	236.5 mg kg^{-1}

5. Analyses of the designer egg

The weights of the shell, yolk, and albumen were measured. All of the collected eggs were weighed and the yolks of 10 eggs were separated from the albumen. All of the shells were collected, grouped by sampling date, and lyophilized. For analyses of the L-carnitine and GABA in the yolk, 4.5 mL of triple distilled water was added to 1 g of the yolk and vortexed for 30 s, followed by addition of 4.5 mL of acetonitrile and mixing for 30 s. The mixture was centrifuged at 12,600 x g for 5 min. The supernatant was collected and filtered using a 0.2 μm syringe filter and 1 μL of the sample was injected into LC/MS/MS. L-carnitine and GABA standards were prepared under the same procedure. The LC/MS/MS system was a Waters Acquity H-class with a Waters Xevo TQ-S detector (Waters, MA. USA). The column and elution solvents were the same as those used for LC/MS. 12% of solvent The following elution gradient was applied: 0–3.5 min, 12% A; 3.6–5.5min, 12–30% A; then a 3.5min equilibrium step ESI positive with multi reaction monitoring (MRM) was used. Selected MRM transitions were

162.14>103.02 and 104.11>86.99 for L-carnitine and GABA. The cone voltage was 30 V and 15 V for L-carnitine and GABA and the collision energy was 16 eV and 10 eV. The capillary voltage was 0.2 kV. Sample temperature was 10°C and column temperature was 40°C. Preparation of blank (50% acetonitrile), evaluation of linearity of calibration curves range from 0.01 to 1 μg mL^{-1} for L-carnitine and GABA (r^2>0.99) and recovery test of eggs by standard addition method with t-test ($p < 0.05$) were performed as same purpose in LC/MS system (Table S2). Total cholesterol and triglyceride were analyzed using Asan total cholesterol and triglyceride kits (Asan Pharm Co., Ltd., Hwasung, Korea). For lipid analysis of sample preparation, 1 g of each yolks from the ten same day eggs was mixed and 20 μL of mixture was transferred into 15 mL conical tubes and analyzed in triplicates.

6. Statistical analysis

The data obtained from the egg assay was analyzed using two sample t-test in SPSS (V.23.0 for Windows, SPSS, Chicago, IL). Significant differences between the groups were evaluated and indicated by different superscript lower-case letters ($p < 0.05$).

III. RESULTS

1. Analysis of L-carnitine and GABA by modified LC/MS or LC/MS/MS

Figure 1 shows the chromatogram of the standard (Figure 1a, b), fermented buckwheat extract (Figure 1c, d), and blank (Figure 1e, f). Standard GABA and L-carnitine peaks were eluted at 3.96 min and 5.04 min (Figure 1a, b). The chromatogram of the fermented buckwheat extract exhibited GABA (3.89 min) and L-carnitine (5.05 min) peaks (Figure 1c, d). Figure 2 shows the chromatogram of the standard (Figure 1a, b), egg samples (Figure 1c, d), and blank (Figure 1e, f): MRM transition was at 162.14>103.02 for L-carnitine and 104.11>86.99 for GABA. The GABA peak eluted at 2.99 min and the L-carnitine peak eluted at 4.72 min were detected in the standard and at 3.00 and 4.73 min in the egg samples (Figure 2a-d).

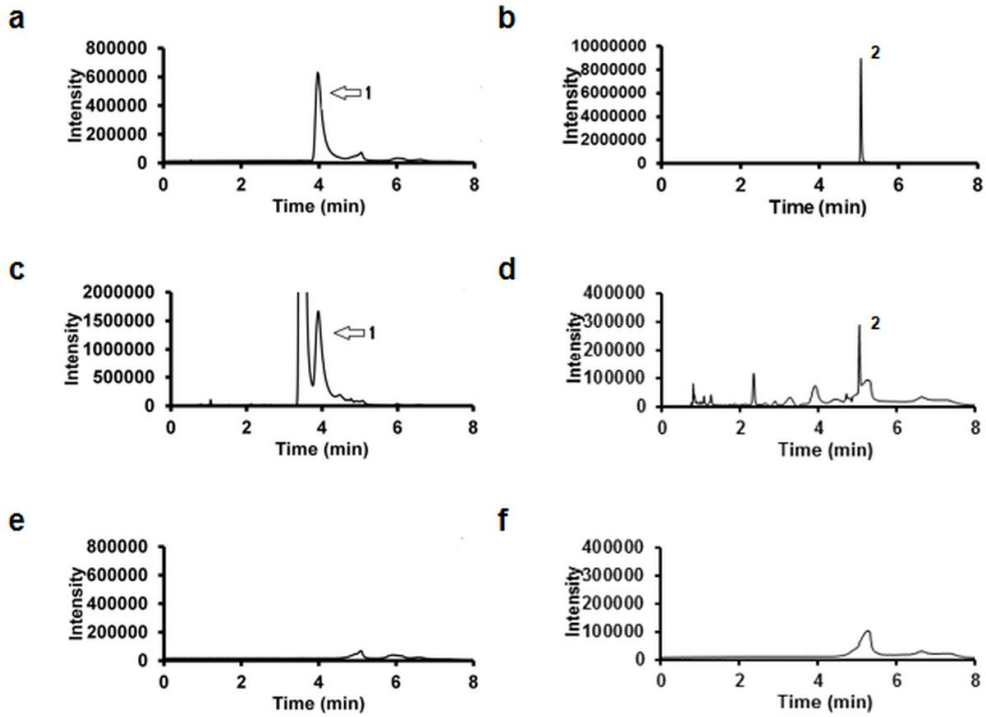

Figure 1. Chromatograms of L-carnitine and GABA of fermented buckwheat extract by LC/MS.

(a) GABA standard (SIR: 104 m/z), (b) L-carnitine standard (SIR: 162 m/z), (c) fermented buckwheat extract (SIR: 104 m/z), (d) fermented buckwheat extract (SIR: 162 m/z), (e) blank (SIR: 104 m/z), and (f) blank (SIR: 162 m/z). Peak 1 is GABA and peak 2 is L-carnitine.

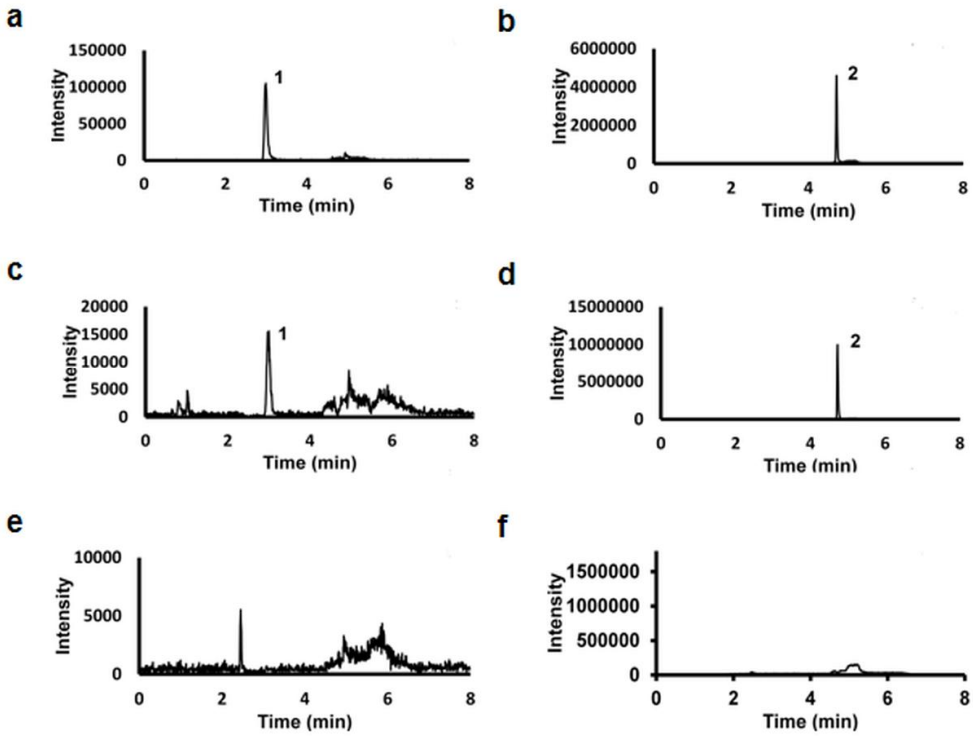

Figure 2. Chromatograms of L-carnitine and GABA of eggs by LC/MS/MS..

(a) GABA standard (MRM transition 104.11>86.99), (b) L-carnitine standard (MRM transition 162.14>103.02), (c) egg (MRM transition 104.11>86.99), (d) egg (MRM transition 162.14>103.02), (e) blank (MRM transition 104.11>86.99), and (f) blank (MRM transition 162.14>103.02). Peak 1 is GABA and peak 2 is L-carnitine.

2. The amount of L-carnitine and GABA in fermented buckwheat was increased

In 1 kg of unfermented buckwheat, the amounts of L-carnitine and GABA were 168.6 μg and 18.6 mg. However, after fermentation, the amounts of L-carnitine and GABA significantly increased to 680.0 μg and 632.3 mg. The amounts of L-carnitine and GABA produced through buckwheat fermentation using *R. oligosporus* was 4 and 34 times higher than those in untreated buckwheat, respectively.

3. Daily egg production was increased with fermented buckwheat feed

The average daily egg production rates were 87.3% and 95.5% for the control and treatment groups (fed with fermented buckwheat extract), respectively (Table 1). The treatment group had 8.2% higher average daily egg production than the control group, with statistical significance ($p < 0.05$). Except for the days 6 and 7^{t} of sampling, daily egg production in the treatment group was higher than that of the control group (Table 1).

4. Weights of albumen and egg shell were increased with fermented buckwheat feed

There was no significant difference in the yolk weight on each sampling date between the control and treatment groups ($p < 0.05$) (Table 1). Average yolk weight was 11.8 g for both the control and treatment groups (Table 1). The albumen weight of the treatment group significantly increased in the days 1 and 6 of sampling compared to that of the control ($P < 0.05$) (Table 1). Average albumen weight was 33.5 g for the control and 34.2 g for the treatment group (Table 1). Average albumen weight was significantly increased by 2.1% in the treatment group compared to the control ($p < 0.05$). Average shell weight was 5.2 g for the control and 5.5 g for the treatment group, which corresponds to a 5.8% increase in the treatment group (Table 1). These results for the shell weight had statistical significances ($P < 0.05$).

Table 1. Effect of fermented buckwheat extract on egg production and egg quality

Type	Egg production(%)												
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	M	n
C	95	75	80	90	85	95	95	85	85	85	90	873[b]	11
T	100	90	95	95	95	95	95	100	100	90	95	955[a]	11
Type	Yolk weight(g)												
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	M	n
C	10.6	11.2	11.6	10.9	11.3	12	12	11.8	12.5	12.8	12.4	11.8 ± 1.1	110
T	10.9	11.5	11	11.7	11.4	12.1	12	12.6	12.2	12.6	12.3	11.8 ± 1.0	110
Type	Albumen weight(g)												
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	M	n
C	31.2[b]	32	33.6	33.8	33.3	32.7[b]	33.9	33.4	34.1	35.6	34.7	33.5 ± 2.1[b]	110
T	33.6[a]	32.4	33.4	35.1	33.4	34.9[a]	33.9	34.6	34.3	35.6	34.8	34.2 ± 1.8[a]	110
Type	Shell weight(g)												

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	M	n
C	4.9	4.9	5.2	5.2	5.2	5.2	5.2	5.3	5.5	5.4	5.4	5.2 ± 0.2[b]	11
T	5.3	5.4	5.3	5.6	5.5	5.6	5.6	5.6	5.8	5.7	5.5	5.5 ± 0.1[a]	11

[ab]Values in the same row with different superscript letters were significantly different ($p < 0.05$). S1-S11 indicate the date of sampling. M is mean value and represented as Mean ± STD. C and T indicate control and treatment groups, respectively. n is the number of observations for the mean value.

$$\text{Egg production } (\%) = \frac{\text{Number of daily eggs}}{\text{Total laying hens}} \text{ x } 100$$

5. The amount of total cholesterol and triglyceride in yolk with fermented buckwheat feed showed slight decrease

The average amount of total cholesterol in the yolk was 16.0 mg g^{-1} and 15.7 mg g^{-1} for the control and treatment groups, respectively (Table 2). The total cholesterol amount was decreased by 1.9% in the treatment group compared to that of the control with no statistical significance ($p < 0.05$). The amount of total cholesterol in the treatment group during days 4 to 7 sampling decreased significantly compared to that of the control. This was not the case on the days 5 or 6 of sampling ($p<0.05$) (Table 2). The average concentration of triglyceride in the yolk was 284.2 mg g^{-1} and 270.2 mg g^{-1} for the control and treatment groups, respectively (Table 2). The average triglyceride was 4.9% less in the treatment group than in the control, but this difference was not statistically significant ($p < 0.05$). The amount of triglyceride in the yolks of the treatment group on the days 1, 3, 4, 6, 7 and 9 of sampling was significantly lower than that of the control from the

same dates. In contrast, the triglyceride in the yolk of the treatment group on the days 5, 8, 10 and 11 of sampling was significantly higher than that of the control ($p < 0.05$) (Table 2).

Table 2. Effect of fermented buckwheat extract on lipid contents of eggs

Type	Total Cholesterol in Yolk (mg g^{-1})												
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	M	n
C	19	14.3	14.5	21.9 [a]	15.3	17.5	20.9 [a]	14.9 [b]	12.8	12.2	13.3	16 ± 3.3	33
T	18.5	13.9	13.9	20.5 [b]	16.4	15.9	17.3 [b]	18.3 [a]	13.4	12.1	12.9	15.7 ± 2.7	33
Type	Triglyceride in Yolk (mg g^{-1})												
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	M	n
C	385.2 [a]	244.5	373.1 [a]	323.8 [a]	290.8 [b]	360.8 [a]	277.4 [a]	230.2 [b]	295.4 [a]	179.9 [b]	165.5 [b]	284.2 ± 72.1	33
T	277.0 [b]	245.4	310.6 [b]	305.0 [b]	399.2 [a]	244.9 [b]	255.7 [b]	234.4 [a]	263.9 [b]	211.4 [a]	225.1 [a]	270.2 ± 51.3	33

[ab]Values in the same row with different superscript letters were significantly different ($p < 0.05$). S1-S11 indicate the date of sampling. M is mean value and represented as

Mean ± STD. C and T mean control and treatment groups, respectively. n is the number of observations for the m

6. The concentrations of L-carnitine and GABA in egg yolk were increased by feeding fermented buckwheat

The average L-carnitine in the yolk was 13.6% higher for the treatment group than that of the control and this difference was statistically significant ($p < 0.05$) (Table 3). L-carnitine in the yolk of the treatment group on the day 3 of sampling was much higher than that of the control ($p < 0.05$) (Table 3). The average concentration of GABA in the yolk was only numerically 8.4% higher for the treatment group than that of the control ($p < 0.05$) (Table 3). GABA in the yolk of the treatment group from the day 6 of sampling was significantly higher than that of the control ($p < 0.05$) (Table 3).

Table 3. Effect of fermented buckwheat extract on L-carnitine and GABA of yolk

Type	L-carnitine in Yolk(mg·kg^{-1})												
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	M	n
C	1.8	2.1	2.2^{b}	2.2	2.2	2.1	2.1	2.0	2.6	2.6	2.8	2.2 ± 0.5^{b}	109
T	1.9	2.0	3.7^{a}	2.3	2.2	2.4	2.3	2.2	3.0	2.8	2.9	2.5 ± 0.7^{a}	107
Type	GABA in Yolk(μg·kg^{-1})												
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	M	n
C	NA	NA	NA	143.7	140.3	119.4^{b}	120.6	113.2	102.1	68.6	57.7	108.2 ± 48.4	80
T	NA	NA	NA	132.6	163.5	146.1^{a}	146.9	112.5	106.7	72.6	57.3	117.3 ± 53.0	77

^{a,b}Values in the same row with different superscript letters were significantly different ($p < 0.05$). NA means no analysis. S1-S11 indicate the date of sampling. M is mean value and represented as Mean ± STD. C and T mean control and treatment groups, respectively. n is the number of observations for the mean value.

IV. DISCUSSION

In this study, our first goal was to enhance L-carnitine in plant species that generally lack L-carnitine. We chose buckwheat due to its high content of precursors for L-carnitine synthesis, lysine and methionine, when compared to those of other crops. Buckwheat with no additional nutrients was fermented using *R. oligosporus* and produced 4 times higher amount of L-carnitine than the original buckwheat. Previous studies have reported that L-carnitine could be produced by microbial fermentation.[35,36] Takeda Chemical Japan[36] developed a method for L-carnitine production by culturing *Cephalosporin* or *Acremonium* with a complex medium using carbon sources (milk sugar, sucrose, dextrin, etc.), various fatty acids and nitrogen sources (meat extract, yeast extract, soybean flour), salts, and other components. Through this method, 258 μg mL^{-1} of L-carnitine could be produced after 16 days of fermentation. Yakult Japan[35] disclosed another

method for preparing L-carnitine: *Aspergillus*, *Mucor*, *Neurospora*, *Penicillium,* and *Rhizopus* were cultivated in complex medium that especially included the depolymerized organic nitrogen, legumes, beans, or fats and oils. After culture, about 1 mg g^{-1} media of L-carnitine was produced. The amount of GABA also increased after buckwheat fermentation because the glutamate decarboxylase of *R. oligosporus* converted glutamate into GABA.[32]

The second goal was to develop a sensitive and efficient analytical method to confirm the increase of L-carnitine and GABA following the fermentation experiment described above and also to compare the variation of these compounds in the eggs. A modified analytical method that detects both compounds simultaneously was developed. Because only small amounts of L-carnitine and GABA are normally found in most food sources except in meat or fermented

foods, a sensitive assay method was essential. Before utilizing LC/MS and LC/MS/MS, we tried using the two analytical methods described in the aforementioned reports: [33,34] L-carnitine enzyme assay performed with the protocol and HPLC-UV with p-bromophenacyl bromide (p-BPB). Although the L-carnitine enzyme assay was a simple and fast method to check for L-carnitine, it could not simultaneously detect for GABA due to enzyme specificity. In addition, the preprocessing step was time-consuming and the reproducibility using eggs was not enough to compare the small difference among eggs due to various unknown factors attributed to assay enzyme activity. The other initially considered method was HPLC-UV. Because L-carnitine and GABA do not have chromophores, the HPLC-UV method required an additional derivatization step with proper reagents for both compounds. Instead of ninhydrin, p-bromophenacyl bromide was adopted as it could theoretically interact with both L-carnitine and GABA simultaneously. However, the derivatizing step was still tiresome. Therefore, we finally developed a modified

analytical method with LC/MS and LC/MS/MS. Because L-carnitine and GABA could be ionized and the mass spectrometer could detect 100 compounds or fewer at the same time, these techniques were appropriate to detect both L-carnitine and GABA simultaneously. In the newly modified method, simple extraction with aqueous solution and simple deproteinization step with acetonitrile made prederivatization and neutralization unnecessary. Because we used a silica-based Hydrophilic Interaction Chromatography (HILIC) column, a high acetonitrile fraction in the final sample was appropriate for column maintenance. The sensitivity and reproducibility of LC/MS and LC/MS/MS were appropriate to precisely detect nano- to micro-scale amounts of L-carnitine and GABA in the samples. Therefore, LC/MS and LC/MS/MS were applied in our experiment. Especially, the limit of quantification (LOQ) for LC/MS/MS was lower than that of LC/MS, which made the assay of GABA in eggs possible.

For our final goal of producing L-carnitine and GABA designer eggs, we fed laying hens with either normal forage or forage with 1.6 % (w/w) fermented buckwheat extract. The amount of L-carnitine and GABA in the control group were fed 2,475.5 μg kg^{-1} and 236.5 mg kg^{-1} feed, respectively. In the treatment group, the L-carnitine content in the diet was fed with 2,490.4 μg kg^{-1} feed, resulting in an additional 14.9 μg kg^{-1} of L-carnitine. The GABA content in the feed for the treatment group was 283.5 mg/kg feed, which was 47 mg higher per kg of feed than that of the control group. The average L-carnitine content in the yolk in the treatment group was significantly increased compared to that of the control ($p < 0.05$). Previous studies have reported that L-carnitine as a feed supplement could be transferred into eggs to some degree. Adabi *et al*[37] fed 275 broilers with 2 different levels of L-carnitine (60 mg kg^{-1} feed for females and 500 mg kg^{-1} feed for males) and the resultant eggs contained 66.7% (g g^{-1}) higher L-carnitine than the control. Zhai *et al*[22] used 125 mg kg^{-1} feed of L-carnitine for tree groups (only males, only females, and

both) and approximately 38% (mg mg^{-1}) increase in L-carnitine was reported when L-carnitine (125 mg kg^{-1} feed) was fed to only females. Compared to previous reports, our current results are interesting in that there was 13.6% L-carnitine increase in the yolk of the treatment group. This was much higher than expected because we added only 0.6% (mg mg^{-1}) L-carnitine in the diet of the treatment group more than that in the control. These results suggest the presence of additional unknown compounds, precursors of L-carnitine that promote L-carnitine synthesis in poultry, and acylated carnitine, another form of L-carnitine-produced during fermentation.

GABA in the yolk was also increased in the treatment group (8.4%, w/w), but no statistical significance was observed ($p < 0.05$). Compared to L-carnitine, the GABA content in normal forage was already high, thus it was unclear whether the added GABA (additional 47 mg higher per kg of feed than that of the control group) in the diet of the treatment group was transferred significantly. There has not been a research on the transfer of GABA from feed to eggs, but it has

been previously reported that consumption of a diet with supplemented GABA increased the concentration of GABA in breast muscle of broilers under heat stress.[38] This report is indirectly related to our current research finding that GABA may have been transferred into the eggs.

From these results, the current study presents for the first time the potential of fermented buckwheat as feed additive to produce designer eggs enhanced with L-carnitine and GABA. These L-carnitine and GABA enriched designer eggs hold significant potential for large-scale food and pharmaceutical industry applications. (These L-carnitine and GABA enriched designer eggs pose significant potential to be utilized in superfood production and supplement industries). The average amounts of triglyceride and total cholesterol in the yolk were reduced in the treatment group compared to the control. These results were similar to previous experiments utilizing L-carnitine[23,37,39] or GABA[40] in the feed. Adabi *et al.*[37] and Kazaemifard *et al.*[23] reported that L-carnitine reduced egg yolk cholesterol ($p < 0.05$). Oso *et al.*[39] demonstrated how L-

carnitine reduced plasma lipid, total egg yolk cholesterol, and triglyceride ($p < 0.05$). It is possible that L-carnitine is associated with increased systemic breakdown of cholesteryl esters, increase in reverse cholesterol transport and excretion, and the stabilization of a phospholipid-based structure for VLDL and LDL particles.[41] Zhang *et al.* showed the effect of GABA on the reduction of serum cholesterol.[40]

The average amount of daily egg production, albumen weight, and shell weight during the 3 weeks of the study were significantly increased in the treatment group ($P < 0.05$). In previous reports, L-carnitine increased egg production and albumen weight significantly ($p < 0.05$).[23,42] Since L-carnitine plays a well-established role in the metabolism of lipids, it may induce some favorable modification in poultry products.[26] Rabie *et al.*[43] also reported that albumen weight was higher, probably due to the increased metabolic rate in the magnum or activity of the shell glands of the treated birds. The increased egg production and quality were in agreement with other

previous reports using GABA or GABA-producing lactic acid bacteria.[27,44] In addition, GABA can affect appetite and improve the utilization of nutrition, resulting in enhanced egg production and quality.[40,44] Zhang *et al.* reported that calcium and phosphate in the serum of laying hens were enhanced after feeding with supplementary GABA and attributed the results to the increase in total protein concentration with their egg production, and modulation of the electrolyte balance.[40] Also, the increase of nutrition utilization and antioxidant activities by GABA is likely to be the main cause of the improved laying egg performance and egg quality.[27]

V. Conclusion

The current study presents for the first time the production of L-carnitine and GABA enriched designer eggs using fermented buckwheat and demonstrates their transfer from feed into eggs. The amount of L-carnitine and GABA produced using buckwheat was increased by simple fermentation using *R. oligosporus*. Because no additional nutrients were required, the L-carnitine and GABA enriched designer eggs pose significant potential to be utilized in superfood production and supplement industries. While only 1.6% (g g^{-1}) of fermented buckwheat extract was fed to the Hy-Line brown hens, the egg production and the quality including the weight of the shell and albumen from these hens were higher and the lipid contents of the yolk were slightly improved. Further studies of L-carnitine and GABA production by fermenting both whole grain

buckwheat and its milling byproducts, such as hull, and possible mechanism for transferring L-carnitine and GABA into eggs are in progress.

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VII. 국문초록 (Abstract)

본 연구에서는 L-카르니틴과 감마-아미노뷰티릭산(가바)가 향상된 디자이너 달걀을 생산하기 위해 사료 첨가제로서 발효 메밀의 가능성이 연구되었다. 메밀에는 L-카르니틴의 합성에 필요한 리신과 메티오닌, 그리고 가바의 합성에 필요한 글루탐산이 풍부하게 함유되어 있으며, *Rhizopus oligosporus* 가 L-카르니틴과 가바의 생산을 위해 메밀의 발효에 사용되었다. 본 실험의 중요한 물질인 L-카르니틴과 가바는 체내 대사 촉진, 항산화효과, 면역력 증강, 혈압 조절 등의 효능이 있다고 알려져 있다.

또한, L-카르니틴과 가바의 동시 분석을 위한 새롭게 수정된 분석 방법을 LC/MS 와 LC/MS/MS 를 이용하여 개발하였다. 위의 방법으로 분석을 실시하였을 때, 발효 메밀 추출물은 일반 메밀 추출물에 비하여 4 배의 L-카르니틴과 34 배의 가바를 함유하였다. 그리고 발효 메밀을 사료 첨가제로 처리한 양계 집단 달걀의 경우 일반 달걀 집단에 비하여 L-카르니틴(13.6%)과 가바(8.4%)가 난황 내에 증가한 것을 확인하였다. 그러나, 오직 L-카르니틴의

경우에만 통계적으로 유의하게 증가하였다 ($p<0.05$). 이에 더해 달걀의 생산성(9.4%)과 흰자의 무게(2.1%), 껍질 무게(5.8%)에서도 유의하게 증가하였다 ($p<0.05$). 난황의 무게와 전체 콜레스테롤, 트리글리세리드의 경우에는 일반 달걀과 유의한 차이를 확인할 수 없었다 ($p<0.05$).

결과적으로 발효 메밀을 사로 첨가제로 사용하여 L-카르니틴과 가바가 향상된 디자니어 달걀의 생산 가능성을 확인 할 수 있었다. 이렇게 생산된 디자이너 달걀의 경우 기능성 식품 생산 시장에 긍정적인 영향을 나타낼 것으로 기대한다.

주요어: 메밀, 발효, *Rhizopus oligosporus*, L-카르니틴, 감마-아미노뷰티릭산 (가바), 디자이너 달걀

학번: 2015-20006: