Master's Thesis of Science in Agriculture

The Effect of Fermented Buckwheat on Producing L-carnitine Enriched Oyster Mushroom

L-카르니틴이 증가된 느타리 생산에 대한
발효 메밀의 영향

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Abstract

L-carnitine is biological compound which serves intake of long chain fatty acids into mitochondria to metabolize. L-carnitine is considered as a nutritious supplement for weight-loss. It is synthesized in human organ, but most of L-carnitine which human intakes is originated from meat based foods. In this study, for the first time we produced L-carnitine enriched mushroom using fermented buckwheat prepared by *Rhizopus oligosporus* fermentation. Oyster mushroom (*Pleurotus ostreatus*) was used to cultivate L-carnitine enriched mushroom. The mushroom grown on buckwheat medium contained 9.9% to 23.9% higher L-carnitine concentration than basal medium without any buckwheat addition. Fermented common buckwheat added medium grown mushroom contained the highest L-carnitine content of 201.2 $mg \cdot kg^{-1}$. The size index and the lightness of mushroom pileus (L*) were the highest, respectively 100.7 and 50.6, with fermented common buckwheat (20%, w/w) added medium. The antioxidant activities of mushroom extracts (1.5 mg/ml) against DPPH radicals were 38.7 % at mushroom grown with the common buckwheat and fermented common buckwheat added medium. The cytotoxicity test against Raw 264.7 cell of mushroom ethanol extracts resulted that fermented buckwheat mushrooms showed EC_{50} 307.2 $\mu g \cdot mL^{-1}$, but on normal media showed 255.1 $\mu g \cdot mL^{-1}$.

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Introduction

1. L-carnitine

L-Carnitine (β-hydroxy-γ-*N*-trimethylaminobutyric acid) is non-essential amino acid and natural compound occurring most in red meat such as ground beef (870.5 $mg \cdot kg^{-1}$), lamb chop (403.0 $mg \cdot kg^{-1}$), pork ham (532.0 $mg \cdot kg^{-1}$), and veal sirloin (1289.6 $mg \cdot kg^{-1}$) [1]. Mushrooms are another L-carnitine contained foods ranged from 132.2 to 528.7 $mg \cdot kg^{-1}$ [2]. Main function of L-carnitine is related to transfer of fatty acid into mitochondria as fatty acids carrier in lipid metabolism. In addition, L-carnitine modulates the ratio of Coenzyme A and sustains its homeostasis by forming acyl-carnitine under abnormal condition [3]. It has two isomers but only L-isoform has biological functionality in organism. L-carnitine is considered as weight-loss product because of its function related to fat oxidation. Clinical researches showed that regular L-carnitine intake lose the weight in human [4]. In addition, Acetyl-L-carnitine is known to improve cognitive function [5]. And L-carnitine is effective antioxidants and increase sperm mobility and numbers for men [6, 7]. In mammals, L-carnitine is biosynthesized from two essential amino acids, lysine and

methionine [8].

2. Mushroom

Due to its functional properties, mushroom has been used as traditional foods and medicine in eastern Asia. The world production of mushroom has been grown rapidly since late 20th century and China is accounted for over 70% of whole world production in 2014 [9]. *Pleurotus ostreatus,* the second most cultivated mushroom in the world, is commonly known as “Oyster mushroom” and “Hiratake” [10]. The oyster mushroom showed various biological functions such as an antimicrobial activity against *Candida albicans, Escherichia coli,* or *Bacillus subtilis*, antineoplastic activity against Ehrlich ascetic tumor [11], antioxidant activity [12] and antitumor activity of mycelium proteoglycan [13], and anti-inflammatory activity of pleuran which is polysaccharides extracted from fruit body.

3. Buckwheat

Buckwheat, classified into family *Polygonaceae,* is mainly produced in China and Russia. [9] The origin of buckwheat was estimated in southwestern china [14]. Buckwheat contains so many nutrients related to anti-oxidant activity. Most of its anti-oxidant ability is attributed to flavonoid compounds such as rutin and quercetin [15]. In addition, buckwheat has been known for large amount of viatamin B_1, B_2 and balanced protein compound and polyphenol [16, 17]. Buckwheat is classified into two classes, which are common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.). Tartary buckwheat is known that it has much more rutin than common buckwheat [18]. Buckwheat has advantage to produce L-carnitine in that it has much more methionine and lysine composition, which is precursor of L-carnitine, than rice and other pseudo cereals [19, 20]. In previous study, L-carnitine-enhanced fermented buckwheat was produced with *Rhizopus oligosporus*, which is main inoculum to produce Indonesian traditional fermented soybean (Tempeh) [21].

4. Solid-state fermentation

Fermentation is divided into two major technology. One is submerged fermentation and the other one is solid-state fermentation. Recent trend moves from submerged fermentation to solid-state fermentation, because solid state fermentation has been known to be more efficacy [22]. Compared to submerged culture, solid-state fermentation has advantage of costs and its simplicity. Numerous bacteria, yeast, and fungi have been used for solid-state fermentation. [23] During solid-state fermentation, enzymes from microbials were involved in bioconversion such as α-amylase, fructosyl transferase, lipase, and pectinase. [24]

5. Research trends of functional mushroom

There were few studies about functionality improved mushroom. da Silva et al. reported transfer of selenium from coffee husk to oyster mushroom and production of selenium enriched mushroom [25]. de Assunção et al. and Fontes Vieira et al reported that metal ion such as iron, zinc, litium were transferred from substrates to fruit bodies. [26,

27]

Ergosterol is one of the main active compound found in mushroom. It is composed of fungal cell wall and can be transformed to vitamin D when it is exposed to UV. With this principle, Kristensen et al. reported that vitamin D in mushroom was enhanced after UV exposure. Villares et al. also reported that ergosterol in mushroom is also enriched after UVB exposure.

Materials and Methods

1. Microbial strain and culture condition

For buckwheat fermentation *R. oligosporus* was maintained on potato dextrose agar (PDA) (Difco, USA) and incubated at 28ºC until spore formation [21]. *P. ostreatus* was supplied from mushroom research institute (Gwangju, Gyeonggi, Korea) [28].

2. Preparation of fermented buckwheat

Tartary buckwheat and common buckwheat were purchased in Bongpyeong (Pyeongchang bongpyeong memil, Gangwon-do, Korea). Fermented buckwheat was prepared with the modified method of previous study [21]. Each whole buckwheat (750 g) was soaked in water (1.5 L) for 6 h in metal tray (height × width × length = 5.5 cm × 15.5 cm × 28.5 cm) and sterilized at 121˚C for 25 min. A 7.5 mL of *R. oligosporus* spore solution (1×10^6 spores·mL^{-1}) was inoculated into each sterilized buckwheat tray after cooling to room temperature. It was incubated at 30˚C until mycelium covered the surface of tray (76 h) with relative humidity maintained above 90%.

The fermented buckwheat was lyophilized at -10 to 0˚C under 10 Pa for four days (Eyela, Japan) and it was milled with blender (Hanil, Korea). For further analysis, it was stored at – 20˚C.

3. Analyses of fermented buckwheat

Total phenolic content (TPC) of buckwheat was measured with Folin-Ciocalteu method [29]. Buckwheat and fermented buckwheat were suspended with 70% ethanol at 50 $mg \cdot mL^{-1}$, and supernatant was separated at 13,500 × g for 10 min. A 120 μL of water diluted sample was mixed with 15 μL of Folin-Ciocalteu reagent for 3 min at 300 rpm with microplate shaker (Thermo Fisher Scientific, Waltham, MA). A 15 μL of 10% (w/v) Na_2CO_3 was added to mixture and shaken for 30 min. TPC was determined at 760 nm with microplate reader (Molecular Devices, Sunnyvale, CA) using gallic acid as the standard (10 to 100 $\mu g \cdot mL^{-1}$).

Total flavonoids content (TFC) of the extracts was measured using aluminum chloride method. A 2 mL of methanol diluted sample was mixed with 10% (w/v) $AlCl_3$ (100 μL) dissolved in water and 0.1 mM Potassium acetate (100 μL). TFC was determined at 415 nm,

using quercetin as the standard (10 to 100 $\mu g \cdot mL^{-1}$).

4. Mushroom spawning and fruiting in field scale

The cultivation of *P.* ostreatus was performed with slight modified version of previously reported procedure [30]. For the field scale experiment, *P. ostreatus* was pre-cultured on substrate mixture composed of 80% douglas fir sawdust and 20% rice bran packaged in heat-resistant bottle (1,100 mL, φ75 mm) at 20˚C for 30 days. After mycelium was totally grown, 4 g of hyphae attached substrate was transferred into each buckwheat medium. The buckwheat medium contains milled whole buckwheat seeds in basal medium as an ingredient. The basal medium was composed of 66.7% (w/w) poplar saw dust (Korea), 16.7% (w/w) cotton-seed meal (China), and 16.7% (w/w) beet pulp (China). The moisture content was adjusted to 65% (w/w) before sterilization. Each milled buckwheat was mixed with basal medium at 20% (w/w) of nutritious substrate. Each medium prepared was basal medium (G), common buckwheat medium (C), fermented common buckwheat (FC), tartary buckwheat medium (T), fermented tartary buckwheat medium (FT) (Table 1). The medium (620 g) was

packed in heat-resistant bottle and sterilized serially at 100˚C for 30 min and 121˚C for 90 min. After sterilization, each medium was cooled down to room temperature, and pre-cultured *P. ostreatus* (4 g of wet weight) was inoculated to each buckwheat containing medium. Each medium was incubated at 20˚C under 65% relative humidity in dark room. Carbon dioxide was controlled ranged from 3,000 to 5,000 ppm to induce balanced shape of pileus and stipe [10]. After 30 days incubation of mycelium to induce fruit body, old spawn was removed and temperature was maintained at 15˚C. Relative humidity and carbon dioxide were maintained over 90% and 500 ~ 3,000 ppm, respectively. The space per bottle was 0.056 m^3 per one bottle. The 8^{th} day after inducing fruit body, all fruit bodies were harvested and 10 fruit bodies of each group were randomly selected. Then, fruit bodies were lyophilized and stored at -80˚C for further analyses.

Table 1 Composition and ratio of mushroom medium.

	Medium composition (g/bottle)							Medium weight per bottle (g)	Buckwheat proportion to nutrient medium (%, w/w)	Buckwheat proportion to total medium weight (%, w/w)
	Poplar saw dust	Cotton seed meal	Beet pulp	Buckwheat						
				CB	FCB	TB	FTB			
G	144.7	36.2	36.2	-	-	-	-	620	0	0
C	135.3	33.8	33.8	14.0	-	-	-	620	20.74	2.26
FC	135.3	33.8	33.8	-	14.0	-	-	620	20.74	2.26
T	135.3	33.8	33.8	-	-	14.0	-	620	20.74	2.26
FT	135.3	33.8	33.8	-	-	-	14.0	620	20.74	2.26

*G: Basal medium; C: Common buckwheat medium; FC: Fermented Common buckwheat medium: T: Tartary buckwheat medium; FT: Fermented Tartary buckwheat medium

5. Analyses of mushroom morphological characteristics

Morphological characteristics such as fruit body weight, pileus diameter, and stipe length were measured. Halogen lamb analyzer MB35 (Ohaus Inc., USA) was used to measure moisture content of fruit body. Color change of mushroom pileus was measured with Hunter's color value (L*, a*, b*) by color difference meter (Konica Minolta, Tokyo, Japan). Total size index (TSI), representing comprehensive morphological size, was used to combine each morphological value. It was obtained by multiplication of weight (g), pileus diameter (cm), stipe length (cm), and stipe thickness (cm), and each obtained value was rescaled (divided by 100) and its unit were omitted. The equation of TSI was the following.

Total Size Index =

$$\frac{(Mshroom\,weight \times \quad ileus\,diameter \times Stipe\,length \times Stipe\,thickness)}{00}$$

6. Liquid chromatography analysis

L-carnitine content in buckwheat and mushroom was analyzed with LC-ESI-MS [21]. Each sample (100 mg) was extracted with water and centrifuged for 10 min at 13,500 × g. The supernatant was diluted 10-fold with acetonitrile and the mixture was centrifuged again. And then the supernatant was filtered using 0.2 μm pore size syringe filter (Sartorius, Germany). For quercetin and rutin analyses, each sample (100 mg) was extracted with 70% ethanol (1 mL) and centrifuged and filtered as described above. The filtrate (1 μL) was used for component analyses as follows. Carnitine analysis was performed on Acquity UPLC system equipped with ESI-MS and BEH 1.7 μm HILIC column (2.1 mm x 150 mm, Waters, USA). Mobile phase A was 15 mM ammonium formate with 0.1% formic acid, and B was acetonitrile with 0.1% formic acid. Flow rate was 0.4 $mL \cdot min^{-1}$, sample manager temperature was sustained on 20˚C, and column temperature was 40˚C. Mobile phase A was sustained at 10% for initial 3 min, 30% for next 2 min, 60% for 1 min, and it was back at 10% for last 4 min. Each compound was recorded and quantified at specific ion mass $[M+H^+]$. ESI-MS condition was set to the followings: Positive ion mode;

capillary voltage +1.5 kV; cone voltage -10 V; single ion recording 162.00 $g \cdot mol^{-1}$. Quercetin and rutin analyses were separated in Kromasil 1.8 μm C18 UHPLC column (2.1 mm x 50 mm, Kromasil, USA). Flow rate was 0.3 $mL \cdot min^{-1}$, mobile phase A was 0.1% formic acid and B was acetonitrile with 0.1% formic acid. Temperature was the same as above. Mobile phase B was gradually increased from 30% to 100% for initial 5 min, then back to 30% for last 3 min. ESI-MS condition was set to the followings: Positive ion mode; capillary voltage +1.5 kV; cone voltage -25 V (rutin), -10 V (quercetin); single ion recording 609.50 $g \cdot mol^{-1}$ (quercetin), 303.20 $g \cdot mol^{-1}$ (rutin).

7. Preparation of mushroom fruit body extract

The powdered mushroom fruit body (1 g) of each treatment (G, C20, FC20, T20, FT20) was suspended with ethanol (10 mL) and extracted at 20˚C (200 rpm) for 24 h. The mushroom powder and supernatant was separated by centrifugation at 3,500 × g for 10 min, and the supernatant was filtered using Whatman paper filter No 1 (Whatman, USA). The filtrate was evaporated at 45˚C for 1 h and freeze-dried (Eyela, Japan). The ethanol extract was re-dissolved in

DMSO solution (10 $mg \cdot mL^{-1}$) and used for *in vitro* cell cytotoxicity and antioxidant assays.

8. Antioxidant effects of mushroom fruit body extract

The antioxidant activity of mushroom ethanol extract was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method with slight modification [31]. The mushroom extracts dissolved in DMSO were mixed with 1 mM DPPH reagent dissolved in ethanol. The final concentration of mushroom extracts was ranged from 0.01 $mg \cdot mL^{-1}$to 1 $mg \cdot mL^{-1}$. The mixture was incubated for 30 min at room temperature and the absorbance of each mixture was obtained at 517 nm on microtiter plate (Molecular Devices). DMSO solution was used for negative control. The relative radical scavenging activity was obtained with the following equation:

The relative radical scavenging activity (%)

$$= \frac{(Absorbance\ of\ negative\ control - Absorbance\ of\ the\ sample)}{sorbance\ of\ negative\ control} \times 100$$

The results were expressed with mean ± standard error of the mean (SEM). All analyses were carried out in triplicates. Trolox was used as a positive control.

9. Cell viability assay of oyster mushroom ethanol extracts

RAW264.7 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (GenDEPOT, USA), 100 units·mL^{-1} penicillin (GenDEPOT, USA) and 100 μg·mL^{-1} streptomycin (GenDEPOT, USA) at 37 ˚C under 5% CO_2. The ethanol extracts of oyster mushroom were prepared in DMSO solution (10 mg·mL^{-1}). RAW264.7 cells were dispensed into 96 well plate at cells·well^{-1}. Then, the cells were incubated at 37℃ in a humidified atmosphere containing 5% CO_2 for 24 h. The medium was discarded, and then each sample diluted with medium (1.17 μg·mL^{-1} to 1.2 mg·mL^{-1}) was added to each well, and incubated for 24 h. For evaluating the cytotoxicity, WST-1 assay was performed by EZ-cytox kit (Daeil Lab service, Korea) with 1/10 dilution. After 1 h reaction, the cell viability was evaluated at 450 nm. And EC50 value were computed by

sigmoidal interpolation using GraphPad Prism 7.02 (GraphPad Software,USA).

10. Statistical analysis

All the data were obtained after repeated three times experiments except morphological value analyses. The morphological characteristic values were mean of 10 randomly selected samples. Mean value was given with standard error of the mean (SEM). The differences between groups were determined by Tukey HSD methods ($P < 0.05$ or $P < 0.01$). Statistical analysis was performed on SPSS statistics 23 (IBM, USA).

Results

1. Amounts of L-carnitine and phenolic compounds in fermented buckwheat

In 1 kg of common buckwheat and tartary buckwheat, the amounts of L-carnitine were 11.3 mg and 6.2 mg, respectively (Table 2). After *R. oligosporus* fermentation, the amounts of L-carnitine in both buckwheats were increased, from 11.3 mg to 26.2 mg in common buckwheat and from 6.2 mg to 38.4 mg in tartary buckwheat, as previously reported. And the rate of L-carnitine increase was bigger with tartary buckwheat fermentation (620.2%) than common buckwheat fermentation (232.4%)

Total phenol content and flavonoids content in fermented buckwheat were higher than those of non-fermented buckwheat. However, quercetin was decreased after fermentation in both common and tartary buckwheats. On the contrary, rutin, glycosides of quercetin, in tartary buckwheat was increased from 3923.2 $mg \cdot kg^{-1}$ to 5148.1 $mg \cdot kg^{-1}$ after fermentation.

Table 2 L-carnitine and phenolic compound composition in buckwheat after *R. oligosporus* fermentation

Buckwheat	L-carnitine ($mg \cdot kg^{-1}$)	Total Phenolics Content (mg GAE$\cdot kg^{-1}$)	Quercetin ($mg \cdot kg^{-1}$)	Rutin ($mg \cdot kg^{-1}$)
Common buckwheat	11.3 ± 0.4	1470.1 ± 57.9	19.3 ± 0.4	75.9 ± 4.5
Fermented Common buckwheat	26.2 ± 0.6**	3466.8 ± 102.6**	14.7 ± 0.8**	71.4 ± 2.0
Tartary buckwheat	6.2 ± 0.1	5372.3 ± 114.5	3155.6 ± 68.3	3923.2 ± 90.7
Fermented Tartary buckwheat	38.4 ± 0.5**	7279.3 ± 176.2**	2619.0 ± 89.7**	5148.1 ± 188.5**

* TPC is expressed by gallic acid equivalent (GAE)
* Mean ± Standard error of the mean (SEM)
* Asterisk star (**) means difference at 0.01 of significant level from non-fermented common buckwheat or tartary buckwheat.

2. Amounts of L-carnitine in oyster mushroom

The amount of L-carnitine in 1 kg of dried oyster mushroom, grown on each medium (C, FC, T, or FT), was compared. (Table 3) When oyster mushroom was grown on FC media, the amounts of L-carnitine were significantly increased 22.31% as compared with G ($P < 0.01$). But the oyster mushroom grown on FT medium had smaller amount increase of L-carnitine, 12.90% increase.

Table 3 L-carnitine content in oyster mushroom grown on various buckwheat medium

	Mean ($mg \cdot kg^{-1}$)	% increase		Mean ($mg \cdot kg^{-1}$)	% increase
Basal medium					
G	164.5 ± 4.9				
Common buckwheat medium			*Tartary buckwheat medium*		
C	186.3 ± 8.1	13.3	T	180.8 ± 6.2	9.9
Fermented Common buckwheat medium			*Fermented Tartary buckwheat medium*		
FC	201.2 ± 3.6**	22.3	FT	185.7 ± 4.9	12.9

* Each mean value was written with standard error of the mean (SEM)
* Asterisk star (**) means difference at 0.01 of significance level.

3. The morphological characteristics of mushroom grown on buckwheat containing medium

The mushroom weight and moisture content were similar in all samples, and there were no significant differences ($P > 0.01$) compared to mushroom grown on basal medium. (Table 4) Mushroom size represented with total size index (TSI). Oyster mushroom grown on FC medium was significantly increased ($P < 0.01$) compared to basal medium. And other size factors such as pileus diameter, stipe length and stipe thickness were no significant changes except pileus diameter in FC and Stipe length in C.

Table 4 Morphological characteristics of oyster mushroom

	Cultivation Number	Sampling number	Formation rate (%)	Mushroom weight (g/bunch)	Moisture (%)	Pileus diameter (cm)	Stipe length (cm)	Stipe thickness (cm)	Color			Total size index
									L*	a*	b*	
Basal medium												
G	48	10	97.9	170.6	89.7	3.0	8.1	1.2	41.8	2.6	4.1	52.1
Common buckwheat medium												
C	32	10	96.9	187.3	88.1	3.0	10.3**	1.3	50.1**	3.6**	6.3**	78.9
Fermented Common buckwheat medium												
FC	32	10	93.8	181.4	88.3	4.0**	9.5	1.4	50.6**	3.6**	6.5**	100.7*
Tartary buckwheat medium												
T	32	10	96.9	180.9	89.7	3.2	10.0	1.3	48.2**	3.5**	5.8	80.9
Fermented Tartary buckwheat medium												
FT	32	10	96.9	182.6	89.5	3.2	10.1	1.3	49.2**	3.6**	6.6**	82.1

* For each parameter, values in the same column with asterisk star (**) are significantly different ($p < 0.01$) from basal medium.

4. The color index of mushroom grown on buckwheat containing medium

The lightness (L*) in all the samples was significantly increased ($P < 0.01$) as compared with G. (Table 4) The yellowness (b*) also increased in all the buckwheat added mushrooms but T wasn't significantly different ($P > 0.01$) from G.

5. Antioxidant activities in ethanol extracts of oyster mushroom

The antioxidant effects of mushroom ethanol extracts against DPPH were evaluated at the concentration from 0.2 $mg \cdot mL^{-1}$ to 1.5 $mg \cdot mL^{-1}$ (Figure 1). The radical scavenging activities showed concentration-dependent manner in the all samples. At 1.5 $mg \cdot mL^{-1}$ of final concentration, the radical scavenging activity of oyster mushroom was ranged from 25.9% to 38.7%. At this concentration, G was found to be 25.9%. C and FC showed the same scavenging activity (38.7%), and T and FT medium grown mushrooms contained 28.7% and 30.9% of scavenging activity, respectively.

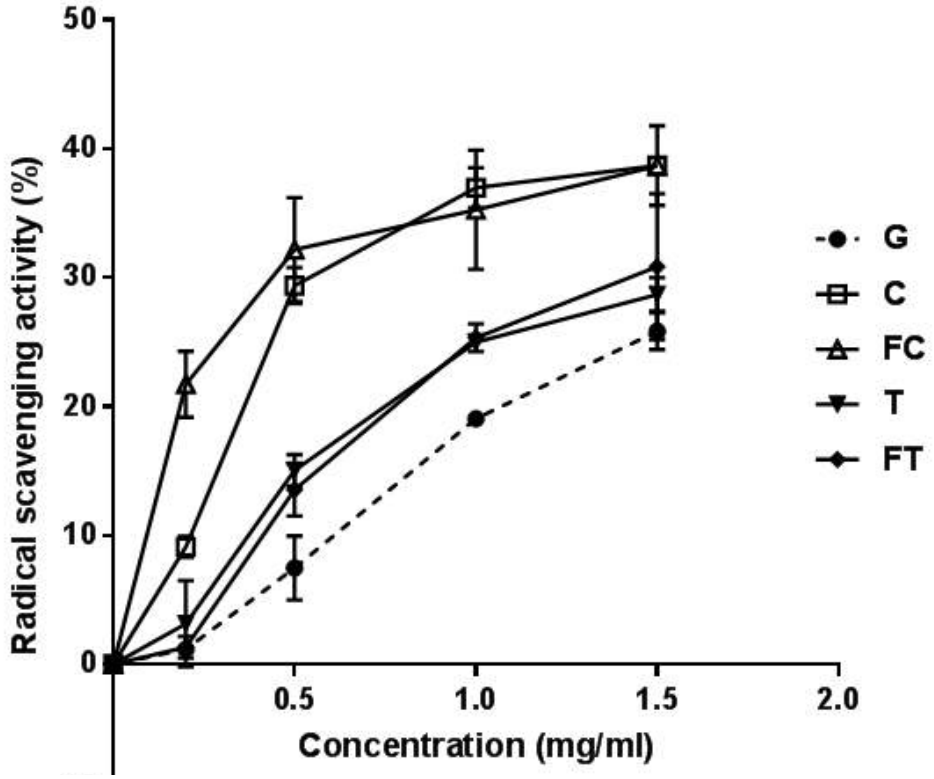

Figure 1. DPPH radical scavenging activities in ethanol extract of oyster mushroom fruit body

6. Cell cytotoxicity assay of ethanol extract of oyster mushroom against Raw 264.7

The cell cytotoxicity of ethanol extract of fruit body was evaluated based on final concentration at from 18.75 μg·mL^{-1} to 1,200 μg·mL^{-1} (Figure 2). From 0 to 75 μg·mL^{-1} final concentrations, the Raw 264.7 cell was viable above 90% in all samples. Upto 150 μg·mL^{-1}, the samples showed no significant differences in cell viability compared to control ($P > 0.05$). At 300 μg·mL^{-1}, extracts of oyster mushroom grown on buckwheat medium showed cell viability from 58.9% to 67.8%. However, the extracts of oyster mushroom grown on basal medium shows under 50% of cell viability. EC50 of mushroom grown on buckwheat medium was 307.2 μg·mL^{-1} to 329.5 μg·mL^{-1}, but mushroom on basal medium was 255.1 μg·mL^{-1}.

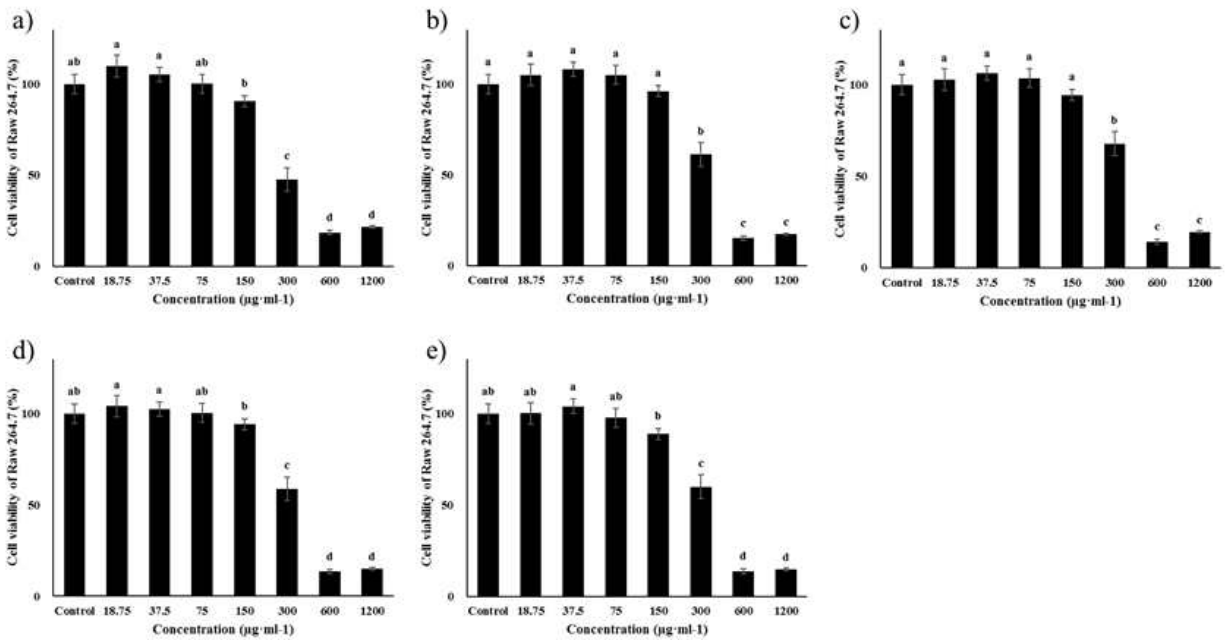

Figure 2 Antiproliferative activities of ethanol extract of oyster mushroom against Raw 264.7 cell. (a): Oyster mushroom grown on basal medium (G); (b): Oyster mushroom grown on common buckwheat medium (C); (c): grown on fermented common buckwheat medium (FC); (d): grown on tartary buckwheat medium (T); (e): grown on fermented tartary buckwheat medium (FT). The different alphabet means significant differences at 95% of confidence level.

Discussion

Although L-carnitine is synthesized from essential amino acids, lysine and methionine, in the human, still 75% of L-carnitine is obtained exogenously from foods, especially meat and pork [32]. Because most L-carnitine is supplied from meat diet, vegetarians have to uptake enough amount of L-carnitine through other plant derived food sources [33, 34]. In the previous study, fermented buckwheat with *R. oligosporus* was reported to have 4.4 times from 1.05 μmol·kg^{-1} of dried buckwheat to 4.22 μmol·kg^{-1} of dried buckwheat. L-carnitine enhancement. Buckwheat contains more lysine and methionine, L-carnitine precursor amino acids, than other pseudocereals and cereals [19]. *R. olgiosporus* could synthesize L-carnitine from these amino acids. Furthermore, when fermented buckwheat was fed to laying hens, L-carnitine content in egg yolk was increased 13.6%. Thus, L-carnitine enhanced foods can be substitutes for meat based diets which is related to severe health problems such as cancer, diabetes, and obesity [35-37]. By using fermented buckwheat as a mushroom medium, we produced upto 20% higher L-carnitine enhanced oyster mushroom. To extent fermentation substrate, we used both common

buckwheat and tartary buckwheat.

The amount of L-carnitine in tartary buckwheat was enhanced six times after fermentation, whereas common buckwheat was two times. Bonafaccia et al reported that lysine composition in protein was similar between common buckwheat and tartary buckwheat, but protein content was 21.6% (w/w) in common buckwheat bran and 25.3% in tartary buckwheat bran [20]. If both buckwheat are the same dry weight, Lysine which is L-carnitine precursor is more abundant in tartary buckwheat.

L-carnitine content in common buckwheat and fermented common buckwheat was higher compared to results from previous study [21]. Firstly, the producing area of common buckwheat was different. While this study used products from Korea, Park et al. used products from China. Also the—differences of L-carnitine production was possibly because of procedures. In this study, compared to the previous fermentation procedure, over 90% of relative humidity was maintained for optimumal environment for *R. oligosporus*. Furthermore, whole grain was used for fermentation. Unlike buckwheat dough, it probably made easier for *R. oligosporus* contact with air.

As shown in table 2, the phenolics content in buckwheats significantly increased after fermentation ($p < 0.05$). Fungal fermentation increases bioactive phenolic compounds because fungal enzyme breaks ester links and releases bound phenolic compounds from plant cell wall. The enhancement of phenolic compounds in fava bean and quinoa after *R. oligosporus* fermentation was also reported [38, 39].

While rutin content in tartary buckwheat increased to 5.1 $g \cdot kg^{-1}$ after fermentation, quercetin content decreased to 2.6 $g \cdot kg^{-1}$ in both buckwheat. (Table 2) Microbial metabolism of flavonoids is various such as glycosylation, deglycosylation, ring cleavage, methylation, glucuronidation, and sulfate conjugation Quercetin and rutin had possibility to transform to other compounds. *R. oligosporus* could synthesize quercetin glucuronides from quercetin by using β -glucuronidase. [40]. However, the reason why total rutin content increased after fermentation could be found in tartary buckwheat bran. Wang et al reported that rutin content (541.3 mg/g) in tartary buckwheat bran was almost 10 times higher than quercetin (66.3 mg/g) [41]. In this reason, more rutin could be degraded from bran, and its

total amount could increase.

L-carnitine content was significantly increased 9.9% to 24.0% in all the treatments. (Table 3) Pork muscle was known to contain 209.6 $mg \cdot kg^{-1}$ of L-carnitine [1]. L-carnitine content in fermented common buckwheat medium increased to 203.1 $mg \cdot kg^{-1}$ which is similar amount of pork muscle. L-carnitine content in oyster mushroom grown on each of fermented buckwheat medium was higher than non-fermented buckwheat medium. The process of L-carnitine synthesis was already done by *R. oligosporus*. Because of this process, various L-carnitnie derivatives could be synthesized. These *R. oligosporus* metabolites could be supplied to mycelium of oyster mushroom and it makes easier to produce more L-carnitine.

The weight and moisture contents of fruit bodies were no significant differences in each treatment (Table 4). And the formation rate was above 90%. Total size index was used to compare weight, pileus diameter, stipe length and stipe thickness. The index was the highest in fermented common buckwheat containing medium. All the treatment had higher total size index than basal medium, but only fermented common buckwheat medium was significantly higher ($P <$

0.01).

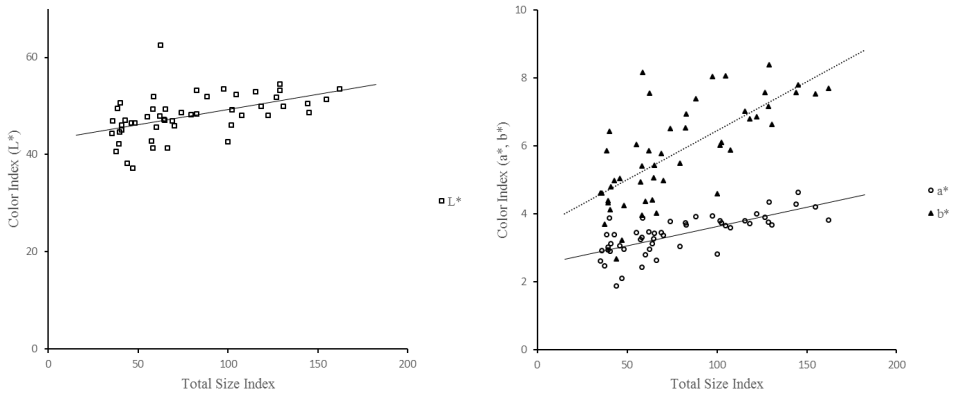

Figure 3 Correlation graph between total size and color index

Table 5 Equation of correlation between total size and color index

Value		Coefficient of correlation (R)	Equation	*P*
X	Y			
Total Size Index	L*	0.4960	Y = 0.06215·X + 43.09	<0.001
	a*	0.7151	Y = 0.01141·X + 2.485	<0.001
	b*	0.7174	Y = 0.02890·X + 3.553	<0.001

Gulpinar et al reported that ethyl acetate fraction of buckwheat seeds had inhibitory activity against mushroom tyrosinase [42]. Some compounds in buckwheat might affect the color of pileus. In this study, the correlation analysis between two variables was carried out. In Figure 3 and Table 6, there was positive correlation between color and total size index ($P < 0.001$). When the total size of fruit body is bigger, the black pigment was dispersed through pileus so that the color was brighter.

The radical scavenging activity was higher in common buckwheat medium (C, FC) than tartary buckwheat medium (T, FT). (Figure 1) This tendency was also observed in L-carnitine concentration of fruit body. Oyster mushroom grown on common buckwheat had higher L-carntine than tartary buckwheat. (Table 3) L-carnitine has been known to be one of the strong antioxidant [6]. Gülçin et al reported that the L-carnitine had an effective DPPH radical scavenging activity, superoxide anion radical scavenging, hydrogen peroxide scavenging, total reducing power and metal chelating on ferrous ions activities. In some cases, L-carnitine shows superior antioxidant activities than trolox. L-carnitine might be one of the factors

influencing antioxidant activity of fruit body extracts in Figure 1.

Raw 264.7 cell viability of oyster mushroom grown on basal medium showed EC50 under 300 $\mu g \cdot mL^{-1}$. However, oyster mushroom grown on buckwheat medium showed higher EC50 than basal medium. There were no accurate ideas why cell cytotoxicity against Raw 264.7 cell decreased. But, the possible answer could be found in above description. It was reported that L-carnitine reduced oxidative stress *in vitro* cell culture, such as Raw 264.7 cells and HK-2 cells [43, 44]. L-carnitine was known to have anti-inflammatory activity due to suppressing iNOS (Inducible nitric oxide synthase), which produces nitric oxide, in transcriptional level. In this reason cytotoxicity of mushroom grown on buckwheat have more resistance power against oxidative stress. However, in that the pattern of L-carnitine concentration in mushroom didn't follow entirely that of cytotoxicity against Raw 264.7, L-carnitine might not be the only one factor.

Conclusion

Buckwheat containing medium seems to increase L-carnitine contents in fruit bodies of oyster mushroom. When the fermented common buckwheat, over 20% increase (164.5 to 201.2 $mg \cdot kg^{-1}$) was observed. Also, its size was the biggest in the treatments and antioxidant ability was higher than basal medium. Therefore, fermented common buckwheat were expected to produce L-carnitine enhanced functional mushroom.

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Abstract in Korean

L-카르니틴은 지방산을 미토콘드리아로 전이시켜 지방산 산화를 보조하는 물질으로, 체중 조절에 효과가 있는 것으로 알려져 있으며 시중에선 건강기능 소재로서 판매되고 있다. L-카르니틴은 인체 내에서 일부 생합성되지만, 대부분 육식 위주의 식단으로부터 얻고 있다. 본 연구에서는 발효 메밀을 이용하여 최초로 L-카르니틴이 강화된 버섯을 생산하였다. 발효는 *Rhizopus oligosporus* 를 이용하였고, 메밀은 단메밀(*Fagopyrum esculentum* Moench)과 쓴메밀(*Fagopyrum tataricum* (L.) Gaertn)을 이용하였다. 느타리 버섯(*Pleurotus ostreatus*)은 세계에서 두 번째로 많이 생산되고 있는 버섯으로 본 연구에서는 L-carnitine 강화 버섯을 만들기 위하여 사용되었다. 발효 단메밀을 배지의 20% 비율로 첨가 하였을때, 크기지표 (Toal size index)와 갓의 밝기가 가장 높이 증가하였다. 또한 메밀 함유 배지에서 키운 버섯의 L-카르니틴 함량은 일반 배지에서 자란 버섯 대비 높게 관찰되었다. 발효 단메밀을 배지에 20% 비율로 첨가하였을때 가장 높은 증가폭 (23.9%)을 보였다. DPPH에 대한 항산화능은 단메밀과 발효 단메밀 배지에서 가장 높게 관찰되었다. 자실체 에탄올 추출물의 Raw 264.7 cell에 대한 세포독성 실험 결과 일반 배지에서 자란 버섯보다 메밀 배지에서 자란 버섯의 경우 독성이 감소한 결과를 보였다.