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original scientific paper

The Quality Improvement of Solid-State Fermentation with *Cordyceps militaris* by UVB Irradiation

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Summary

Cordyceps militaris, a medicinal and edible mushroom, was used to ferment buckwheat and embryo rice by solid-state fermentation (SSF). Our aim was to investigate the effect of ultraviolet B (UVB) light irradiation on the content of vitamin D_2 and biologically effective components, and antioxidant properties of buckwheat and embryo rice in SSF with *C. militaris*. Irradiated samples of buckwheat and embryo rice fermented by *C. militaris* had significantly increased vitamin D_2 content, from 0-0.3 to 1.18-16.79 μg/g, while the increase in fresh embryo rice fermented by *C. militaris* was up to 16.79 μg/g. The content of adenosine, cordycepin and polysaccharide in irradiated dry samples fermented by *C. militaris* was 0.08 to 11.15 mg/g, higher than that of the irradiated fresh samples fermented by *C. militaris* (0.07–8.40 mg/g). Samples fermented by *C. militaris* had lower EC_{50} values and higher content of antioxidants than did unfermented samples. When the solid-state fermented sample was irradiated with UVB light, the content of biologically effective and antioxidant components and antioxidant property of sample decreased. However, it still contained enough of these biologically effective and antioxidant components.

Key words: *Cordyceps militaris*, UVB irradiation, solid-state fermentation, vitamin D_2, antioxidant activity

Introduction

The medicinal caterpillar fungus *Cordyceps militaris* (L.) Link (Clavicipitaceae, Ascomycetes) is the only cultivated caterpillar fungus whose fruiting bodies can be formed without the process of caterpillar infection (*1*). It contains many bioactive components, such as adenosine, cordycepin and polysaccharides. Because of its various physiological activities, including anticancer, antiageing, antiviral, anti-inflammatory and hypoglycaemic activities, it is used for multiple medicinal purposes (*2-8*). Currently, cultivation methods of *C. militaris* mainly include solid-state fermentation, submerged fermentation and membrane-surface liquid cultivation (*9,10*). Furthermore, the solid-state fermentation (SSF) of grains by *C. militaris* results in biotransformed grains with high antioxidant activity, DNA damage protection, and angiotensin I-converting enzyme (ACE) inhibitory activity, thereby providing a method to obtain

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oats and chickpeas with enhanced functional value (*11,12*). Therefore, in the present study, SSF of grains by *C. militaris* was studied to find a technological method to potentially produce functional foods and nutraceutical formulations.

The SSF technique involves the growth and metabolism of microorganisms on solid materials, which generally have a low water content to reduce the risk of contamination (*13*). It is particularly suitable for propagating fungi that are used as food or medicine. There are several recent publications describing the development of SSF for utilizing such raw materials for the production of food matrices and value--added fine products such as enzymes, flavonoids, phenols, amino acids, organic acids, and biologically active secondary metabolites, among others (*14-17*).

Vitamin D is important for calcium absorption and bone health. In addition, vitamin D deficiency causes many health problems, including immune-mediated diseases, cardiovascular diseases, diabetes, cancer and infections (*18*). Therefore, vitamin D intake has attracted increasing interest in recent years, and consuming foods rich in vitamin D has become a general preference (*19*). Edible and medicinal mushrooms contain many physiologically active substances, including ergothioneine, ergosterol, vitamin D_2, polysaccharides and triterpenoids (*20*). Mushrooms are a natural and non-animal food source of vitamin D_2. Fortunately, when mushrooms are exposed to UV light, ergosterol undergoes photolysis and results in an increase in the amount of vitamin D_2 (*21-26*).

Most studies have worked on fruiting bodies to enhance their vitamin D_2 content. However, information is lacking about the application of UV light on solid-state fermented products. Since they are easily produced compared to fruiting bodies, there is a great potential for the development of healthy food with high content of vitamin D_2 and enhanced functional value. In this regard, we cultivated *C. militaris* on buckwheat and embryo rice with ultraviolet B (UVB) irradiation, which might provide abundant nutraceutical compounds derived from the biologically active components. Therefore, the objective of this work is to assay the influence of *C. militaris* fermentation with UVB irradiation on the biologically active components, antioxidant properties and antioxidant components of buckwheat and embryo rice. Furthermore, the analysis of biologically active components was conducted to determine the vitamin D_2, ergosterol, adenosine, cordycepin and polysaccharide contents of irradiated and unirradiated samples. The antioxidant properties of the ethanolic and hot water extracts of irradiated samples, including their reducing power, radical scavenging ability and ferrous ion chelating ability, were determined and compared with those of the unirradiated samples. The content of potential antioxidant components (flavonoids and total phenols) was also determined.

Materials and Methods

Materials and microorganism

Buckwheat and embryo rice were purchased from a local market in Taichung City, Taiwan, and the mycelium of *Cordyceps militaris* was obtained from the Star-Ocean International Co., Taichung City, Taiwan. Chemicals and reagents were of analytical grade. *C. militaris* was cultivated and maintained on potato dextrose agar (PDA; Merck, Darmstadt, Germany) slants and subcultured every month. Slants were incubated at 25 °C for seven days, and then stored at 4 °C.

Inoculum preparation and SSF of buckwheat and embryo rice

Preparation of fermented buckwheat and embryo rice started with the propagation of *C. militaris* on a PDA slant at 25 °C for seven days, and then the 0.5-cm^2 mycelial block was inoculated into a 250-mL flask containing 100 mL of liquid culture medium. The liquid culture medium was composed of the following components (in %): glucose 2, yeast extract 0.5, $MgSO_4 \cdot 7H_2O$ 0.05, KH_2PO_4 0.05 and KHP_2O_4 0.05. The culture was incubated at 25 °C for five days on a rotary shaker (100 rpm; Hsin Chien Xiang Precision Industry Co., Kaohsiung, Taiwan). This *C. militaris* seed culture containing mycelia was ready to serve as inoculum for buckwheat and embryo rice fermentation. The buckwheat or embryo rice (15 g) was soaked with 45 mL of water for 2 h and then sterilized at 121 °C for 15 min. The culture was homogenized for 10 s in a Waring blender (7011G; Torrington, CT, USA). Then, 5 mL of the homogenized seed culture (7.4 g of culture per L of dry biomass) were inoculated into the autoclaved buckwheat and embryo rice, mixed thoroughly, placed in a 90 mm×15 mm Petri dish and incubated at 25 °C for 12 days.

UVB irradiation of SSF substrates

An open Petri dish of SSF substrate was placed 19 cm from the source of irradiation, a UVB lamp (λ=280-360 nm, G15T8E; Sankyo Denki, Tokyo, Japan) for 2 h at room temperature (25 °C). The UVB irradiation intensity was measured by a UVX 31 radiometer (UVP, Upland, CA, USA) to be 0.36 mW/cm^2, and the irradiation doses for 2 h were 25.9 kJ/m^2 (*27*). The sample was hot-air-dried at 50 °C, then ground in an RT-08 pulverizing machine (Rong Tsong Precision Technology, Taichung, Taiwan). The samples were sequentially ground and sieved until all particles were <0.4 mm and were then stored in darkness at 4 °C before use.

Samples obtained using three processing methods before UVB irradiation were examined in the following experiments. The first treatment included solid-state fermentation and then drying of buckwheat or embryo rice, followed by UVB irradiation. In the second treatment, after solid-state fermentation, followed by UVB irradiation, the samples were dried. The third approach started with UVB irradiation, followed by solid-state fermentation and finally drying of the samples of buckwheat and embryo rice. Other unfermented samples were used as control groups for comparison, and they included: buckwheat, UVB irradiated buckwheat, buckwheat mycelia, embryo rice, UVB irradiated embryo rice and embryo rice mycelia.

Determination of biologically active components

Adenosine and cordycepin were extracted and analysed according to the method of Masuda *et al.* (*28*) with some modifications. A sample (5 g) was mixed with 100 mL of

distilled water, sonicated at 40 °C for 30 min and centrifuged (CN5101; Hsiangtai Machinery Industry Co., Taipei, Taiwan) at 3000×*g* for 10 min. The residue was then extracted twice and centrifuged as described above. The combined filtrate was dried in a rotary evaporator (N-1000; EYELA, Tokyo Rikakikai Co., Tokyo, Japan) at 40 °C until the solvent was evaporated, redissolved in distilled water, and filtered prior to injection into high-performance liquid chromatograph (HPLC) which consisted of L-2130 pump and L-2400 UV detector (Hitachi, Tokyo, Japan), and a LiChrospher 100 RP-18e column (4.6 mm×250 mm, 5 μm i.d.; Merck). The mobile phase was methanol/0.02 M potassium dihydrogen phosphate (18:85 by volume) at a flow rate of 1.0 mL/min, and UV detection was done at λ=254 nm. The content of adenosine and cordycepin was calculated on the basis of the calibration curves of authentic adenosine and cordycepin (Sigma-Aldrich; St. Louis, MO, USA).

Vitamin D_2 and ergosterol were extracted and analysed according to the method of Huang *et al.* (*23*) with some modifications. A sample (5 g) was mixed with 10 mL of dimethyl sulfoxide (Merck) and sonicated at 45 °C for 30 min. Then, 10 mL of methanol and water (1:1 by volume) and 20 mL of hexane were added, the mixture was sonicated at 45 °C for another 30 min and centrifuged (CN-5101; Jusun Instruments Co. Ltd, Taiwan, ROC) at 3000×*g* for 10 min. The residue was extracted twice with 20 mL of hexane and centrifuged. The combined filtrate was dried in a rotary evaporator (N-1000; EYELA) at 40 °C until the solvent was evaporated, redissolved in 1 mL of methanol (LC grade, Merck) and filtered using a 0.45-μm polyvinylidene difluoride filter (Millipore, Billerica, MA, USA) prior to HPLC injection in the same manner as in the adenosine and cordycepin assay. The used HPLC system and column were the same as for the adenosine and cordycepin assay. The mobile phase was methanol/H_2O (95:5 by volume) at a flow rate of 1.0 mL/min, and UV detection was done at λ=254 nm. The content of vitamin D_2 and ergosterol was calculated on the basis of the calibration curve of authentic vitamin D_2 and ergosterol (Sigma-Aldrich).

Polysaccharides were extracted and analysed according to the method of Huang *et al.* (*23*). A sample (1 g) was refluxed with 50 mL of deionized water for 30 min. The mixture was cooled to room temperature and filtered through Whatman No. 4 filter paper (Sigma-Aldrich). The residue was then refluxed with two additional 10-mL portions of deionized water for 30 min, cooled and filtered. The combined filtrate was dialyzed using a Cellu Sep T2 tubular membrane (MMCO=6000-8000; Membrane Filtration Products, Inc., Seguin, TX, USA) for 24 h. The water soluble polysaccharide content was determined by the phenol-sulfuric acid assay (*29*).

Preparation of extracts

For ethanolic extraction, a subsample (10 g, dry basis) was extracted by stirring with 100 mL of 95 % (by volume) ethanol at 25 °C and 150 rpm for 24 h and filtered through Whatman No. 1 filter paper (Sigma-Aldrich). The residue was then extracted with two additional 100-mL portions of ethanol and filtered. The combined ethanolic extracts were then dried in a rotary evaporator (N-1000; EYELA) at 40 °C until the solvent was evaporated. For hot water extraction, a subsample (10 g) was extracted by stirring with 100 mL of boiling water at 100 °C and 150 rpm for 10 min and filtered. The residue was extracted with two additional 100-mL portions of boiling water as described above. The combined hot water extracts were then freeze dried. The dried ethanolic and hot water extracts were redissolved in ethanol and water respectively to concentrations of 50 mg/mL and stored at 4 °C for further use.

Determination of antioxidant properties

The reducing power was determined according to the method of Oyaizu (*30*). The reducing power assayed the ability of the extracts to form a coloured complex with ferricyanide, which is an electron acceptor. The ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich) radicals was determined according to Shimada *et al.* (*31*). The sample chelating ability was determined according to the method of Dinis *et al.* (*32*). EC_{50} values were obtained by interpolation from linear regression analysis.

Determination of antioxidant components

Flavonoids were determined according to the method of Zhishen *et al.* (*33*). An aliquot (0.5 mL) of an appropriately diluted sample or a standard solution of rutin (Sigma-Aldrich) was added to the flask that contained 0.1 mL of 5 % aqueous $NaNO_2$ solution. After 6 min, 0.1 mL of 10 % aqueous $AlCl_3$ solution was added, and after another 6 min, 1 mL of 5 % aqueous NaOH solution was added to the mixture. Next, the mixture was diluted with 0.8 mL of deionized water and thoroughly mixed. The absorbance of the mixture was determined at 510 nm against a blank using spectrophotometer (U-2900; Hitachi, Tokyo, Japan). The flavonoid content in the sample was expressed in mg of rutin equivalents (RE) per g of sample.

Total phenols were determined according to the method of Taga *et al.* (*34*). Each sample (20 mg) was dissolved in a solution of 5 mL of 1.3 % HCl in methanol/deionized water (60:40 by volume), and the resulting mixture (100 μL) was added to 2 mL of 2 % aqueous Na_2CO_3 solution. After 3 min, 100 μL of 50 % Folin-Ciocalteu's phenol reagent (Sigma-Aldrich) were added to the mixture. After resting for 30 min, the absorbance was measured at 750 nm against a blank using spectrophotometer model U-2900 (Hitachi). The content of total phenols in the sample was calculated on the basis of the calibration curve of gallic acid (Sigma-Aldrich) and was expressed in mg of gallic acid equivalents (GAE) per g of sample.

Statistical analysis

A one-way analysis of variance (ANOVA) for a completely random design was used, with Statistical Analysis System v. 9.4 (SAS Institute, Inc., Cary, NC, USA). The results presented in this paper were the average of three independent assays and expressed as the mean value±standard deviation (S.D.). The difference among the mean values was determined using Duncan's multiple range tests at the level of α=0.05. The EC_{50} values were obtained from linear regression analysis. Principal component analysis (PCA) provided an overview of the relationships between irradiation and

the biologically active components, antioxidant properties and antioxidant components of buckwheat and embryo rice. PCA analysis was performed using XLSTAT-Pro v. 2016 software (Addinsoft, Inc., Brooklyn, NY, USA), which provides the internal structure of the data and gives good dispersion of data (*35*).

Results and Discussion

Biologically active components

Three types of unfermented controls (substrates, substrates containing 5 % mycelia, and UVB irradiated substrates) contained trace amounts of vitamin D_2 (<0.71 μg/g) (Fig. 1). With UVB irradiation of the three differently treated fermented products, the vitamin D_2 content was in the descending order: fresh fermented products>dried fermented products>untreated fresh products. The fermented and UVB irradiated buckwheat and embryo rice contained the highest mass fractions of vitamin D_2 (13.03 and 16.79 μg/g, respectively). According to the results, these samples with higher moisture content absorb more irradiation energy for vitamin D_2 conversion. Drying of buckwheat and embryo rice resulted in less efficient absorption of UVB irradiation. However, pre-fermentation with UVB irradiation could denature proteins and damage the DNA in living organisms, and hence suppress the growth of microorganisms, resulting in lower conversion of vitamin D_2.

The three variously treated fermented products contained higher mass fractions of ergosterol (26.91-107.46 μg/g) than the unfermented controls (0-16.89 μg/g) (Fig. 1). Generally, ergosterol content has been positively correlated with mycelial biomass in products obtained by solid-state fermentation (*36*). Therefore, *C. militaris*-fermented buckwheat samples contained higher mass fractions of ergosterol than *C. militaris*-fermented embryo rice samples, except for the non-irradiated buckwheat sample. Simon *et al.* (*37*) reported that the vitamin D_2 content in mushrooms was due to photosynthetic/thermal processes occurring from the exposure of ergosterol to UV light. UVB irradiation of SSF products resulted in nonsignificant decreases in ergosterol content, whereas vitamin D_2 content significantly increased. Guan *et al.* (*22*) reported that ergosterol may not be the limiting factor of vitamin D_2 conversion since previtamin D_2 can undergo several reversible photoreactions.

Process operating conditions, mushroom morphology and fermentation types have been shown to play important roles in vitamin D_2 accumulation in mushrooms. Jasinghe and Perera (*38*) reported that the optimum moisture content of shiitake for the conversion of ergosterol to vitamin D_2 was approx. 70-80 % on a wet mass basis. Therefore, we used the fresh fermented and UVB irradiated buckwheat and embryo rice that contained 70-80 % moisture, which could enhance such vitamin D_2 conversion. Furthermore, both the fresh and dried samples have the ability to produce vitamin D_2 when they are subjected to the UVB irradiation.

Adenosine and cordycepin are metabolic products resulting from mycelial growth on grains. Polysaccharides are the most abundant and well-known bioactive constituents of edible and medicinal mushrooms. Dried fermented and UVB irradiated buckwheat and embryo rice contained higher mass fractions of adenosine (95.75 and 334.34 μg/g), cordycepin (76.11 and 85.78 μg/g) and polysaccharides (5.73 and 11.15 mg/g), respectively, than the fresh and untreated fermented products (Fig. 1). After UVB irradiation, the adenosine, cordycepin and polysaccharide contents of dried fermented products demonstrated a 1.10 to 1.41-fold greater increase than the fresh products. The UVB irradiation in the presence of 70-80 % moisture might cause the decomposition of adenosine, cordycepin and polysaccharide in fresh products. In other words, refermentation with UVB irradiation could denature proteins and damage DNA in living organisms and hence suppress the growth of microorganisms, resulting in lower conversion of bioactive compounds.

Huang *et al.* (*20*) reported that UVB irradiation could increase the vitamin D_2 content of mushroom fruiting bodies and mycelia. Based on the obtained results, the following experiment on UVB irradiation can be applied in solid--state fermentation of mushroom.

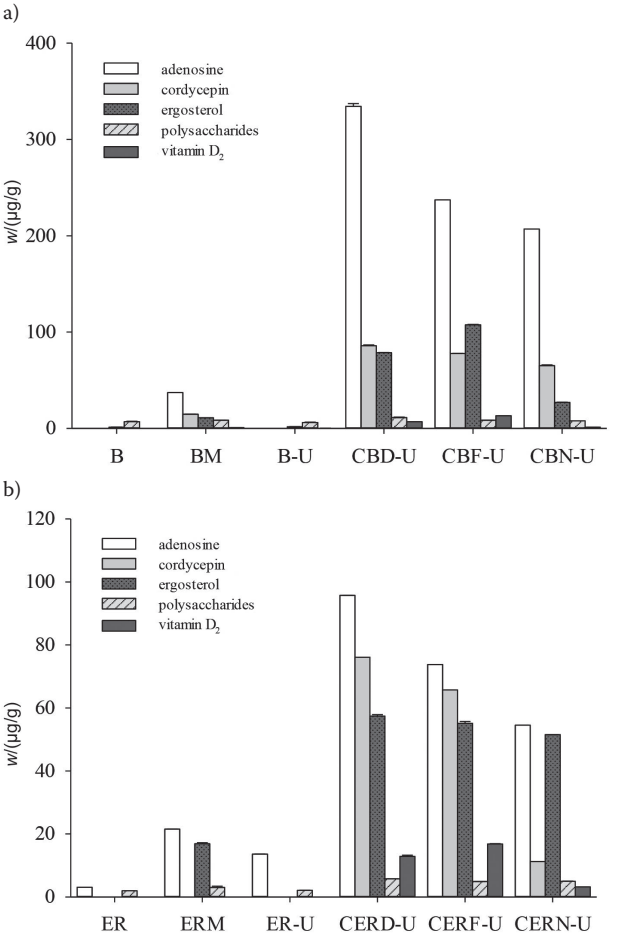


Fig. 1. Effect of UVB irradiation on the functional components of samples fermented by *Cordyceps militaris* and grain substrates: a) buckwheat and b) embryo rice. B=buckwheat, BM=buckwheat mycelia, B-U=UVB irradiated buckwheat, CBD-U=fermented then dried and UVB irradiated buckwheat, CBF-U=fermented and UVB irradiated buckwheat, CBN-U=fermented unirradiated buckwheat, ER=embryo rice, ERM=embryo rice mycelia, ER-U=UVB irradiated embryo rice, CERD-U=fermented then dried and UVB irradiated embryo rice, CERF-U=fermented and UVB irradiated embryo rice, CERN-U=fermented unirradiated embryo rice

Antioxidant properties

The antioxidant properties assayed herein are summarized in Table 1, and the EC_{50} values (mg of ethanolic and hot water extracts per mL) were calculated for comparison. The effectiveness of antioxidant properties correlates inversely with their EC_{50} values. The EC_{50} values of the ethanolic and hot water extracts of *C. militaris*-fermented buckwheat (0.85-6.8 and 0.58-17.4 mg/mL, respectively) were lower than those of both extracts of unfermented buckwheat (1.58-24.0 and 0.68-35.4 mg/mL, respectively). Similarly, the EC_{50} values of the ethanolic and hot water extracts of *C. militaris*-fermented embryo rice (1.3-10.9 and 6.37-26.2 mg/mL, respectively) were lower than those of both extracts of unfermented embryo rice (2.7-31.1 and 8.0-46.8 mg/mL, respectively).

For the reducing power, scavenging ability and chelating ability assays, the EC_{50} values of the ethanolic extracts of fresh fermented and UVB irradiated buckwheat and embryo rice were more effective than those of the dried fermented and irradiated both samples and unirradiated buckwheat, while fermented unirradiated embryo rice was more effective than buckwheat. In addition, the EC_{50} values of the hot water extracts from fermented then dried and UVB irradiated dried buckwheat, and fermented and UVB irradiated embryo rice demonstrated greater effectiveness than those of the fresh fermented and UVB irradiated buckwheat, fermented and unirradiated buckwheat, fermented then dried and UVB irradiated embryo rice and fermented unirradiated embryo rice. Overall, the ethanolic extracts of UVB irradiated fresh buckwheat and embryo rice fermented by *C. militaris* demonstrated greater antioxidant properties than irradiated dried and unfermented substrates.

Zhang *et al.* (*39*) found that the EC_{50} values of the 70 % ethanolic extracts of unfermented wheat and wheat fermented by *C. militaris* were 0.52 and 0.58 mg/mL for reducing power, 0.08 and 0.06 mg/mL for scavenging ability, and 0.61 and 0.26 mg/mL for chelating ability, respectively. Furthermore, regarding the three antioxidant properties assayed, it is clear that both extracts of the *C. militaris*--fermented products were more effective than those of the unfermented substrates. In addition, the hot water extracts were less effective than the ethanolic extracts, as evidenced by their higher EC_{50} values, except that the reducing power of the ethanolic extracts was less effective than that of the hot water extracts. Both phenomena were consistent with previous findings (*40*).

Antioxidant components

Flavonoids act as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellents, and light screening substances in plants. In both extracts, the samples of buckwheat and embryo rice dried after incubation and UVB irradiation had the highest flavonoid content, except for irradiated dried buckwheat (Fig. 2). In addition, in both extracts, the flavonoid content of *C. militaris*-fermented substances (0.57-6.29 mg/mL) was higher than those of both extracts of unfermented substrates (0.48-5.15 mg/mL). Zhang *et al.* (*39*) found that in ethanolic and water extracts, the total phenolic content of *C. militaris*-fermented wheat (32.79-41.63 mg/g) was higher than of unfermented wheat (29.37-36.94 mg/g). Liu *et al.* (*41*) found that postharvest irradiation with UVB enhanced the levels of flavonoids, phenols and total phenols in fruiting bodies on tomato. It appears that UVB irradiation of fresh products could increase

Table 1. Effects of UVB irradiation on the EC_{50} values of ethanolic and hot water extracts of samples fermented by *Cordyceps militaris* and grain substrates

Extract		EC_{50}/(mg extract per mL)					
		B	BM	B-U	CBD-U	CBF-U	CBN-U
Ethanolic	Reducing power	(16.0±0.9)^{b}	(1.94±0.03)^{c}	(24.0±0.9)^{a}	(0.85±0.01)^{d}	(0.96±0.01)^{d}	(0.99±0.02)^{d}
	Scavenging ability	(1.65±0.06)^{b}	(1.94±0.01)^{a}	(1.58±0.02)^{b}	(1.38±0.04)^{c}	(0.95±0.01)^{e}	(1.28±0.05)^{d}
	Chelating ability	(8.2±0.1)^{a}	(6.9±0.4)^{b}	(8.7±0.4)^{a}	(5.4±0.2)^{c}	(3.7±0.1)^{d}	(6.8±0.4)^{b}
Hot water	Reducing power	(21.0±0.7)^{b}	(20.8±0.8)^{b}	(26.5±2.8)^{a}	(10.6±0.5)^{d}	(12.6±0.1)^{d}	(17.4±0.5)^{c}
	Scavenging ability	(14.0±0.7)^{b}	(20.8±0.4)^{b}	(35.4±2.2)^{a}	(7.5±0.4)^{e}	(8.4±0.1)^{d}	(9.7±0.7)^{d}
	Chelating ability	(0.69±0.01)^{b}	(0.68±0.01)^{b}	(0.87±0.01)^{a}	(0.58±0.02)^{d}	(0.65±0.01)^{c}	(0.68±0.02)^{b}
		ER	ERM	ER-U	CERD-U	CERF-U	CERN-U
Ethanolic	Reducing power	(28.5±0.3)^{b}	(14.73±0.71)^{c}	(31.11±0.62)^{a}	(1.3±0.2)^{d}	(1.6±0.9)^{d}	(1.4±0.3)^{d}
	Scavenging ability	(2.72±0.06)^{d}	(2.92±0.04)^{b}	(3.16±0.02)^{a}	(2.84±0.06)^{bc}	(2.79±0.02)^{cd}	(2.79±0.07)^{cd}
	Chelating ability	(23.9±0.6)^{b}	(15.1±0.5)^{c}	(26.0±0.4)^{a}	(10.9±0.8)^{d}	(7.8±0.4)^{e}	(7.6±0.1)^{e}
Hot water	Reducing power	(36.1±1.3)^{b}	(26.5±0.5)^{c}	(46.8±1.1)^{a}	(25.0±0.6)^{d}	(22.9±0.2)^{e}	(26.2±0.2)^{cd}
	Scavenging ability	(26.8±0.7)^{b}	(17.78±0.04)^{c}	(43.7±0.6)^{a}	(12.2±0.7)^{f}	(15.3±0.2)^{e}	(16.2±0.1)^{d}
	Chelating ability	(12.2±0.6)^{a}	(8.3±0.7)^{b}	(8.0±0.2)^{bc}	(7.6±0.1)^{c}	(6.37±0.03)^{d}	(6.8±0.2)^{d}

B=buckwheat, BM=buckwheat mycelia, B-U=UVB irradiated buckwheat, CBD-U=fermented then dried and UVB irradiated buckwheat, CBF-U=fermented and UVB irradiated buckwheat, CBN-U=fermented unirradiated buckwheat, ER=embryo rice, ERM=embryo rice mycelia, ER-U=UVB irradiated embryo rice, CERD-U=fermented then dried and UVB irradiated embryo rice, CERF-U=fermented and UVB irradiated embryo rice, CERN-U=fermented embryo rice without UVB irradiation. EC_{50} values were obtained by interpolation from linear regression analysis $A_{750\,nm}$=0.5 in reducing power assay, 1,1-diphenyl-2-picrylhydrazyl radicals were scavenged by 50 % and ferrous ions were chelated by 50 %. Each value is expressed as the mean±standard error (N=3). Mean values with different superscript letters within a row differ significantly ($p<0.05$)

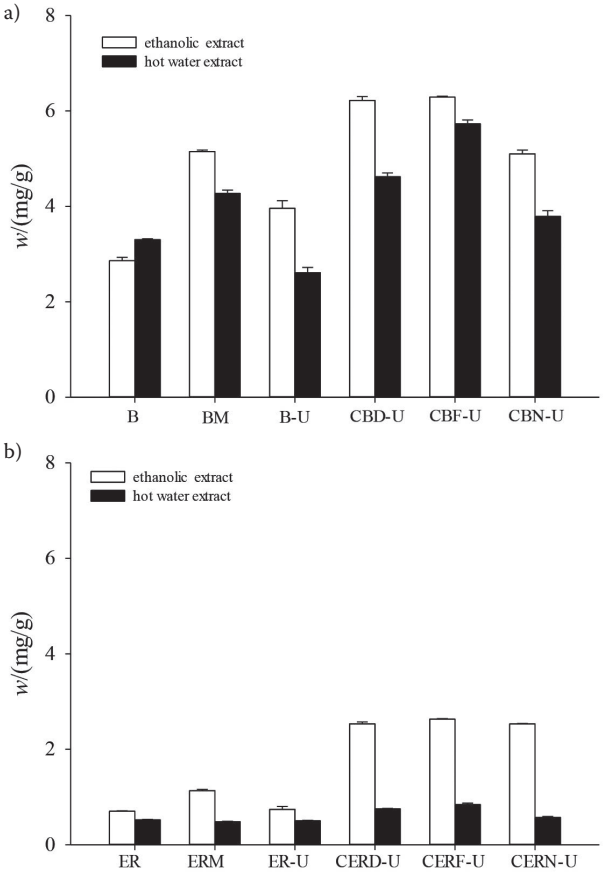


Fig. 2. Effect of UVB irradiation on the flavonoid content of samples fermented by *Cordyceps militaris* and grain substrates: a) buckwheat and b) embryo rice. B=buckwheat, BM=buckwheat mycelia, B-U=UVB irradiated buckwheat, CBD-U=fermented then dried and UVB irradiated buckwheat, CBF-U=fermented and UVB irradiated buckwheat, CBN-U=fermented unirradiated buckwheat, ER=embryo rice, ERM=embryo rice mycelia, ER-U=UVB irradiated embryo rice, CERD-U=fermented then dried and UVB irradiated embryo rice, CERF-U=fermented and UVB irradiated embryo rice, CERN-U=fermented unirradiated embryo rice

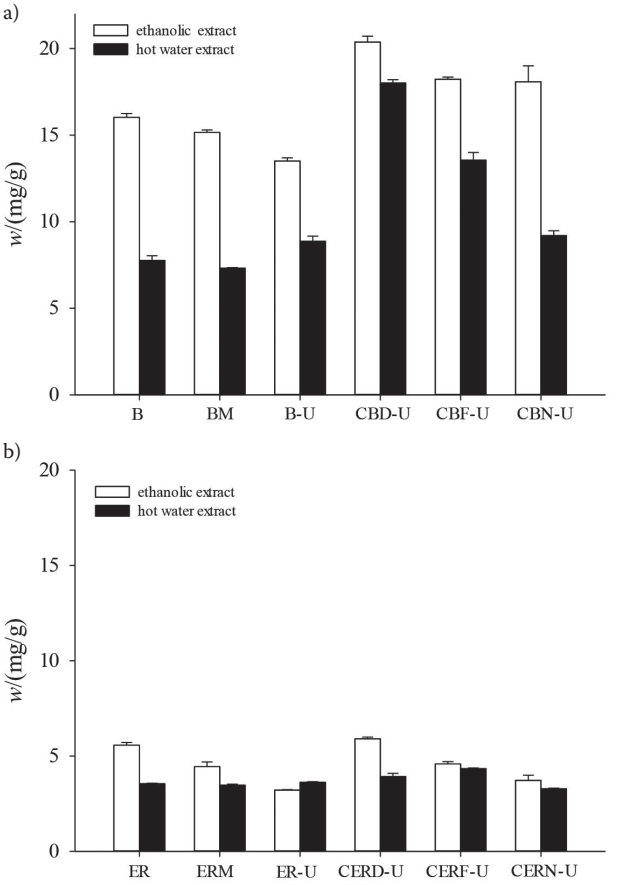


Fig. 3. Effect of UVB irradiation on total phenolic content of samples fermented by *Cordyceps militaris* and grain substrates: a) buckwheat and b) embryo rice. B=buckwheat, BM=buckwheat mycelia, B-U=UVB irradiated buckwheat, CBD-U=fermented then dried and UVB irradiated buckwheat, CBF-U=fermented and UVB irradiated buckwheat, CBN-U=fermented unirradiated buckwheat, ER=embryo rice, ERM=embryo rice mycelia, ER-U=UVB irradiated embryo rice, CERD-U=fermented then dried and UVB irradiated embryo rice, CERF-U=fermented and UVB irradiated embryo rice, CERN-U=fermented unirradiated embryo rice

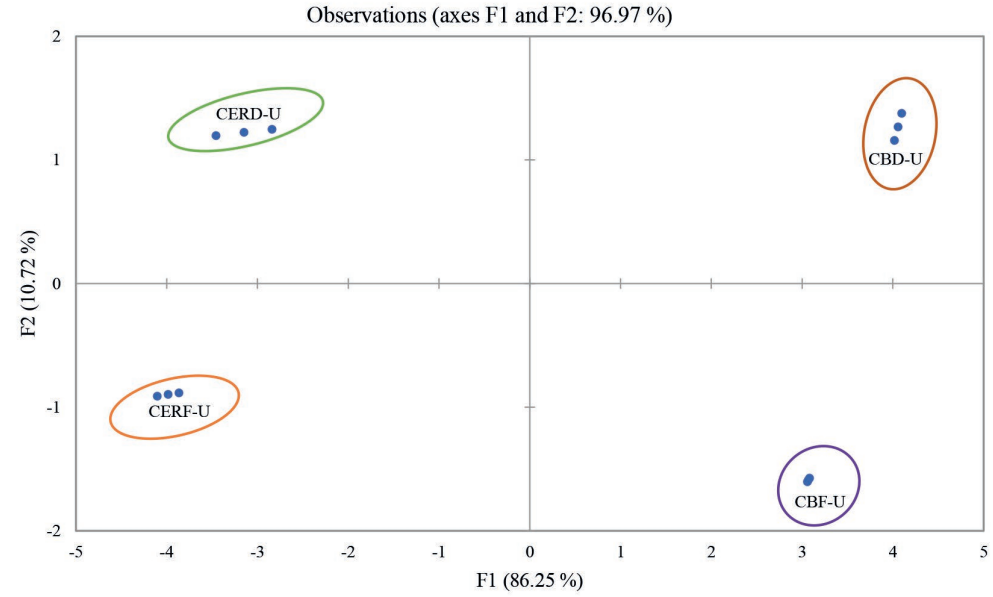
their flavonoid content. UVB irradiation of dried products seems to have had only a slight influence on the flavonoid content.

In both ethanolic and hot water extracts, the total phenolic content (3.28-20.37 mg/g) (Fig. 3) was higher than the flavonoid content (0.48-6.29 mg/g) (Fig. 2). Phenolic compounds are widely distributed in mushrooms. As shown in Fig. 3, in both extracts, UVB irradiated samples of dried buckwheat and embryo rice contained the highest total phenolic contents, except for the embryo rice dried after incubation and UVB irradiation (Fig. 3). The high mass fractions of total phenols and flavonoids in the ethanolic and hot water extracts might explain their higher reducing power and scavenging ability of DPPH radicals. These results are consistent with their assayed effectiveness in antioxidant properties. Phenols such as BHT (butylated hydroxytoluene) and gallate are known to be effective antioxidants (*42*). Due to their free radical scavenging and ferrous ion chelating abilities, phenols may possess good antioxidant, antimutagenic and anticancer properties (*43*). Overall, *C. militaris* can enhance flavonoid and total phenolic contents by solid-state fermentation. Furthermore, UVB irradiation has no influence on the flavonoid and total phenolic content during the solid-state fermentation, as these irradiated samples still contained sufficient quantities of these antioxidant components.

Principal components analysis

To gain an overview of the relationships among the biologically effective components, antioxidant properties and antioxidant components in the irradiated samples, principal component analysis (PCA) was performed, and the results are shown in Fig. 4. The multivariate treatment of the data obtained for the samples permitted the reduction of the variables to two principal components, which together explained 96.97 % of the total variability. The first axis accounted for 86.25 % and the second axis for 10.72 %. A PCA score plot shows that irradiated *C. militaris*-fermented products were quantitatively distinguished as four sets of three replicates, indicating that irradiated samples of both

a)



b)

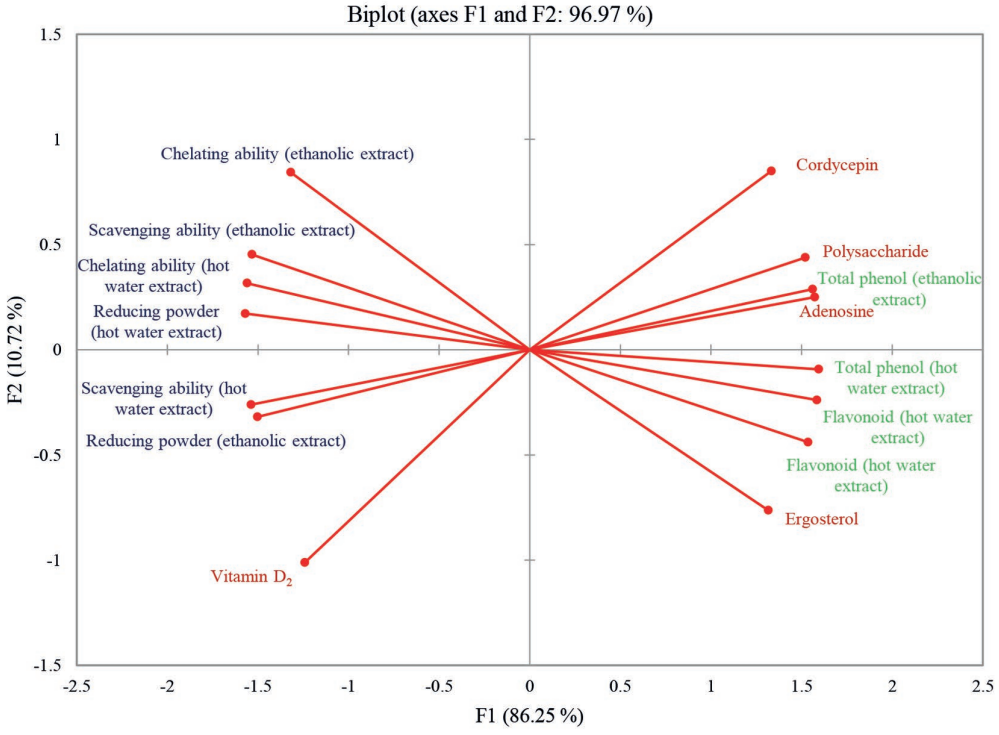


Fig. 4. Principal component analysis of the biologically active components, antioxidant properties and antioxidant components of samples fermented by *Cordyceps militaris*: a) score and b) loading plots. CBD-U=fermented then dried and UVB irradiated buckwheat, CBF--U=fermented and UVB irradiated buckwheat, CERD-U=fermented then dried and UVB irradiated embryo rice, CERF-U=fermented and UVB irradiated embryo rice

dried and fresh buckwheat and embryo rice had entirely different profiles of biologically active components, antioxidant properties and antioxidant components as well as a dispersed score plot. In addition, in plots for components and properties (Fig. 4a), irradiated both fresh and dried buckwheat had positive values in F1, while both irradiated dried samples had positive values in F2. The antioxidant properties and biological and antioxidant components in F1 and F2 were dispersed actinomorphically from the origin (Fig. 4b). Vitamin D_2 and ergosterol seemed to correlate negatively. From the score and loading plots, irradiated dried buckwheat positively correlated with cordycepin, irradiated fresh embryo rice positively correlated with vitamin D_2, irradiated fresh buckwheat positively correlated with ergosterol, and irradiated dried embryo rice positively correlated with chelating ability and scavenging ability with ethanolic extracts. In summary, irradiated products fermented by *C. militaris* were well separated in score plots, and their biologically active components, antioxidant properties and antioxidant components were individually dispersed using

PCA. Therefore, PCA could be helpful to provide valuable information on relationships among the biologically active components, antioxidant properties and antioxidant components of irradiated products fermented by *C. militaris*.

Conclusions

After UVB irradiation, fresh buckwheat and embryo rice fermented by *C. militaris* had higher vitamin D_2 and flavonoid content, antioxidant properties of ethanolic extracts and antioxidant activity than dried fermented products. However, after UVB irradiation, adenosine, cordycepin, polysaccharide, and total phenolic contents were higher in dried products fermented by *C. militaris* than those of fresh fermented products. Therefore, to develop products rich in vitamin D_2, fresh products fermented by *C. militaris* are the best choice; however, when considering cordycepin, polysaccharides or other biologically active components, fermentation of dried products by *C. militaris* may be better choice, because the dried samples are easy to store and process, while fresh samples require additional attention such as cold storage. This study presents an innovative approach to the improvement of biologically active ingredients of buckwheat and embryo rice fermented by *C. militaris* in SSF. Future research will be oriented towards the solid-state fermentation after grinding of buckwheat and embryo rice into a dry powder and then UVB irradiation, and development of functional ingredients for food, cosmetics or animal feed production.

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