Fermentation Technology
ISSN: 2167-7972

Ratha and Jhon, Ferment Technol 2017, 6:2
DOI:~ 10.4172/2167-7972.1000147

Research Article Open Access

Increase of Rutin, Quercetin, and Antioxidant Activity during Germinated Buckwheat (*Fagopyrum esculentum* Moench) Fermentation

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Received date: October 15, 2017; **Accepted date:** October 23, 2017; **Published date:** October 29, 2017

Abstract

Buckwheat is gaining increasing attention as a potential functional food due to its content of bioactive compounds such as rutin, quercetin and other antioxidant substances. In this paper changes in content of rutin, quercetin and antioxidant activity in buckwheat have been measured during germination and fermentation. Buckwheat seeds were germinated for 2 days at 18°C and dried for 20 h at 60°C. Fermentation was carried out at 30°C for *Bif. breve* BV-B and *Bac. subtilis*, 37°C for *Lactobacillus* spp. and *Bif. animalis*, and 40°C for *Bacillus* sp. 224 B4 and *Bac. subtilis* B53 for 48-72 h. When the buckwheat was germinated, the content of rutin decreased from 0.17 g kg^{-1} to 0.1 g kg^{-1} and did not show the effect on increasing the content of quercetin during germination (0.18-0.19 g kg^{-1}). However, when the germinated buckwheat was fermented with *Bifidobacterium breve* BV-B, *Bacillus subtilis* B53, and *Lactobacillus buchneri*, the contents of rutin and quercetin increased to 0.97 g kg^{-1} and 2.53 g kg^{-1}, 0.56 g kg^{-1} and 0.13 g kg^{-1}, 0.37 g kg^{-1} and 0.16 g kg^{-1}, respectively. The antioxidant activities such as total phenolic compounds, total flavonoid content, DPPH radical scavenging activity and ABTS radical scavenging activity of fermented buckwheat were 3.41%, 4.28%, 65.21% and 45.46%, respectively, when *Lac. buchneri* microorganism was used. *Lac. plantarum*, *Bif. animalis*, and *Bif. breve* BV-B showed active fermentation. Overall acceptability of germinated buckwheat fermented by *Bacillus* sp. 224 B4, *Bac. subtilis* B53, *Bac. subtilis* KCCM11315, *Bif. animalis*, and *Bif. breve* BV-B was evaluated as good or very good. This study shows that germinated buckwheat fermented with *Lac. buchneri*, *Lac. plantarum*, *Bif. animalis* and *Bif. breve* BV-B, respectively, is promising as a health functional food.

Keywords: Buckwheat; Germinated; Fermentation; Rutin; Quercetin; Antioxidant activity

Introduction

Buckwheat (*Fagopyrum esculentum* Moench) is a crop belonging to the *Polygonaceae* family and has been cultivated in many countries in Europe, Asia, South Africa, Canada and USA [1-3]. This plant is a rich source of vitamins, large quantity of phenolic compounds and has high protein content with a well-balanced amino acid composition [2,4,5]. In recent years because buckwheat has been perceived as a health promoting grain, it has become a highly appreciated ingredient for the preparation of functional foods [6]. In particular, an important source of rutin and quercetin also were detected in buckwheat grain [7]. Rutin is known to have beneficial health effects such as anti-inflammatory, anti-carcinogenic, cardiovascular benefits, reduced blood pressure, lowered blood sugar concentration, and increased antioxidant activity [8,9]. Quercetin has benefic such as antioxidant, anti-inflammatory, anti-bacterial, anti-coagulative, and anti-hypertensive properties [10-13].

Germination methods are also used to improve the nutritional quality of cereals. It has been reported that the antioxidant activity of germinated barley is higher than that of non-germinated barley [14]. In germinated brown rice, it has been reported that there is 10 times more γ-aminobutyric acid, which has an antihypertensive effect, as compared to non-germinated brown rice [15]. During the past few years, research has been conducted on the change of flavonoids and antioxidant activities of germinated buckwheat seeds. The highest rutin content in germinated buckwheat was found to be 0.158 g kg^{-1} [16]. Germinated buckwheat had better nutritional value and antioxidant activities than non-germinated buckwheat, and it represented an excellent natural source of flavonoids and phenolic compounds, especially rutin and C-glycosylflavones [17].

Various fermented cereal products, such as foods containing probiotic bacteria, rice vinegar, soy sauce, soybean-barley paste, natto and tempeh, are sold in food stores in Asia [18]. New bioactive metabolites in cereals can be produced during fermentation from the starters present in raw materials. Modification of the cereal matrix during fermentation can be tailored to increase the bioaccessibility of bioactive compounds [19]. The fermentation by selected LAB strains can strongly increase the functional benefits of buckwheat by releasing the functional γ-aminobutyric acid in the sourdough [20]. However, there is no article that mentions the increase of rutin and quercetin in fermented buckwheat. This study was performed to determine the antioxidant activity, rutin, and quercetin after fermentation of non-germinated and germinated buckwheat using *Bacillus* spp., *Lactobacillus* spp., and *Bifidobacterium* spp.

Materials and Methods

Microorganisms

Bifidobacterium animalis DY-64 was isolated from human feces [21]. *Bifidobacterium breve* BV-B (KCCM43018), *Lactobacillus* plantarum (KCCM12116), *Lactobacillus buchneri* (KCCM40982), and *Bacillus subtilis* (KCCM11315) were purchased from the Korean Culture Center of Microorganism (KCCM). *Bacillus* sp.224 B4 and *Bac. subtilis* B53 (KCCM 11609P) were isolated from Chungkookjang

in this laboratory. *Bacillus* spp. were cultivated in edible LB broth and *Lactobacillus* spp. and *Bifidobacterium* spp. were cultured in MRS broth.

Germination conditions

Buckwheat (*Fagopyrum esculentum* Moench) grain obtained from North Gwangju, South Korea. Buckwheat seeds were cleaned and soaked in water for 5 h at room temperature. The steeped seeds were drained off, spread out on wet gauze placed in baskets. Germination was carried out in the dark in a germination cabinet for 2 days at 18°C. After germination buckwheat was dried for 20 h at 60°C and was stored at 4°C until its use.

Fermentation conditions

Buckwheats were autoclaved in same amount of water (w/v 1:1) at 121°C for 15 min. After autoclaving, the samples were cooled to 40-50°C and *Bacillus* spp., *Lactobacillus* spp. and *Bifidobacterium* spp. starter culture were inoculated in 10% of the sample buckwheat weight. The fermentation temperature was 30°C for *Bif. breve* BV-B and *Bac. subtilis*, 37°C for *Lactobacillus* spp. and *Bif. animalis*, and 40°C for *Bacillus* sp. 224 B4 and *Bac. subtilis* B53 and incubate for 48-72 h.

Rutin and quercetin analysis

Rutin and quercetin were analyzed based on the method of Yoo et al. [22]. Fermented non-germinated or germinated buckwheat was dried in 60°C oven for 12 h and pulverized using a grinder. Sample powder 1 g was extracted with 20 mL of methanol at 80°C, cooled overnight at 4°C, and filtered through No. 41 Whatman filter papers. After the extracts were filtered through 0.45 μm syringe filters, the amounts of rutin and quercetin were quantitatively measured using HPLC (Shimadzu LC-20, Japan) with a UV detector and Capcell Pak C18 column (UG120 S-5, Shiseido Co., Ltd., Japan). The mobile phase for HPLC analysis was consisted of methanol/acetic acid (v/v 95:5) (solvent A) and water (Solvent B). A linear gradient of water was applied from 85% to 90% for 35 min, followed by an increase to 100% in 15 min of (Solvent B). The flow rate was 0.5 mL/min, the column temperature was set at 45°C, and the components were detected at 350 nm.

Determination of antioxidant activity

Buckwheat flour extracts were prepared according to the method described by Prior et al. [23]. The buckwheat flour was treated with hexane (w/v 1:10, two successive 1 h extractions in the dark) to get rid of hydrophobic compounds. Then the residues extracted with hexane were sonicated and extracted twice with a mixture of acetone, water, and acetic acid (v/v/v 70:29.5:0.5) for 15 min (w/v 1:20). The extracts were combined, filtered and concentrated by a rotary evaporator. The obtained phenolic extracts were re-dissolved in methanol at the concentration of 1.0 mg/mL for the antioxidant activity and phenolic content analysis.

The phenolic content was measured using the Folin-Ciocalteu method. One mL of the sample was added to 9 mL of distilled water and vortexed. Then 1 mL of Folin-Cialteu reagent was added, vortexed and stood for 5 min at room temperature. 7 mL of 7% sodium carbonate and 4 mL distilled water were added, allowed to stand for 90 min at room temperature and absorbance was measured at 750 nm with a spectrophotometer. The flavonoid content was determined by a colorimetric method. Aliquots of extract (1 mL) were reacted with distilled water (4 mL) and 5% sodium nitrite (0.3 mL). After 5 min, a 10% aluminum chloride solution (0.3 mL) and 1M sodium hydroxide (2 mL) were added and allowed to stand 5 min, followed by adjusting the volume to 5 mL with distilled water. After 15 min, the absorbance was measured at 510 nm with a spectrophotometer.

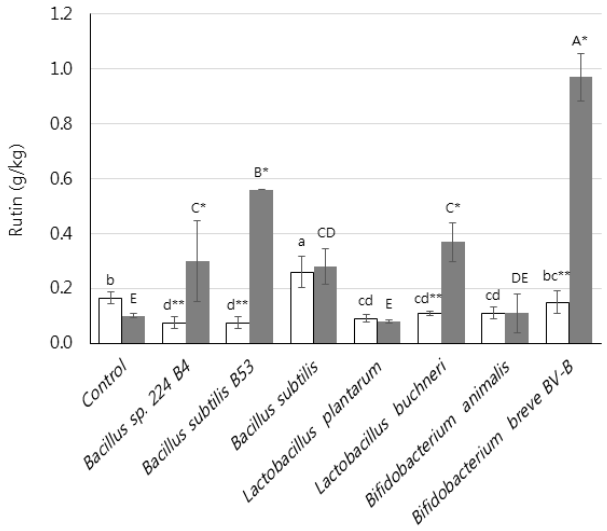

Figure 1: Rutin amount in non-germinated or germinated buckwheat fermented with different kind of microorganisms. Open bar indicates non-germinated and filled bar indicates germinated sample. For each set of samples, mean values of bars with different letters (a, b, c for non-germinated and A, B, C for germinated) are significantly different (P<0.05). *indicate significant differences (P<0.05) in non-germinated and germinated buckwheat. Error bars represent for sample n=3.

A working solution of 0.1 mM DPPH (2, 2-diphenyl-1-picrylhydrazyl) was prepared in absolute ethanol. Before the assay, the absorbance of the working solution was read at 515 nm. 380 μL of the DPPH solution and 20 μL of the sample were added to a 96 wall micro plate reader, with shaking for 5 s, and allowed to stand for 30 min at room temperature. Absorbance was read at 515 nm.

$$DPPH(\%) = 1 - \frac{absorbance\ of\ sample}{absorbance\ of\ control} \times 100\%$$

ABTS stock was prepare by addition of 3 mL potassium phosphate buffer to 7 mM ABTS (0.012 g/3 mL+2.45 mM potassium persulfate 0.018 g), vortexed and kept in the refrigerator for 12 h. Stock solution was diluted 70 times to make working solution in water. Working solution (v/v 70:1) was prepared by addition of 500 μL stock solution to 38 mL phosphate buffered saline and vortexed. 10 μL of the sample and 390 μL of the working solution of ABTS were added to a 96 wall microplate reader, allowed to stand for 2 min and the absorbance was read at 734 nm.

$$ABTS(\%) = 1 - \frac{absorbance\ of\ sample}{absorbance\ of\ control} \times 100\%$$

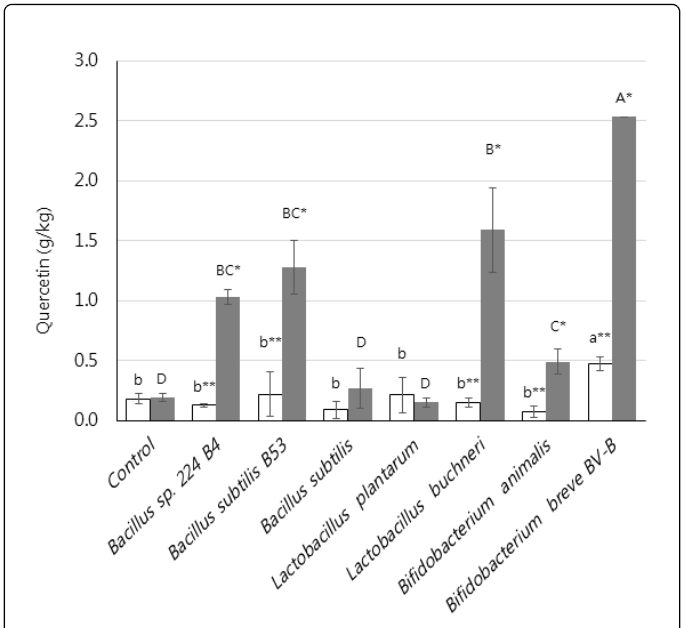

Figure 2: Quercetin amount in non-germinated or germinated buckwheat fermented with different kind of microorganisms. Open bar indicates non-germinated and filled bar indicates germinated sample. For each set of samples, mean values of bars with different letters (a, b, c for non-germinated and A, B, C for germinated) are significantly different (P<0.05). *indicate significant differences (P<0.05) in non-germinated and germinated buckwheat. Error bars represent for sample n=3.

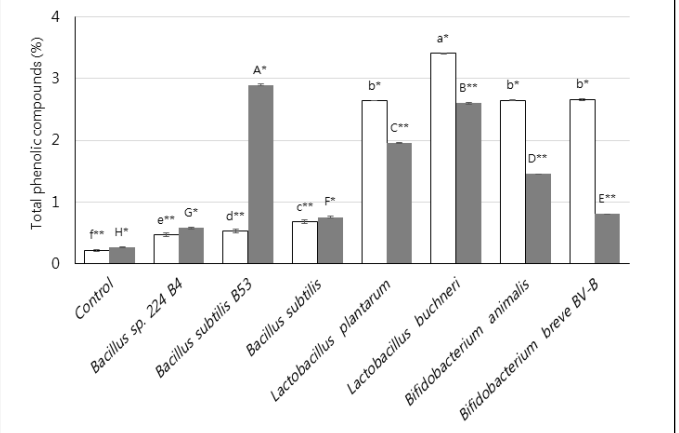

Figure 3: Total phenolic compounds in non-germinated or germinated buckwheat fermented with different kind of microorganisms. Open bar indicates non-germinated and filled bar indicates germinated sample. For each set of samples, mean values of bars with different letters (a, b, c for non-germinated and A, B, C for germinated) are significantly different (P<0.05). * indicate significant differences (P<0.05) in non-germinated and germinated buckwheat. Error bars represent for sample n=3.

Sensory evaluation

Non-germinated and germinated fermented buckwheat samples, sensory evaluation was performed by student. Sensory evaluations of bitter taste, odor, savory taste and overall acceptability were performed using a 5 point scale. The scale level was consisted of "very strong" or "very good" (5 points), "strong" and "good" (4 points), "moderate" (3 points), "weak or poor" (2 points) and "very weak" or "very poor" (1 point).

Statistical analysis

All data were expressed as a mean ± standard deviation. Duncan's multiple range test was used to test the difference between mean values of data. A p-value of <0.05 was considered statistically significant.

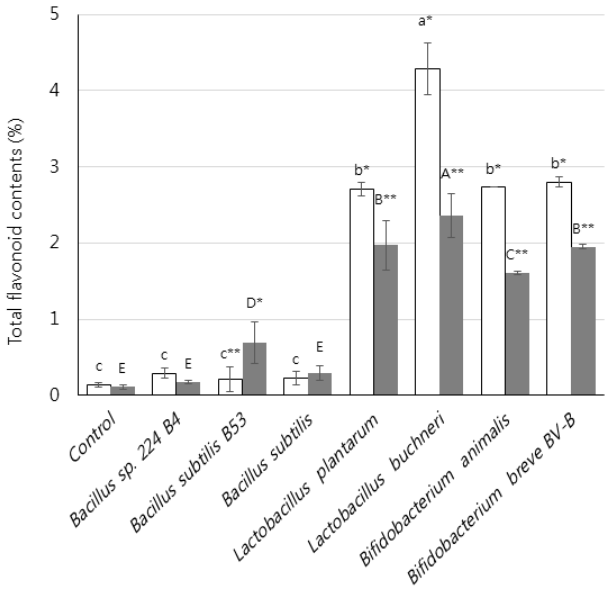

Figure 4: Total flavonoid contents in non-germinated or germinated buckwheat fermented with different kind of microorganisms. Open bar indicates non-germinated and filled bar indicates germinated sample. For each set of samples, mean values of bars with different letters (a, b, c for non-germinated and A, B, C for germinated) are significantly different (P<0.05). * indicate significant differences (P<0.05) in non-germinated and germinated buckwheat. Error bars represent for sample n=3.

Results and Discussion

Rutin and quercetin

Rutin level in non-germinated buckwheat decreased to approximately 0.1 g/kg following fermentation with *Bacillus* sp. 224 B4, *Bac. subtilis* B53, *Lac. plantarum*, *Lac. buchneri*, and *Bif. animalis* (Figure 1). *Bif. breve* BV-B fermentation of non-germinated buckwheat resulted in the same amount of rutin, and *Bac. subtilis* increased the level (0.26 g kg^{-1}). Rutin amount was the same as the control (0.17 g kg^{-1}) in germinated buckwheat fermented with *Lac. plantarum* and *Bif. animalis*. The amounts increased to 0.3-0.97 g kg^{-1} by fermentation with *Lac. buchneri*, *Bif. breve* BV-B, *Bacillus* sp. 224 B4, *Bac. subtilis*

B53, and *Bac. subtilis*. In addition, rutin amount in fermented germinated buckwheat was different from fermented non-germinated buckwheat as shown in Figure 1. Previous studies have reported a wide range of rutin content in common buckwheat. They were 0.2-0.3 g kg^{-1} [24] and 0.1 g kg^{-1} [25] in groats of buckwheat. Thus, these results are comparable to ours. However, our results showed that germinated buckwheat fermented by *Lac. buchneri*, *Bif. breve* BV-B, and *Bac. subtilis* B53 contained 0.37, 0.97, and 0.56 g/kg, respectively.

Quercetin concentration was 0.18-0.47 g kg^{-1} in buckwheat as shown in Figure 2. When buckwheat was fermented with *Bac. subtilis* or *Bif. animalis* the quercetin contents was similar to the control (0.18 g kg^{-1}). The fact of the matter is that there was no significant difference in quercetin content of non-germinated buckwheat control and the non-germinated buckwheat fermented with all the bacterial cultures used except *Bif. breve* BV-B. Quercetin amount in germinated and fermented buckwheat increased to 0.49-2.53 g kg^{-1}, except the buckwheat fermented with *Lac. plantarum* or *Bac. subtilis*, that were not significantly different from the control (0.19 g kg-1). *Bif. breve* BV-B produced the highest quercetin amount (2.53 g kg^{-1}) by fermentation of germinated buckwheat.

In previous studies, the levels of quercetin were determined, and values of 0.3, 1.2, and 0.2 g kg^{-1} were reported for buckwheat hull, bran, and flour, respectively [26], with 4.3 g kg^{-1} for buckwheat noodle [22]. Thus, our results showed that either germinated or non-germinated buckwheat fermented with *Bif. breve* BV-B contain higher quercetin amount than those in buckwheats reported previously [26]. Figure 2 shows that quercetin amount in fermented non-germinated buckwheat was lower than fermented germinated buckwheat.

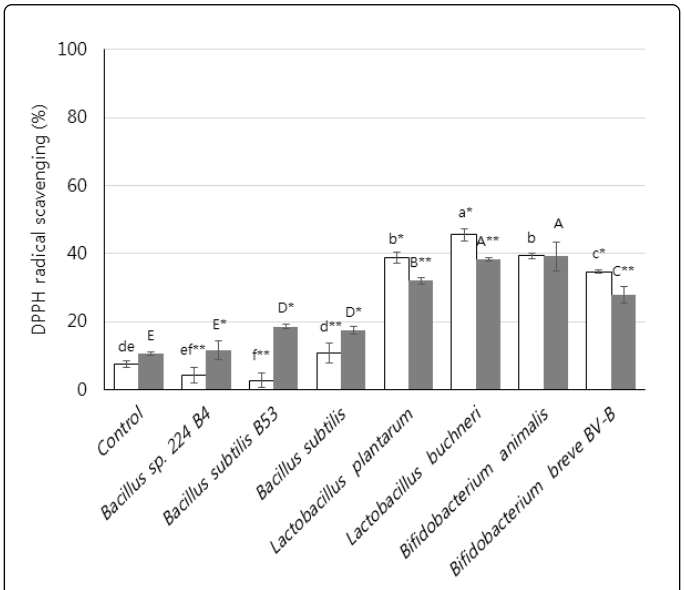

Figure 5: DPPH radical scavenging activity in non-germinated or germinated buckwheat fermented with different kind of microorganisms. Open bar indicates non-germinated and filled bar indicates germinated sample. For each set of samples, mean values of bars with different letters (a, b, c for non-germinated and A, B, C for germinated) are significantly different (P<0.05). *indicate significant differences (P<0.05) in non-germinated and germinated buckwheat. Error bars represent for sample n=3.

Total phenolic compounds

Total phenolic compounds of non-germinated buckwheat was 0.2%. And the value was 0.27% in germinated buckwheat. When the non-germinated buckwheat was fermented with the starter microorganisms total phenolic compounds increased to 2.2-3.41%. The amounts in non-germinated buckwheat fermented with *Lac. buchneri*, *Bif. animalis*, and *Bif. breve* BV-B was 3.41%, 2.65%, and 2.66%, respectively. The phenolic compounds in fermented germinated buckwheat were 0.27-2.9%. The highest value of 2.9% was obtained from buckwheat fermented with *Bac. subtilis* B53. According to Chlopicka et al. [27], the total phenolic compounds in buckwheat was 0.73% in dry weight. Figure 3 shows that by the fermentation total phenolic compounds increased higher in non-germinated buckwheat than in germinated buckwheat.

Total flavonoid content

Total flavonoid contents of non-germinated buckwheat was 0.14% while the value of germinated buckwheat was 0.11%. The range of total flavonoid contents in fermented non-germinated buckwheat was 0.14-4.28% and the highest value was found in products fermented with *Lac. buchneri*. Fermented germinated buckwheat contained 0.11-2.36% flavonoids, with the highest value in buckwheat fermented with *Lac. buchneri* (2.36%). Total flavonoid contents was reported previously as 0.015% in dry weight [27]. This value is lower than that obtained in this work. By the fermentation the total flavonoid contents increased higher in non-germinated buckwheat than in germinated buckwheat, as shown in Figure 4.

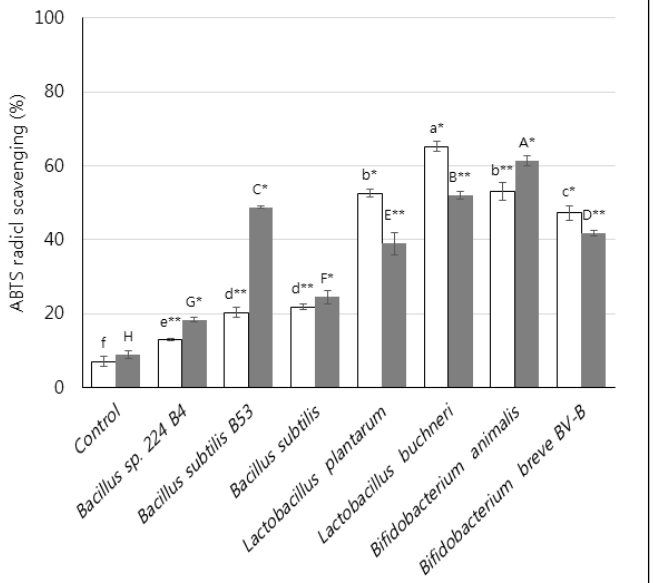

Figure 6: ABTS radical scavenging activity in non-germinated or germinated buckwheat fermented with different kind of microorganisms. Open bar indicates non-germinated and filled bar indicates germinated sample. For each set of samples, mean values of bars with different letters (a, b, c for non-germinated and A, B, C for germinated) are significantly different (P<0.05). *indicate significant differences (P<0.05) in non-germinated and germinated buckwheat. Error bars represent for sample n=3.

DPPH radical scavenging activity

DPPH radical scavenging activity of non-germinated buckwheat was 7.75% while the value of germinated buckwheat was 10.73%. The range of DPPH radical scavenging activity was 7.75-45.56% in fermented non-germinated buckwheat. Germinated buckwheat fermented with some edible bacteria showed 10.73-39.19% DPPH radical scavenging activity. The amount in buckwheat fermented with *Lac. buchneri*, *Bif. animalis*, *Lac. plantarum*, and *Bif. breve* BV-B was 45.56%, 39.42%, 38.97% and 34.75%, respectively. For the germinated buckwheat the higher DPPH radical scavenging activity was obtained by fermentation with *Lac. buchneri*, *Bif. animalis*, and *Lac. plantarum* and the value was 38.48%, 39.19%, and 38.97%, respectively. Sun and Ho reported buckwheat DPPH radical scavenging activity as 78.6% [28]. This value is higher than that obtained in this work. Fermentation increased DPPH radical scavenging activity compared to the control (7.45-10.73%) as shown in Figure 5. The activity was heavily dependent on the starter microorganisms such as *Lac. buchneri*, *Bif. animalis*, and *Lac. plantarum*.

ABTS radical scavenging activity

ABTS radical scavenging activity of non-germinated buckwheat was 7.1% while the value of germinated buckwheat was 8.83%. ABTS radical scavenging activity was 7.1-65.21% in fermented non-germinated buckwheat. The ABTS radical scavenging activity in fermented non-germinated buckwheat with *Lac. buchneri*, *Bif. animalis*, *Lac. plantarum* and *Bif. breve* BV-B was 65.21%, 53.14%, 52.56% and 47.25%, respectively. In germinated buckwheat fermented with some edible bacteria showed 8.83-61.41% ABTS scavenging activity. The germinated fermented buckwheat the higher amount was obtained by *Bif. animalis*, *Lac. buchneri*, *Lac. plantarum*, *Bac. subtilis* B53, and *Bif. breve* BV-B and the value was 61.41%, 52.04%, 38.97%, 48.84% and 41.78%, respectively. Fermentation increased ABTS radical scavenging activity of non-germinated and germinated fermented buckwheat compared to the control (7.1-8.83%) as shown in Figure 6.

Microorganisms	Buckwheat (B) and Germinated Buckwheat (GB)	[a]Bitter taste	[a]Sour taste	[a]Savory taste	[b]Odor	[b]Overall acceptability
Bacillus sp.224 B4	B	1	-	4.5	4.5	4.5
	GB	1	-	4.5	3.5	4.5
Bacillus subtilis B53 (KCCM 11609P)	B	1	-	3	2	4
	GB	1	-	3	1.5	4
Bacillus subtilis (KCCM11315)	B	1	-	4.5	3.5	4.5
	GB	1.25	-	4	4	4
Bifidobacterium animalis DY-64	B	-	3	3.5	4	3.8
	GB	-	3.4	3.6	4.5	4.5
Bifidobacterium breve BV-B (KCCM43018)	B	-	3.25	2.75	3	3.1
	GB	-	1.8	4	4.5	4
Lactobacillus plantarum (KCCM12116)	B	-	3.5	3	3	3.0
	GB	-	3	2	3	3
Lactobacillus buchneri (KCCM40982)	B	-	3	3	3.5	3
	GB	-	3	3	3.5	3.0

[a]very weak-very strong (1-5), [b]very poor-very good (1-5)

Table 1: Sensory evaluation of fermented buckwheat.

Sensory scores of fermented buckwheats

The result of sensory evaluation by *Bacillus* spp., *Lactobacillus* spp., and *Bifidobacterium* spp. in buckwheat fermentation was show in Table 1. The savory taste level of fermented non-germinated buckwheat and germinated fermented buckwheat by *Bacillus* sp. 224 B4 or *Bac. subtilis* KCCM11315 was higher than the score of *Bif. animalis*, *Lac. buchneri*, *Lac. plantarum*, and *Bac. subtilis* B53. The savory taste of geminated buckwheat fermented by *Bif. breve* BV-B was evaluated better than non-germinated one. All the products was not evaluated below level "moderate" in overall acceptability. The high score over 4 in overall acceptability was obtained in fermented buckwheat, whether it was germinated or not, by *Bacillus* sp. 224 B4, *Bac. subtilis* B53, and *Bac. subtilis* KCCM11315. By the way, only germinated buckwheat was evaluated as good or very good when it was fermented by *Bif. animalis* or *Bif. breve* BV-B.

Conclusion

This study demonstrates that fermented buckwheat exhibit significant amount of rutin, quercetin, and high antioxidant activities based on studies of total phenol contents, total flavonoid contents, DPPH radical scavenging activities, and ABTS radical scavenging activities. These findings suggest that fermented buckwheat based

foods may contain important antioxidants that could be beneficial to human health. Fermentation processes can enhance the levels of many bioactive compounds in cereals and can be used to improve functional food. The type of fermentation clearly had an effect on the potentially bioactive constituents of buckwheat. However, studies on microbial population changes and activities of relevant enzymes during fermentation of cereals are required in order to establish the precise mechanisms that cause fermented cereals to improve their nutritional value. In this study, we found that high level of rutin and quercetin were present in fermented germinated buckwheat compared to the control. In particular, rutin and quercetin in germinated buckwheat fermented with *Bif. breve* BV-B, *Bac. subtilis* B53 , *Lac. buchneri* contain 0.97 and 2.53, 0.56 and 0.13, and 0.37 g kg^{-1} rutin and 0.16 g kg^{-1} quercetin, respectively. For antioxidant activity such as total phenol content, total flavonoid content, DPPH, and ABTS radical scavenging activities, the fermented buckwheat had higher values than the germinated buckwheat fermented with *Lac. buchneri*, with values of 3.41%, 4.28%, 65.21% and 45.46%, respectively. Sensory score of germinated buckwheat fermented by *Bacillus* sp. 224 B4, *Bac. subtilis* B53, *Bac. subtilis* KCCM11315, *Bif. animalis*, and *Bif. breve* BV-B was very high.

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