Master's Thesis of Science in Agriculture

Production of L-carnitine Enhanced Fermented Quinoa with *Rhizopus oligosporus* and its Bioactive Properties

***Rhizopus oligosporus*로 발효한 L-카르니틴이 증가된 퀴노아의 생리활성적 특성**

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Production of L-carnitine Enhanced Fermented Quinoa with *Rhizopus oligosporus* and its Bioactive Properties

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Abstract

Quinoa is a pseudocereal that contains high quality protein, minerals, vitamins, polyphenols, and phytosterols. In this study, quinoa was fermented by *Rhizopus oligosporus* (*R.oligosporus*) up to 5 days and degradation of quinoa starch granules were observed with a scanning electron microscope. After optimization of fermentation based on L-carnitine production, L-carnitine and GABA amounts were 3.14 mg/kg and 1.04 g/kg at the third day. Total phenolic contents and total flavonoids contents were 8.0 mg gallic acid (GAE)/100 g and 1.9 mg quercetin equivalent (QE) /100 g for five days fermented quinoa (5F), and 4.1 mg GAE/100 g and 1.3 mg QE/100 g for regular quinoa (NF), respectively. Antioxidant activity (SC_{50}) was 2.3 mg/mL for 5F and 3.6 mg/mL for NF. Nitric oxide production on RAW264.7 macrophages of fermented quinoa revealed 29.3% inhibition of nitric oxide production of NF and 56.4% for 5F, resulting in improvement 1.9 times in anti-inflammatory activity.

Fatty acids composition in fermented quinoa was increased; myristic acid (68%), palmitic acid (48%), palmitoleic acid (264%), margaric acid (25%), stearic acid (129%), linoleic acid (33%) and linolenic acid (32%), compared to the regular quinoa. As for lipid soluble extract, wound healing effects on scratched HaCaT keratinocyte were enhanced from 24.8% wound closure effects of NL to 67.5% and 58.3% of 3L and 5L, respectively.

Keywords: Anti-inflammatory activity; Antioxidant activity; Fermentation; L-carnitine; *Rhizopus oligosporus*; Quinoa.

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List of Abbreviations

R. oligosporus, *Rhizopus oligosporus*; SEM, scanning electron microscope; TPC, total phenolic contents; TFC, total flavonoid contents; 5F, 5 days fermented quinoa; 3F, 3 days fermented quinoa; NF, regular quinoa; GAE, gallic acid; QE, quercetin equivalent; GABA, ɣ-aminobutyric acid; KCCM, Korean Culture Center of Microorganism; PDA, potato dextrose agar; SE, secondary electron; LC/MS, liquid chromatography-mass spectrometry; ESI, electrospray ionization; SIR, selective ion recording; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

Introduction

1. Quinoa

Quinoa (*Chenopodium quinoa*) has been widely cultivated in South America for 5,000 -7,000 years (Vega Gálvez et al. 2010). It has high nutritional properties and contents of antioxidant compounds essential amino acids, vitamins, minerals, and unsaturated fatty acids (Carciochi et al. 2016). Lysine and methionine contents are high in quinoa, deficient in many legumes and rice, and also can be a precursor of L-carnitine synthesis(Koeth et al. 2013).

2. L-carnitine

L-carnitine is synthesized from lysine and methionine, serving an essential function in transportation of fatty acids into the mitochondrial compartment for β-oxidation and subsequent energy production (Bremer 1983). L-carnitine is abundant in red meat (5977 mg/kg beef) but in lesser amounts in crops (0.4 mg/kg wheat seed), and plants (2.9 mg/kg tomatoes, 0.8 mg/kgavocado)(Steiber et al. 2004). In addition, L-carnitine is an antioxidant compound that can prevent oxidative stress and regulates cellular respiration by nitric oxide (Brown 1999). As an antioxidant role of L-carnitine, mainly three enzymes (glutathione peroxidase, catalase, and superoxide dismutase) are prevented from peroxidative damage (Kalaiselvi and Panneerselvam 1998).

3. ɣ - aminobutyric acid (GABA)

Gamma aminobutyric acid (GABA) is a non-protein amino acid and an inhibitory neurotransmitter that affects the personality and the stress management. GABA is mainly produced by microbial fermentation and it is synthesized from glutamic acid catalyzed by glutamic acid decarboxylase (GAD) (Handoyo and Morita 2006).

4. *Rhizopus oligosporus* fermentation

Rhizopus oligosporus (*R. oligosporus*) is a dominant fungus in special fermented soybean products like soybean Indonesia teampeh. During the fermentation by *R. oligosporus*, the macromolecules were hydrolyzed by enzymes and coupled with the metabolism of the corresponding hydrolytic products to changes in the biochemical composition of food substrates (deReu et al. 1997; Handoyo and Morita 2006). Fermented soybean with *Rhizopus oligosporus* (*R. oligosporus*) showed increase amounts of ɣ-aminobutyric acid (GABA), one of main fermented products from synthesizing from glutamate by glutamate decarboxylase (Dhakal et al. 2012; Handoyo and Morita 2006). The quinoa fermentation by using *R. oligosporus* have been reported (Matsuo 2005; Matsuo 2006). However, these studies only focus on antioxidant activity of fermented quinoa.

5. Antioxidant activity of phenolic compounds

Phenolic compounds are abundant in quinoa and had the highest antioxidant activities among the studied cereals and pseudocereals in total phenolics and anthocyanins (Maradini Filho, Pirozi et al. 2015). Flavonoids are polyphenolic compounds that constitute a diverse group of secondary plant metabolites related to antioxidant, antiviral, and anticancer activities, reduction of inflammation, and reduction in the incidence of cardiovascular diseases (Nijveldt, Van Nood et al. 2001). Theses oxidative damages are generated by reactive oxygen species (ROS) and the production of ROS is the critical factor in inflammation (Kim, Yi et al. 2009). In this regard, anti-inflammation effects evaluated with RAW 264.7 macrophage cells by measuring nitric oxide production which is a factor of tissue damage and inflammation and inhibited by iNOS (Kim, Cheon et al. 1999).

6. Research perspective of functional quinoa

In our previous study, *R. oligosporus* was used to produce L-carnitine and GABA in buckwheat by fermentation with no additional nutrients, and feed for chickens to gain high L-carnitine content in eggs(Park et al. 2016). However, there was no report for the improvement of L-carnitine and GABA after fermentation of quinoa and its biological and

biochemical studies. In this regard, we fermented quinoa with *R. oligosporu*s DK1 to improve the quality of the food regarding the enhancement of L-carnitine, GABA, phenolic acid compounds, antioxidant and anti-inflammatory effects. White quinoa was selected for this study because it contains more antioxidative compounds than those of red and black quinoa (Masayo and Watanabe 2010).

Materials and Methods

1. Microbial strain and culture condition

Rhizopus microspores var. *oligosporus* was obtained from our previous study (Park et al. 2016) and deposited as KCCM 11948P (Korean Culture Center of Microorganisms, Seoul, Korea). It was maintained on Potato Dextrose Agar (PDA, Difco, USA) plates.

2. Preparation of fermented quinoa

R. oligosporus was cultured on PDA medium at 30℃for 3days to prepare spores (Park et al. 2016). White quinoa was purchased from KtFood (Seoul, Korea). 150 g quinoa was soaked in 150 mL water for 12 h and steamed for 20 min at 121℃ Fermentation was conducted by inoculating 1×10^4 spores/g steamed quinoa at 30℃for 3-5 days. Fermented quinoa was lyophilized at -10 to 0℃under 20 Pa (Tokyo Rikakikai Co., Tokyo, Japan) for further study.

3. Sample extraction

300 g lyophilized quinoa was extracted with 1 L of ethanol for 1 h at 28℃in the shaking incubator and repeated 7 times, then filtered using filter paper (8 micron, 11 cm) (Whatman LTD., Maidstone, England). Ethanol in the sample was removed by evaporation (Heidolph Instruments, Schwabach, Germany) with addition of 400 mL distilled water. The lipid layers from extracted sample were removed and the extracted sample were lyophilized for further study. Extraction yield was calculated as follows

$$\text{Yield (g/100 g) \%} = \frac{extract\ mass\ (g)}{quinoa\ mass\ (300\ g)} \times 100\ (\%)$$

4. Scanning electron microscopy image analyses

Quinoa surfaces of sample prepared before and after fermentation were observed using a Scanning Electron Microscope (SEM, TM 3030plus, Hitachi, Tokyo, Japan). Whole grain images were taken at magnification of x 100, 5.0 kV of accelerate voltage in secondary electron (SE) mode. At magnification of x 1.0 k, fungi hypha was removed and observed at 15 kV of accelerate voltage in SE mode.

5. Analyses of L-carnitine and GABA

LC/MS analysis was conducted by using the method as our previously described (Park et al. 2016). Samples were dissolved in water for analysis of L-carnitine or GABA. Samples were diluted with acetonitrile for L-carnitine and GABA, and all filtered using a 0.2 μm membrane (Sartorius AG, Gottingen, Germany). A 1 μL sample was injected into the LC/MS system (Waters, Milford, MA, USA); Waters Acquity H-Class system with Waters QDa detector, Waters Acquity UPLC BEH HILIC 1.7 μm, 2.1 mm×100 mm column. Solvent A was 15 mM ammonium formate with 0.1% formic acid in distilled water and solvent B was 0.1% formic acid in acetonitrile. The temperature of column was maintained at 40℃ The following elution gradient was applied for L-carnitine analyses; 0-3 min, 10% A; 3.1-5 min, 10-30% A; 5.1-6 min, 30-60% A; then a 4 min for equilibrium step. The following elution gradient was applied for GABA analyses; 0-3 min, 10% A; 3.1-5 min, 10-30% A; 5.1-6 min, 60% A; 6.1-10 min, 10% A then a 4 min for equilibrium step. Electrospray ionization (ESI) was conducted with a positive with selective ion recording (SIR) (m/z 162 for L-carnitine and m/z 104 for GABA). Capillary energies were 1.5 kV. Cone voltage was 10 V for L-carnitine and 5 V for GABA. Acetonitrile (90%, v/v) was used as a blank. The samples were analysed as triplicated. Calibration curves were prepared using the external standard method with L-carnitine concentrations ranged from 0.01-1 μg/mL, GABA ranged from 0.02-1 μg/mL.

Linearity between concentrations of standards vs area was evaluated ($r^2 > 0.99$).

6. Analyses of phenolic acids

LC/MS analysis was conducted by using the method as previously described (Park et al. 2016). Samples were dissolved in DMSO for vanillic acid, chlorogenic acid, or gallic acid as 10 mg/mL. Samples were diluted with methanol for phenolic acids, and all filtered using a 0.2 μm membrane (Sartorius AG, Gottingen, Germany). A 1 μL sample was injected into the LC/MS system (Waters, Milford, MA, USA); Waters Acquity H-Class system with Waters QDa detector, Waters Acquity UPLC BEH HILIC 1.7 μm, 2.1 mm×100 mm column. Solvent A was distilled with water and solvent B was acetonitrile with 1 mL formic acid/L. The temperature of column was maintained at 40℃ The following elution gradient was applied for phenolic acids analyses; 0-0.5 min, 95% A; 0.5-3 min, 95-70% A; 3-5 min, 70-0% A; 5-6 min, 0% A; then a 4 min for equilibrium step. ESI was conducted with a negative with SIR (m/z 169 for gallic acid, m/z 353 for chlorogenic acid, and m/z 167 for vanillic acid). Capillary energy was 0.8 kV for phenolic acids. Cone voltage was 10 V for phenolic acids and methanol was used as a blank. The samples were analysed as triplicated. Calibration curves were prepared using the external standard method with phenolic acids concentration ranged from 0.1-10

μg/mL. Linearity between concentrations of standards vs area was evaluated ($r^2 > 0.99$).

7. Total phenolic contents analyses (TPC)

The total phenolic contents were determined by Folin ciocalteu's method (Masayo and Watanabe 2010) with gallic acid as the standard (Sigma). 10 mg quinoa or fermented quinoa extracted powder was dissolved in 1 mL water. Each 120 μL of sample or gallic acid (0-50 μg/mL) was added into 96 wells plate and 15 μL of Folin ciocalteu's reagent(Sigma) was mixed together for 3 min in dark condition. Then, 15 μL of 10% (w/v) Na_2CO_3 was added and reacted for 30 min in dark condition. The TPC were determined by spectrophotometry at 750 nm (SpectraMax M3, Molecular Devices, USA) and presented as gallic acid equivalent (GAE).

8. Total flavonoids contents (TFC) analyses

TFC was determined by aluminum chloride colorimetric method (Chang et al. 2002) with quercetin as the standard (Sigma). 20 mg quinoa or fermented quinoa extracted powder was dissolved in 1 mL DMSO, and then 5-folds diluted in methanol to give final concentration as 1 mg/mL and volume

of 2 mL. Each sample or quercetin (0-15 μg/mL) was mixed with 100 μL of 10% (w/v) $AlCl_3$ and 100 μL of 0.1 mM CH_3CO_2K. TFC was determined by spectrophotometry at 415 nm using SpectraMax M3 and presented as quercetin equivalent (QE).

9. Determination of DPPH radical scavenging activity

Antioxidant activities of quinoa or fermented quinoa were evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method (Nguyen et al. 2015). Quinoa extracted powder were diluted in 70% ethanol and centrifuged at 13,572 x g for 10 min. Supernatants were reacted with 100 μM DPPH (Sigma)in ethanol solution to give a final concentration of 0.2-7 mg quinoa extract/mL, then, kept at room temperature for 30 min in darkness. Absorbance of each sample was measured at 517 nm on a microplate reader, SpectraMax M3. DPPH radical-scavenging activity was converted into percentage of antioxidant activity using the following equation:

$$DPPH\,radical\,scavenging\,activity\,(\%) = \frac{(Absorbance\,of\,control - Absorbance\,of\,test\,sample)}{Absorbance\,of\,control} \times 100$$

A linear regression curve was established to determine the amount of sample necessary to decrease 50% of the absorbance of DPPH (SC_{50} value). All analyses were conducted in duplicate. Results were expressed as mean ± standard error (SEM).

10. Cell cytotoxicity tests

RAW264.7 mouse macrophage cell line and HaCaT was purchased from Korean Cell Line Bank (Seoul, Korea) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gendepot, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gendepot, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, USA) at 37℃in 5% CO_2 atmosphere. RAW264.7 macrophage cell and HaCaT were seeded on 96 wells plate at 2×10^4 cells/well and cultured for 48 h. Cells were rinsed with phosphate-buffered saline (PBS) and then treated with quinoa or fermented quinoa extract in DMEM medium without Fetal bovine serum (FBS) ranging from 1.56-1,600 μg/mL obtained by diluting quinoa or fermented quinoa extract with the culture medium. Cells cultured in a medium without adding samples were used as controls. After 24 h at 37°C, 90 μL of medium was mixed with 10 μL of Ez-CyTox

solution (Daeil Lab Service, Seoul, Korea) and then incubated at 37℃for 1 h. Absorbance was measured at 450 nm using SpectraMax M3. Percent viability was calculated as cell viability relative to the control.

11. Measurement of nitric oxide production

Nitric oxide production was determined as previously described method (Kim et al. 1999). RAW 264.7 cells were seeded to 96 wells plate at 2×10^4 cells/well and cultured at 37℃for 48 h. The sample treated with 1 μg LPS/mL and 100 μM indomethacin was used for positive control. Cells were then treated with quinoa or fermented quinoa extract in DMSO ranging from 12.5-50 μg/mL without effects on cytotoxicity under testing, and cultured at 37℃for 24 h. Then, the 80 μL of culture supernatant was mixed with 80 μL of Griess reagent containing 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid, and0.1% (w/v) naphthylethylenediamine, for 20 min, and absorbance was measured at 540 nm using SpectraMax M3. The amount of nitrite in the sample was evaluated from a standard curve generated with a sodium nitrite standard curve (0 - 500 μM in cell culture medium).

12. GC-FID analyses

Fatty acids of quinoa and fermented quinoa was analysed as previously described method (Grace and Manuel 1993). The lyophilized sample were methylated with 2 mL methylation mixture (MeOH:Benzen:DMP:H_2SO_4=39:20:5:2) and 1 mL heptane and extracted for 2 h at 80℃. The supernatant was analyzed by Agilent 7890 (Agilent, USA). DB-23 (Agilent, 60mm * 0.25mm * 0.25μm) column were used and injector condition was 250℃ and detecter was 280℃ with H_2 35, Air 350, and He 35 mL/min. 1 μL were injected and 0-1 min, 50℃ hold; 130℃, 8℃/min 0 min hold; 170℃, 8℃/min, 0 min hold; 215℃, 2℃/min 10 min hold.

13. Scratch Wound Healing Assay

HaCaT cells were seeded on 24 wells plate at 1×10^5cells/well and cultured for 24h. The medium was changed for starvation without FBS and cultured for 24h. Full confluent cells were scratched with yellow tips and cells were rinsed with phosphate-buffered saline (PBS) and then treated with quinoa or fermented quinoa extract in

DMEM cultured for 72h. The wound closure was photographed by Zeiss Z1 and the wound healing ratio was calculated as following equation;

$$Wound\,closure\,(\%) = \frac{(Blank - Sample)}{(Blank - Control)} \times 100$$

Control and Sample treated cell wound closure area were photographed after 72 h cultivation, and Blank area were measured just after scratching.

14. Statistical analyses

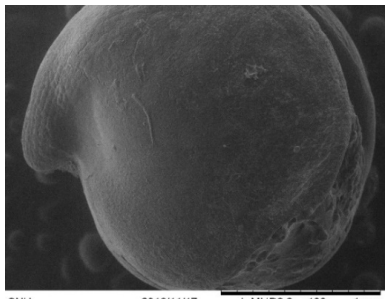
Experimental results were statistically analyzed by t-test (IBM SPSS Statistics 22, IBM, USA). Values are presented as means ± standard error of the mean (SEM).Significant differences between the groups were evaluated and indicated by different lower-case letters ($p<0.05$ and $p<0.01$)

Results

1. Scanning electron microscopic observation

The general quinoa and degradation of quinoa after fermentation were observed by the SEM. Figure 1 shows the surface of general and fermented quinoa. General quinoa (Fig. 1a) was observed with smooth surface. Fermented quinoa (Fig. 1b, c) was covered by *R. oligosporus* and mycelium was observed on fermented quinoa. The washed off fermented quinoa (Fig. 1e, f) revealed surface degradation comparable to the general quinoa (Fig. 1d), and exposed starch granule.

(a)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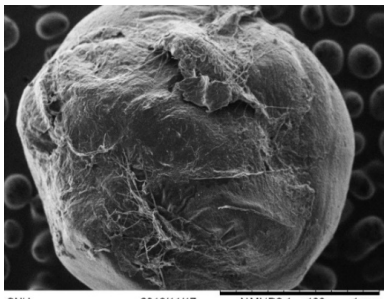
quinoa w/o fermentation

(b)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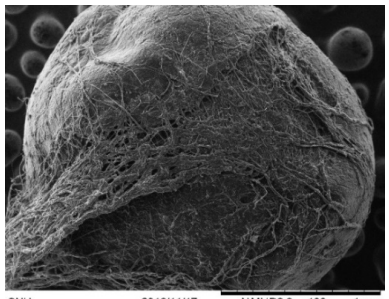
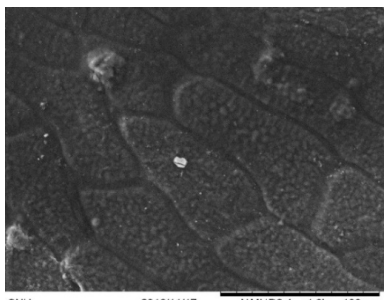
quinoa day3 fermentation

(c)


SNU 2016/11/17 NMUD8.8 x100 1 mm
quinoa day5 fermentation

(d)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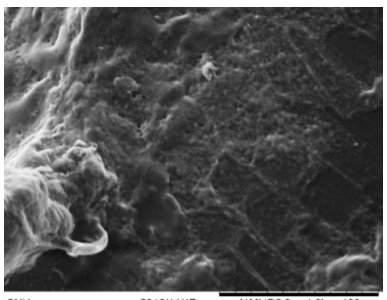
quinoa w/o fermentation

(e)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quinoa day3 fermentation

(f)

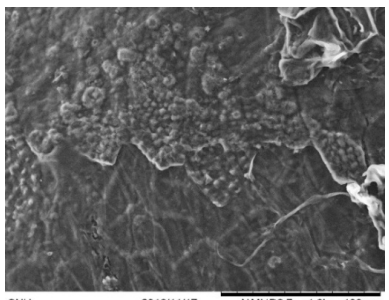

SNU 2016/11/17 NMUD8.7 x1.0k 100 µm
quinoa day5 fermentation

Figure 1. Scanning electron microscope of fermented quinoa: (a) NF, 100x; (b) 3F, 100x; (c) 5F, 100x; (d) NF, 1,000x; (e) hypha removed 3F, 1,000x; (f) hypha removed 5F, 1,000x.

(NF; Non-fermented, 3F; 3 days, 5F; 5 days of fermented quinoa extracts)

2. Ethanol extraction of fermented quinoa

The extraction yields of NF, 3F, and 5F were 23.4%, 45.9%, and 39.1%, respectively. Among then, 3 days fermented quinoa showed highest extraction yield.

Table 1. Quinoa extract yields

Extracts	Yield (%, g/g)	
	Non-lipids	Lipids
NF	0.23	1.71
3F	0.46	3.73
5F	0.39	2.05

(NF;Non-fermented, 3F; 3 days, 5F; 5 days of fermented quinoa extracts)

3. Analyses of phenolic acids, L-carnitine and GABA

Concentration of vanillic acids was increased during fermentation as 1.3, 1.55, and 1.83 mg/kg in NF, 3F, and 5F extracts, respectively (Table 1). Concentration of gallic acid was 0.01, 2.37, and 0.84 mg/kg in NF, 3F, and 5F extracts, respectively (Table 2). Chlorogenic acid was found 0.002 mg/kg for NF and 5F, but 0.03 mg/kg was detected at 3F extract (Table 2). The L-carnitine content was enhanced from 0.13 mg/kg to 3.15 and 1.54 mg/kg of quinoa extracts at 3F and 5F, respectively (Table 2). GABA was produced by the *R. oligosporus* fermentation, from 0.54 mg/kg to 1.04 and 0.81 mg/kg of quinoa extracts at 3F and 5F, respectively.

Table 2. L-carnitine, GABA and phenolic acids in regular and fermented quinoa

Group	NF	3F	5F
L-carnitine (mg/kg of extracts)	0.13	3.15 ± 0.06**	1.54 ± 0.06**
GABA (mg/kg of extracts)	0.54	1.04**	0.81**
Phenolic acids			
Vanillic acid (mg/kg of extracts)	1.30 ± 0.04	1.55 ± 0.06**	1.83 ± 0.06**
Gallic acid (mg/kg of extracts)	0.01	2.37 ± 0.08**	0.84 ± 0.02**
Chlorogenic acid (mg/kg of extracts)	0.002	0.03	0.002

(NF;Non-fermented, 3F; 3 days, 5F; 5 days of fermented quinoa extracts)

(NF vs 3F and 5F, **p0.01)

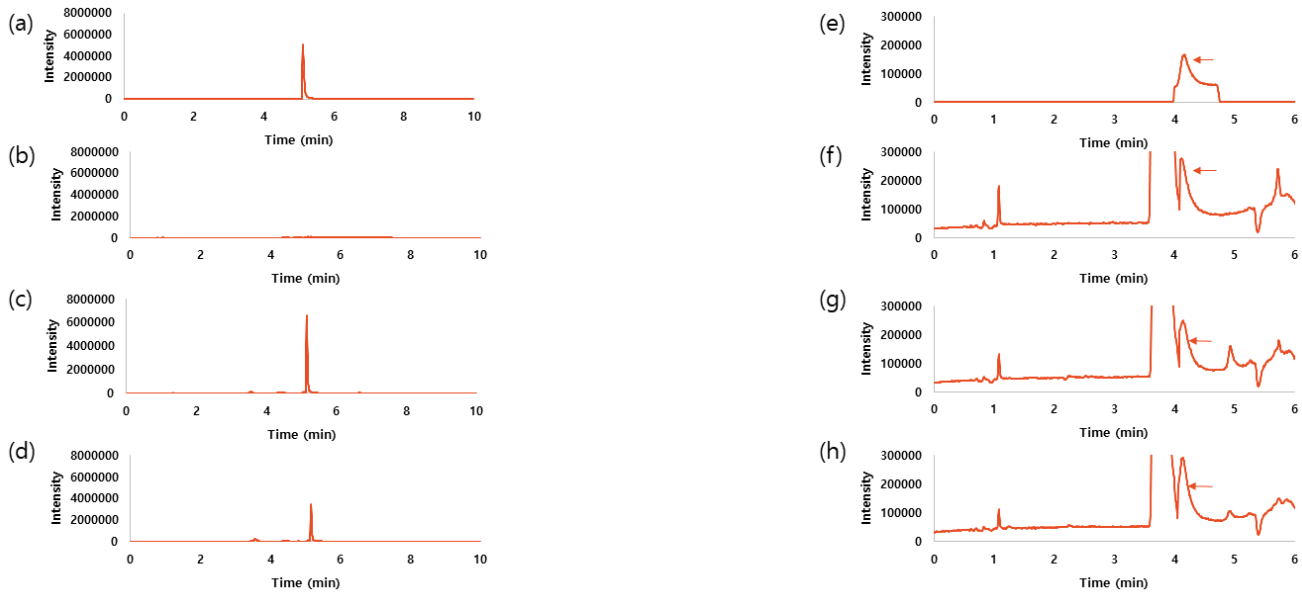


Figure 2. UPLC/MS L-carnitine and GABA analyses chromatogram: L-carnitine ((a) Standard, (b) NF, (c) 3F, (d) 5F); GABA ((e) Standard, (f) NF, (g) 3F, (h) 5F)

(NF; Non-fermented, 3F; 3 days, 5F; 5 days of fermented quinoa extracts)

* Representative data

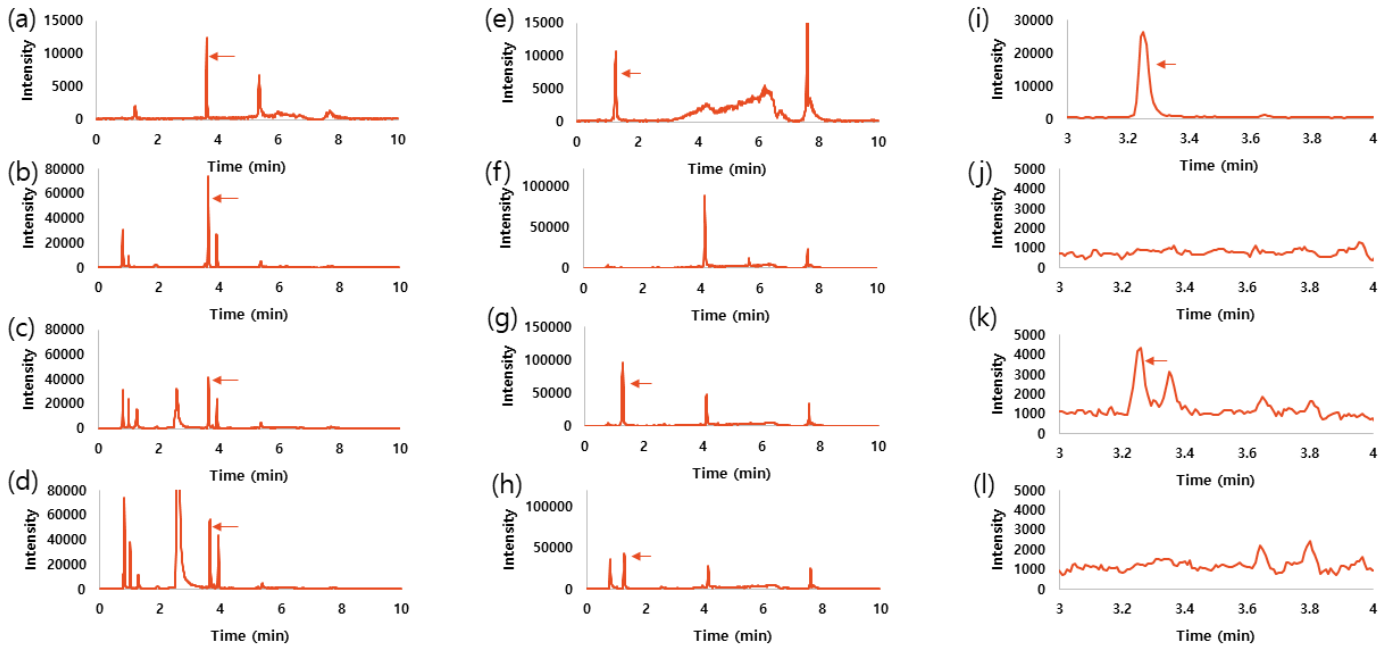


Figure 3. UPLC/MS phenolic compounds analyses chromatogram: vanillic acid ((a) Standard, (b) NF, (c) 3F, (d) 5F); gallic acid ((e) Standard, (f) NF, (g) 3F, (h) 5F); chlorogenic acid ((i) Standard, (j) NF, (k) 3F, (l) 5F))

(NF; Non-fermented, 3F; 3 days, 5F; 5 days of fermented quinoa extracts)

* Representative data

4. Total phenolic content, total flavonoid contents, and antioxidant activity of quinoa

Antioxidant activity was mainly investigated based on analysis of TPC, TFC, or DPPH radical-scavenging activity of each sample. After fermentation, TPC was increased from 41 mg GAE/kg to 74 and 80 mg GAE/kg of quinoa extracts at 3F and 5F, respectively (Table 3). TFC was increased from 13 mg QE/kg to 16 and 19 mg QE/kg of quinoa extract at 3F and 5F, respectively (Table 3). Antioxidant activity (SC_{50}) of quinoa extracts prepared with NF, 3F, and 5F were 3.6, 3.4, and 2.3 mg/mL, respectively (Table 3).

Table 3. Total phenolic and flavonoids contents and DPPH-radical scavenging activity of fermented quinoa extract

	NF	3F	5F
Total phenolic contents (mg GAE/100 g of quinoa extract)	4.1 ± 0.1	7.4 ± 0.0**	8.0 ± 0.1**
Total flavonoid contents (mg QE/100 g of quinoa extract)	1.3 ± 0.0	1.6 ± 0.0**	1.9 ± 0.0**
DPPH radical-scavenging activity [SC_{50}(mg/mL)]	3.6 ± 0.0	3.4 ± 0.5	2.3 ± 0.1**

(NF;Non-fermented, 3F; 3 days, 5F; 5 days of fermented quinoa extracts)

(NF vs 3F and 5F, **p0.01)

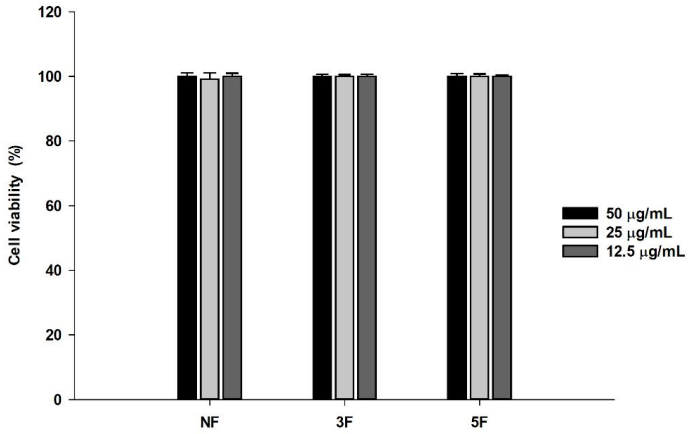
5. Cell viability of RAW264.7 cells

Cell viabilities of RAW 264.7, macrophages cells, are shown in Figure 2a. Cell viabilities were reached at 100% at the concentration of 100 μg/mL so that the nitric oxide assay was conducted from 50, 25, and 12.5 μg/mL (Fig. 3a).

6. Nitric oxide production

Production of nitric oxide was investigated by lipopolysaccharides (LPS) stimulation. Atthe concentration of 50 μg/mL, the 5F extracts had significantly high anti-inflammatory activities. Production of nitric oxide was decreased 22.8, 19,7 and 14.0 μM in NF, 3F, and 5F extracts, respectively (Figure 3b). These patterns were also shown at 25 μg/mL as 27.4, 24.5 and 23.6 μM of nitric oxide production in NF, 3F, and 5F extracts, respectively. As for 12.5 μg/mL, the nitric oxide was produced 30.8, 28.0 and 29.8 μM at NF, 3F, and 5F extracts, respectively. Since nitric oxide production is generated from inflammation, fermented quinoa had therapeutic abilities by reducing nitric oxide production.

(a)



(b)

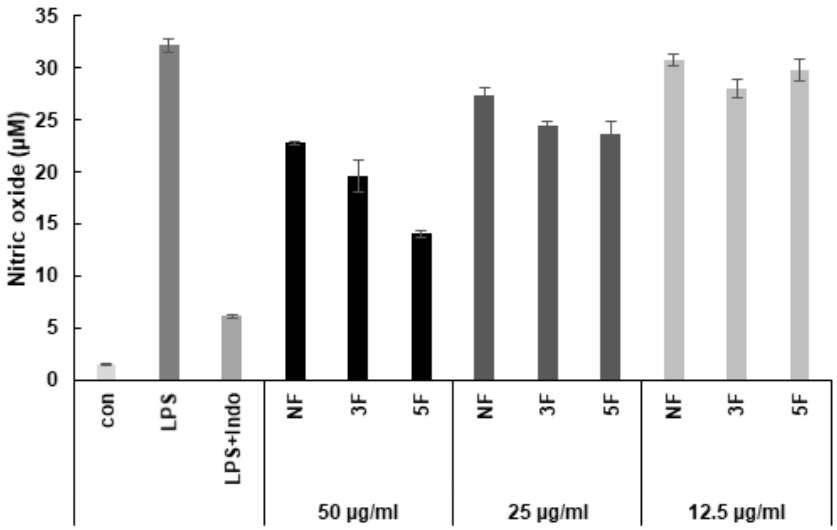


Figure 4. Cell viability on RAW264.7 of quinoa and fermented quinoa (a) and Nitric oxide production (%) of quinoa extracts (b).

(NF; Non-fermented, 3F; 3 days, 5F; 5 days of fermented quinoa extracts)

7. GC-FID analyses

Fatty acids composition in fermented quinoa was increased; myristic acid (68%), palmitic acid (48%), palmitoleic acid (264%), margaric acid (25%), stearic acid (129%), linoleic acid (33%) and linolenic acid (32%), compared to the regular quinoa. All kinds of fatty acids present in quinoa were increased after the fermentation, especially at 5 days of ferment.

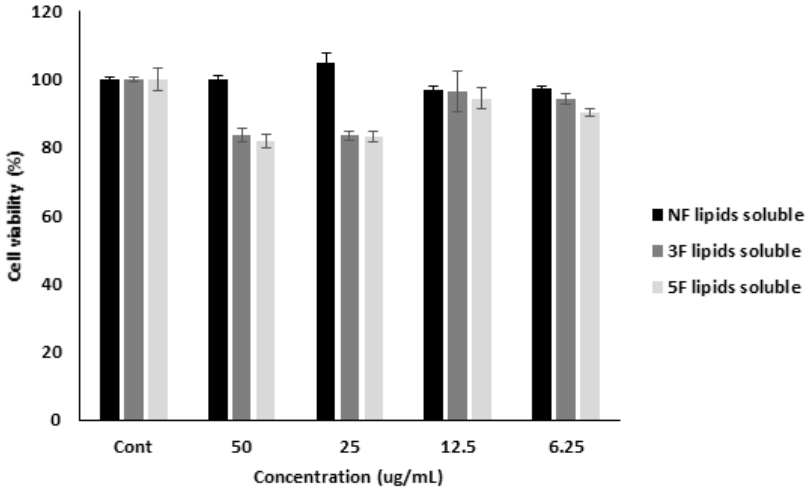
Table 4. Fatty acids content of lyophilized quinoa and fermented quinoa

mg/g	NF	3F	5F
C14 0	0.07	0.10	0.12
C16 0	4.75	6.35	6.70
C16 1	0.04	0.12	0.15
C17 0	0.06	0.07	0.07
C18 0	0.33	0.75	0.76
C 18 1n9 c	10.73	14.96	15.85
C 18 2n6 c	20.82	26.52	27.73
C18 3n3	1.95	2.41	2.57
C20 0	0.24	0.33	0.34
C20 1	0.76	0.94	1.01
C20 2	0.06	0.08	0.09
C22 0	0.34	0.46	0.48
C22 1n9	0.85	1.02	1.10
C22 2	0.05	0.06	0.06
C24 0	0.20	0.29	0.31
C24 1	0.10	0.12	0.13
Total	41.36	54.59	57.47

8. Wound closure effect on HaCaT cell

As for lipid soluble extract, wound healing effects on scratched HaCaT keratinocyte were enhanced from 24.8% wound closure effects of NL to 67.5% and 58.3% of 3L and 5L, respectively.

(a)



(b)

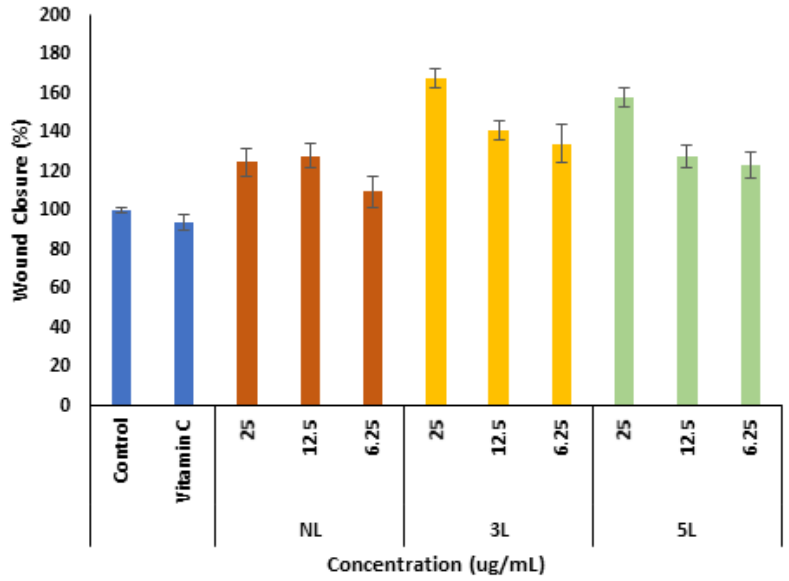


Figure 5. Cell viability on HaCaT of lipids layer of quinoa and fermented quinoa (a) and Wound closure (%) of quinoa lipids extracts (b).

(NL; Non-fermented, 3L; 3 days, 5L; 5 days of fermented quinoa extracts)

Discussion

Morphological characteristics by using the SEM reveals degradation of quinoa by *R. oligosporus* after fermentation (Fig. 1 a-c). Raw quinoa seeds had polygonal granules (0.6-2.0 μm diameter) present singly and as spherical aggregates (Ruales and Nair 1993). After fermentation, the polygonal granule was hardly observed and degraded into low-molecule-substances that brought different biological values to fermented quinoa (Fig. 1b-c). Degradation of cell structures of fermented quinoa was probably due to lipases, amylases, protease, and glucoamylase that produced from *R. oligosporus* (Handoyo and Morita 2006) (Jin et al. 1999).

The 3F and 5F quinoa extracts had higher content of L-carnitine (24.1 and 11.8 times) than non-fermented quinoa. Handoyo and Morita reported that *R. oligosporus* hydrolyzed protein into amino acids and small peptides (Handoyo and Morita 2006; Matsuo 2006). In other study, Matsuo reported that the amount of lysine was increased from non-detection to 0.31 g/kg dry quinoa and lysine from 0.11 g/kg to 0.51 g/kg dry quinoa during *R. oligosporus* fermentation (Matsuo 2006). L-carnitine is synthesized from lysine and methionine thus the synthesis of L-carnitine depends on the amount of lysine and methionine in

quinoa. The GABA contents in fermented quinoa showed 1.9 and 1.5 times higher than that of NF. GABA is synthesized from glutamate catalyzing by glutamate decarboxylase while quinoa contained high glutamate (0.71 g/kg dry quinoa) (Matsuo 2006).

Fermented quinoa extract revealed 36.1% enhanced anti-oxidative activity in DPPH-radical scavenging activity level. This result was agreed with previous reported by Mastuo (Matsuo 2006). The enhancement of antioxidant activities probably resulted in the increased amounts of phenolic compounds by the fermentation. Contents of phenolic acids such as vanillic acids, gallic acids, chlorogenic acids, and TPC, TFC were also changed and possibly effected to improvement of DPPH-radical scavenging activity. Vanillic acid was known for main phenolic acids in quinoa, and other phenolic acids were also analyzed such as gallic acids and chlorogenic acids. Vanillic acids contents were increased by fermentation as 1.3-1.83 mg/kg by NF and 5F, respectively. In case of gallic acids were increased at 3F by 2.37 mg/kg from 0.01 mg/kgof NF, but decreased at 5F by 0.84 mg/kg compared to 3F. As for chlorogenic acid, the pattern was similarly revealed as gallic acids for increased amount at 3F by 0.03 mg/kg from 0.02 mg/kg of NF, and decreased at 5F as 0.02 mg/kg from 3F. It is probable that phenolic acids are derivatives for other phenolic acids (Rice-Evans et al. 1996). In

this study, TPC (Table 3) were enhanced (1.8 and 2.0 times increase at 3F and 5F) during the *R. oligosporus* fermentation and were probably obtained from formation of higher contents of phenolic compounds such as vanillic acids, gallic acids and chlorogenic acids. McCue and Shetty reported that α-amylase and endogenous carbohydrate-cleaving enzymes activities hada role to generate polyphenols from carbohydrates-conjugated phenolic compounds (McCue and Shetty 2003). *R. oligosporus* is a known strain to produce β-glucosidase, β-glucuronidase and xylanase when degrade the cell wall matrix (Huynh et al. 2014). Thus, it was probably metabolized with the bioconversion of phenolic compounds by the fermentation leads the cell-wall degrading enzymes to hydrolysis of glycosidic bonds and produces bound phenolics and aglycone forms (Huynh et al. 2014). Also, TFC (Table 3) were enhanced by 1.2 and 1.5 times increase at 3F and 5F. The fermentation processes releasing phenolic compounds from plant matrixes followed by the metabolic pathways of flavonoids: glycosylation, deglycosylation, ring cleavage, methylation, glucuronidation, and sulfate conjunction which are way of producing new bioactive compounds (Huynh et al. 2014). These increases in phenolic compounds effected to the enhancement of antioxidative activity (Kaur and Kapoor 2002). Increase of flavonoids contents has also influenced on

DPPH-radical scavenging activity resulting in higher antioxidant activity of fermented quinoa extracts than non-fermented ones.

Since antioxidant properties of fermented quinoa extracts were found, anti-inflammatory effect on mammalian cells was further investigated. Anti-inflammatory function of fermented quinoa extract was studied on RAW 264.7, macrophages, with LPS-stimulation. Nitric oxide production was inhibited 29.3% for NF, 38.9 for 3F and 56.4% for5F, resulting in improvement of 192.6% in anti-inflammatory activity (Fig. 2b). Kim et al reported that the flavonoids inhibit NO production in lipopolysaccharide-activated RAW264.7 cells and reduce of iNOS enzyme expression (Kim et al. 1999).

Fatty acids composition in fermented quinoa was increased; myristic acid (68%), palmitic acid (48%), palmitoleic acid (264%), margaric acid (25%), stearic acid (129%), linoleic acid (33%) and linolenic acid (32%), compared to the regular quinoa. As for lipid soluble extract, wound healing effects on scratched HaCaT keratinocyte were enhanced from 24.8% wound closure effects of NL to 67.5% and 58.3% of 3L and 5L, respectively. *R. oligosporus* also has lipase which increases lipid composition in 3F and 5F of 3.72 and 2.05% from 1.71 % of NF, respectively. Phytosterols are group of biologically active compoments present

in pseudocereal lipids and have effects such as anti-inflammatory, antioxidative, anticarcinogenic, and cholesterol-lowering capacity (James 2009). Thus, the increase anti-inflammatory effect and wound healing effect of fermented quinoa compared to the regular quinoa due to increasing of new products from fermentation, and total phenol and total flavonoids contents and fatty acids in fermented quinoa by *R. oligosporus* are representatively studied for the research.

In this regard, in quantitative aspects, the short-term *R. oligosporus* quinoa fermentation was necessary for L-carnitine, GABA, vanillic acid, gallic acid and chlorogenic acid. In functional aspects of antioxidant, anti-inflammatory, and wound healing effects, the long-term quinoa fermentation had stronger activities.

Conclusion

In conclusion, fermented quinoa extract has effective antioxidant and anti-inflammatory activities. These activities may be due to presence of phenolic compounds, flavonoids and the other products produced during fermentation by *R. oligosporus*. Although the 3 day fermentation revealed optimal conditions to produce L-carnitine and GABA, the 5 day fermented quinoa extract had higher TPC, TFC, antioxidant activity and improved reduction in inflammation and wound healing than regular quinoa extract. In this regard, fermented quinoa can be applied to industrial level with short term fermentation for increases in new substances or long term fermentation for functional effects.

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Abstract in Korean

퀴노아는 단백질, 미네랄, 비타민, 폴리페놀, 파이토스테롤이 풍부한 곡물이다. 퀴노아를 5일 차까지 발효하여 주사전자현미경 (SEM) 으로 퀴노아 표면의 전분이 분해됨을 촬영하여 발효 전후의 물리적 특성을 관찰하였다. 본 연구에서는 *Rhizopus oligosporus* (*R. oligosporus*) 로 발효한 퀴노아를 에탄올 추출하여 수용성과 지용성 성분을 나누었다.

수용성 추출물에서는 최적화된 L-카르니틴 함량의 발효 기간을 설정하여 발효 3일 차가 발효 전보다 L-카르니틴은 24배, 가바는 1.9배로 그 양이 증가하였다. 항산화능 확인을 위해 총 페놀 함량, 총 플라보노이드 함량, DPPH 라디컬 소거능 연구가 진행되었다. 발효 5일 차 퀴노아에서 총 페놀 함량과 총 플라보노이드 함량은 각각 8.0 mg gallic acid (GAE)/100 g 그리고 1.9 mg quercetin equivalent (QE) /100 g로 나타났다. 이는 일반 퀴노아의 총 페놀 및 플라보노이드 함량인 4.1 mg GAE/100 g 그리고 1.3 mg QE/100 g 보다 증가한 것이다. 항산화능 (SC_{50})은 발효 5일 퀴노아에서 2.3mg/mL, 발효하지 않은 퀴노아에서는 3.6 mg/mL로 나타났다. 항염증 효능 확인을 위한 RAW264.7 대식세포에서의 산화질소 생성의 저해는 발효 전 29.3%에서 5일 발효에서 56.4%로 염증 발생이 감소하였다.

지용성 추출물에서는 발효 5일 퀴노아 지방산 증가량이 myristic acid(68%), palmitic acid (41%), palmitoleic acid(264%), margaric acid (25%), stearic acid (129%), linoelic acid (33%), linolenic acid (32%)로 나타났다. HaCaT 각질세포로 확인한 상처치료 효과는 발효 전, 발효 3, 5일 퀴노아에서 각각 24.8%,

67.5%, 58.3%로 확인되었다.

이러한 결과로 보아, 발효 퀴노아의 L-carnitine, GABA, Phenolic acids, Fatty acids 함량 증가를 위해 3일 발효가 선호되며 항산화, 항염증, 상처치료의 기능적 측면에서는 이보다 긴 5일 발효가 더 우수한 능력을 보였다. 산업적으로도 위 연구 결과에 따라, 특성에 맞게 장단기 발효로 우수한 고부가가치 산물을 얻어 적용할 수 있을 것으로 기대된다.

Keywords: Anti-inflammatory activity; Antioxidant activity; Fermentation; L-carnitine; *Rhizopus oligosporus*; Quinoa.