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Soybean fermentation with basidiomycetes (medicinal mushroom mycelia)

Kohei Suruga^{*} , Tsuyoshi Tomita and Kazunari Kadokura

Abstract

Background: Edible mushroom fruiting bodies and their mycelia have become attractive functional foods. Mushroom mycelia have been investigated for their potential food applications. Here, soybeans were fermented using medicinal mushroom mycelia from *Ganoderma lucidum*, *Hericium erinaceus*, and *Hericium ramosum* to develop novel functional food materials for human health.

Results: Wild mushroom fruiting bodies were collected from nature to isolate their mycelia. Soybeans were fermented using mushroom mycelia for 4 weeks. The antioxidant activity of fermented soybeans was analysed, and fermented soybean compounds were determined using HPLC and LC/MS analysis. Antioxidant and alpha-glucosidase inhibitory activities of fermented soybean mycelia were more potent than the control group. The volume and type of isoflavones significantly differed between soybean fermentation by *Ganoderma lucidum*, *Hericium erinaceus*, and *Hericium ramosum* mycelia, based on HPLC and LC/MS analysis.

Conclusion: We used mushroom mycelia to uncover new information regarding fermented soybean. Soybean fermentation using mushroom mycelia could be useful as a novel bioactive food material or nutritional supplement.

Keywords: Soybean, Fermentation, Mushroom mycelia, Antioxidant, Alpha-glucosidase inhibition, Isoflavone

Background

Soybean is a legume and a major protein source crop in East Asian countries. In general, soybeans have various useful nutrients, including proteins, oils, carbohydrates, minerals, and vitamins [1]. Protein is one of the major compounds in soybean, and dried soybean contains 36% protein [2]. Soybean proteins, including glycinin and β -conglycinin, contain most of the essential amino acids required for human nutrition [3]. Soybean protein is regarded as a good substitute for animal proteins [4]. There are many non-fermented and fermented soybean products, including soybean sprout, soy milk, tofu, soy meat, tempeh, miso, natto, soy sauce, and doenjang [5]. Moreover, many active compounds are isolated from soybean and soybean products, and isoflavones are one group of active compounds [6].

Isoflavones are a subgroup of flavonoids, and the major isoflavones in soybean include genistein, daidzein, and glycitein. They are found in glycosylated forms in nature like genistin, daidzin, and glycitin. In general, they cannot be easily absorbed in the intestines, and they undergo hydrolysis catalysed by beta-glucosidase from intestinal microflora [7]. Isoflavones can be used as alternative therapies against hormone-related cancers like breast and prostate cancer [8], cardiovascular diseases [9], osteoporosis [10], and menopausal symptoms [11]. Fermented soybean products including tempeh, miso, and natto contain the isoflavone aglycone form [12]. Miura et al. reported that soybean fermented by basidiomycete mushrooms, *Ganoderma lucidum* (*G. lucidum*), contained isoflavone aglycones [13].

Mushrooms are grown and consumed by humans in many countries. Many people are interested in edible mushroom fruiting bodies and their mycelia as functional foods because they are less toxic and contain bioactive compounds. Investigators have reported the

*Correspondence: kouhei_suruga@kibun.co.jp
Food Function Research & Development Division, International Operation Department, Kibun Foods Inc., 86 Yanokuchi, Inagi, Tokyo 206-0812, Japan

antitumour [14], antimutagenicity [15], antiviral [16], and antioxidant activities [17] of mushroom fruiting bodies and mycelia. Mushroom mycelia have been investigated for their potential food applications, and they are used to create fermented foods like soybeans, bread, cheese, and alcoholic drinks [18].

In our previous study, we assessed the antioxidant activities of 20 mushroom mycelia species that were obtained in nature. The activity of *Hericium ramosum* (*H. ramosum*) mycelia was more potent than other mushroom mycelia species [19]. Moreover, nerve growth factor synthesis in *H. ramosum* was higher than *Hericium erinaceus* (*H. erinaceus*) in the hippocampus of intact mice [19]. There have been various investigations to characterise fermented soybeans using mushroom mycelia [13, 18, 20]. However, very little work is currently available in the published literature on the oxygen radical absorbance capacity (ORAC), alpha-glucosidase inhibitory activity, and liquid chromatography/mass spectrometry (LC/MS) chemical profiles of fermented soybean using mushroom mycelia.

Here, we obtained new information regarding effect of fermented soybean on general health. We prepared the fermented soybeans from *G. lucidum* and *H. erinaceus* mycelia, which are well-known medicinal mushrooms. Moreover, in addition to *G. lucidum* and *H. erinaceus*, we produced fermented soybean using the comb tooth cap medical mushroom, *H. ramosum* mycelia, which has strong antioxidant activity.

Materials and methods

Chemical

Genistein, glycitein, daidzein, genistin, glycitin, and daidzin were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Potato dextrose agar (PDA) medium was obtained from Eiken Chemical Co., Ltd. (Tochigi, Japan). Folin and Ciocalteu's phenol reagent (2N) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 2-Morpholinoethanesulfonic acid monohydrate (MES) was obtained from Dojindo Laboratories (Kumamoto, Japan).

Mushroom collection and mushroom mycelia separation

We collected *G. lucidum*, *H. erinaceus*, and *H. ramosum* wild mushroom fruiting bodies from Akita and Iwate prefectures in Tohoku in northern Japan. Pieces of mushrooms fruiting bodies collected from the natural field were plated onto a 90 mm petri dish with PDA medium and incubated at 25 °C for 2 days until the mycelia germinated on PDA medium. The germinated mycelia were cultured for 14 days at 25 °C. After the termination of cultivation, they were maintained

on PDA medium at 3 °C in a refrigerator. The medium contained 3% sucrose, 0.3% polypeptone, 0.3% yeast extract, 0.05% sodium phosphate, and 0.05% potassium phosphate in distilled water. The initial pH of this medium was adjusted to 5.5. The culture was performed at 25 °C for 14 days with gentle shaking (80 rpm).

Soybean fermentation using mushroom mycelia

Commercial dried soybeans were soaked overnight in water (whole soybean:water ratio 1:4 by weight) and placed in a culture bottle. After autoclaving at 121 °C for 20 min, the soybeans were cooled and cultured with fresh *G. lucidum*, *H. erinaceus*, and *H. ramosum* mycelia (suspension mycelia solution) at 25 °C for approximately 4 weeks. After cultivation, fermented soybeans were lyophilised to powder by freeze-drying.

Extraction of fermented soybean

Extraction of fermented soybean with ethanol was performed using methods of our previous report [19], with some modification. In brief, the powder of lyophilised fermented soybean was extracted with 10 times the volume of 80% ethanol at 23–27 °C, and the extracted solution was concentrated. The solution was used for later experiments as fermented soybean extract solution.

Total phenolic content

The total phenolic content of fermented soybean was analysed by the Folin and Ciocalteu method with catechin as a standard [21]. The fermented soybean extract solution (20 µL) was mixed with 20 µL of 1 N Folin and Ciocalteu solution and 100 µL of 0.4 M sodium carbonate solution. The reaction mixture was stored at 30 °C for 30 min, and the absorbance was measured using a Synergy HTX Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA) at 660 nm. Results were expressed as milligrammes of catechin equivalent per gramme of dry weight of fermented soybean (mg catechin/g dry powder).

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity

The DPPH radical scavenging activity of fermented soybean was analysed by the methods described by Midoh et al. [22]. The fermented soybean extract solution (60 µL) was mixed with 120 µL of 100 mM MES buffer (pH 6.0)/10% ethanol solution and 60 µL of 400 µM DPPH in ethanol. The reaction was performed at room temperature for 20 min, and the absorbance of the reaction mixture was measured at 520 nm using a microplate reader. The DPPH radical scavenging activity of fermented soybean was estimated using

a 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) standard curve (0, 5, 10, 15, 20, and 25 μM) and expressed as μmol Trolox/g fermented soybean dry powder.

ORAC assay

The ORAC was performed using the OxiSelect™ ORAC Activity Assay Kit (Cell Biolabs Inc., San Diego, CA, USA) [23]. The reaction was performed in 75 mM phosphate buffer (pH 7.4), and the volume of the final reaction mixture was 200 μL . Antioxidant (25 μL) and fluorescein solution (150 μL) were added to the microplate wells. The reaction mixture was incubated at 37 °C for 30 min. The free radical initiator 2,2'-azobis (2-methylpropionamide) dihydrochloride (25 μL) was added using a multichannel pipette. The microplate was immediately placed in the reader, and the fluorescence intensity (excitation at 485 nm, emission at 528 nm) was recorded every 5 min for 60 min using a microplate reader. The area under curve at specific Trolox concentrations (0, 5, 10, 20, 40, and 50 mM) was used to plot the standard curve for ORAC activity. Each extract was quantified and expressed as μmol Trolox equivalents/g of dry fermented soybean powder.

Alpha-glucosidase inhibition assay

The inhibition of yeast alpha-glucosidase activity was measured using a method described by Matsui et al. with minor modifications [24]. The fermented soybean extract solution (10 μL) was pre-incubated at various concentrations for 15 min at 37 °C with 40 μL of an enzyme solution of yeast alpha-glucosidase (0.1 mU/mL) in 67 mM phosphate buffer (pH 6.8). After pre-incubation with the enzyme solution, 50 μL of *p*-nitrophenyl α -D-glucopyranoside (*p*NP-glucoside, 1 mM) was added and incubated for 30 min at 37 °C. The reaction was terminated by adding 0.5 M sodium carbonate (100 μL). The increase in absorbance at 400 nm was measured using a microplate reader. The mammalian alpha-glucosidase inhibitory activity was assessed using crude alpha-glucosidase solution prepared from rat intestinal acetone powder (Sigma-Aldrich, St. Louis, MO, USA) in 0.9% saline [25]. The assay mixture was comprised of 18.5 mM maltose or 74 mM sucrose in 100 mM maleate buffer (pH 6.0, 50 μL) and 25 μL of the fermented soybean extract solution at various concentrations. The mixture was pre-incubated for 3 min at 37 °C. The reaction was initiated by adding crude alpha-glucosidase solution (25 μL) to the reaction mixture. The amount of glucose released in the reaction mixture was determined using LabAssay Glucose (Wako Pure Chemical Co., Osaka, Japan) based on the mutarotase–glucose oxidase method. The reaction mixture (100 μL) and LabAssay Glucose (150 μL) were

mixed and incubated for 10 min at 37 °C, and the absorbance was measured at 505 nm. The inhibition rate (%) of alpha-glucosidase can be calculated as follows: Inhibition rate (%) = $(A_C - A_S)/(A_C - A_B) \times 100$, where A_C , A_S , and A_B represent the absorbance of the control, sample, and blank, respectively [24].

High-performance liquid chromatography (HPLC) analysis

The isoflavone levels in fermented soybean after mushroom mycelia were investigated using the methods described by Kudou et al. [26]. Soybeans fermented using mushroom mycelia were analysed with an LC-6A system (Shimadzu, Kyoto, Japan) equipped with a PEGASIL-ODS (4.6 mm i.d. \times 250 mm) HPLC column (Sensyu Scientific, Tokyo, Japan). Analyses of genistein, daidzein, glycitein, and genistein were performed using acetonitrile–water (20:80) containing 0.1% acetic acid, and the analyses of daidzin and glycitin were conducted using acetonitrile–water (15:85) containing 0.1% acetic acid under isocratic conditions at a flow rate of 1.0 mL/min and UV detection at 260 nm. The injection volume was 10 μL , and the analyses were performed at 30 °C. The amounts of isoflavones in the extracts were calculated from standard curves derived from authentic standards.

LC/MS analysis

LC analysis was performed using Waters ACQUITY UPLC (Waters MS Technologies, Manchester, UK), which was equipped with a reversed-phase Acquity UPLC CHS C18 column with a particle size of 2.1 mm \times 100 mm \times 1.7 μm (Waters MS Technologies). The column oven temperature was set at 40 °C, and the flow rate was 0.4 mL/min. Mobile phases A and B consisted of water containing 0.1% formic acid and 0.1% acetonitrile, respectively. The linear gradient program was set as follows: 0 min, 5% B; 20 min, 30% B; and 34 min, 100% B. The injection volume was 5 μL . Mass spectrometry was performed using a Xevo QToF Mass Spectrometer (Waters MS Technologies). The scan range covered *m/z* from 50 to 1200. For positive electrospray modes, the capillary and cone voltages were set at 3.0 kV and 15 V, respectively.

Statistical analysis

Results are expressed as mean \pm standard deviation (SD). Statistical significance was determined using the paired *t* test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Soybean fermentation using mushroom mycelia

Soybeans were fermented using *G. lucidum*, *H. erinaceus*, and *H. ramosum* mycelia at 25 °C for approximately

4 weeks. The soybeans fermented by mushroom mycelia are shown in Fig. 1. Soybeans fermented using *G. lucidum* mycelia tended to ferment faster than soybean fermented by *H. erinaceus* and *H. ramosum* mycelia.

Total phenolic content and antioxidant activity of soybeans fermented by mushroom mycelia

Table 1 summarises the results of the total phenolic content and antioxidant activity of soybeans fermented by mushroom mycelia. The total phenolic content (mg/g dry powder) of non-fermented soybeans and soybeans fermented by *G. lucidum*, *H. erinaceus*, and *H. ramosum* mycelia were 1.547 ± 0.068 , 2.304 ± 0.035 , 2.074 ± 0.066 , and 2.160 ± 0.014 , respectively. DPPH radical scavenging activity ($\mu\text{mol Trolox/g dry powder}$) of non-fermented soybean and soybeans fermented by *G. lucidum*, *H. erinaceus*, and *H. ramosum* mycelia were 1.847 ± 0.073 , 4.246 ± 0.010 , 2.246 ± 0.061 , and 2.367 ± 0.173 , respectively. The ORAC values ($\mu\text{mol Trolox/g dry powder}$) of non-fermented soybean and soybeans fermented by *G. lucidum*, *H. erinaceus*, and *H. ramosum* mycelia were 49.763 ± 2.856 , 60.090 ± 1.506 , 66.147 ± 1.898 , and 72.897 ± 2.113 , respectively.

Alpha-glucosidase inhibition assay

We analysed the alpha-glucosidase inhibitory activity of soybeans fermented using mushroom mycelia (Fig. 2). Soybeans fermented using mushroom mycelia inhibited the enzymatic activity of yeast alpha-glucosidase consuming the substrate pNP-glucoside compared to non-fermented soybeans. The inhibition rates of soybeans fermented using *G. lucidum*, *H. erinaceus*, and

H. ramosum mycelia were 62.3%, 71.3%, and 79.5%, respectively (Fig. 2a). The inhibitory effect of fermented soybean on rat small intestinal alpha-glucosidase was determined for maltose and sucrose as substrates compared to non-fermented soybeans. The inhibition rates of soybeans fermented using *G. lucidum*, *H. erinaceus*, and *H. ramosum* mycelia with maltose were 55.9%, 10.6%, and 11.6%, respectively (Fig. 2b). Moreover, the values of soybeans fermented using *G. lucidum*, *H. erinaceus*, and *H. ramosum* mycelia for sucrose were 8.1%, 6.0%, and 1.5%, respectively (Fig. 2c).

HPLC and LC/MS analysis of soybeans fermented using mushroom mycelia

The concentrations of isoflavones in soybeans fermented using mushroom mycelia are listed in Table 2. The glycosylated forms genistin, daidzin, and glycitin comprised 95.6% of the isoflavones in the control group (non-fermented soybean), but these values of soybeans fermented using *G. lucidum*, *H. erinaceus*, and *H. ramosum* mycelia were 52.5%, 15.8%, and 17.6%, respectively. On the other hand, the aglycon forms daidzein, glycitein, and genistein in the control group were 4.4% of the isoflavones, but these values of soybeans fermented using *G. lucidum*, *H. erinaceus*, and *H. ramosum* mycelia were 47.5%, 84.2%, and 82.4%, respectively.

The chemical profiles of non-fermented soybean and soybeans fermented by *G. lucidum*, *H. erinaceus*, and *H. ramosum* mycelia were analysed using LC/MS (Fig. 3). The main 12 peaks were detected in non-fermented soybean and soybeans fermented using mushroom mycelia, and 11 out of the 12 compounds

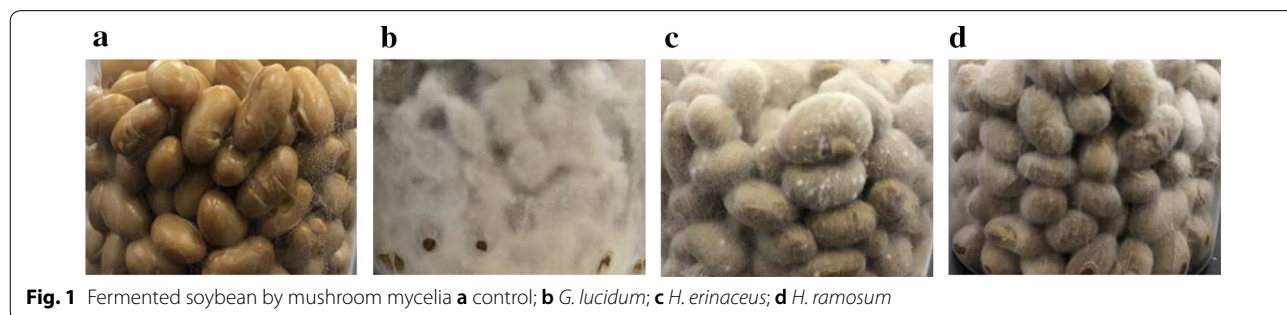


Fig. 1 Fermented soybean by mushroom mycelia **a** control; **b** *G. lucidum*; **c** *H. erinaceus*; **d** *H. ramosum*

Table 1 Total phenolic content and antioxidant activity of soybeans fermented by mushroom mycelia

	Control	<i>G. lucidum</i>	<i>H. erinaceus</i>	<i>H. ramosum</i>
Total phenolic content (mg/g dry powder)	1.547 ± 0.068	2.304 ± 0.035	2.074 ± 0.066	2.160 ± 0.014
DPPH radical scavenging activity ($\mu\text{mol Trolox/g dry powder}$)	1.847 ± 0.073	4.246 ± 0.010	2.246 ± 0.061	2.367 ± 0.173
ORAC ($\mu\text{mol Trolox/g dry powder}$)	49.763 ± 2.856	60.090 ± 1.506	66.147 ± 1.898	72.897 ± 2.113

Results are expressed as mean \pm SD ($n = 3$)

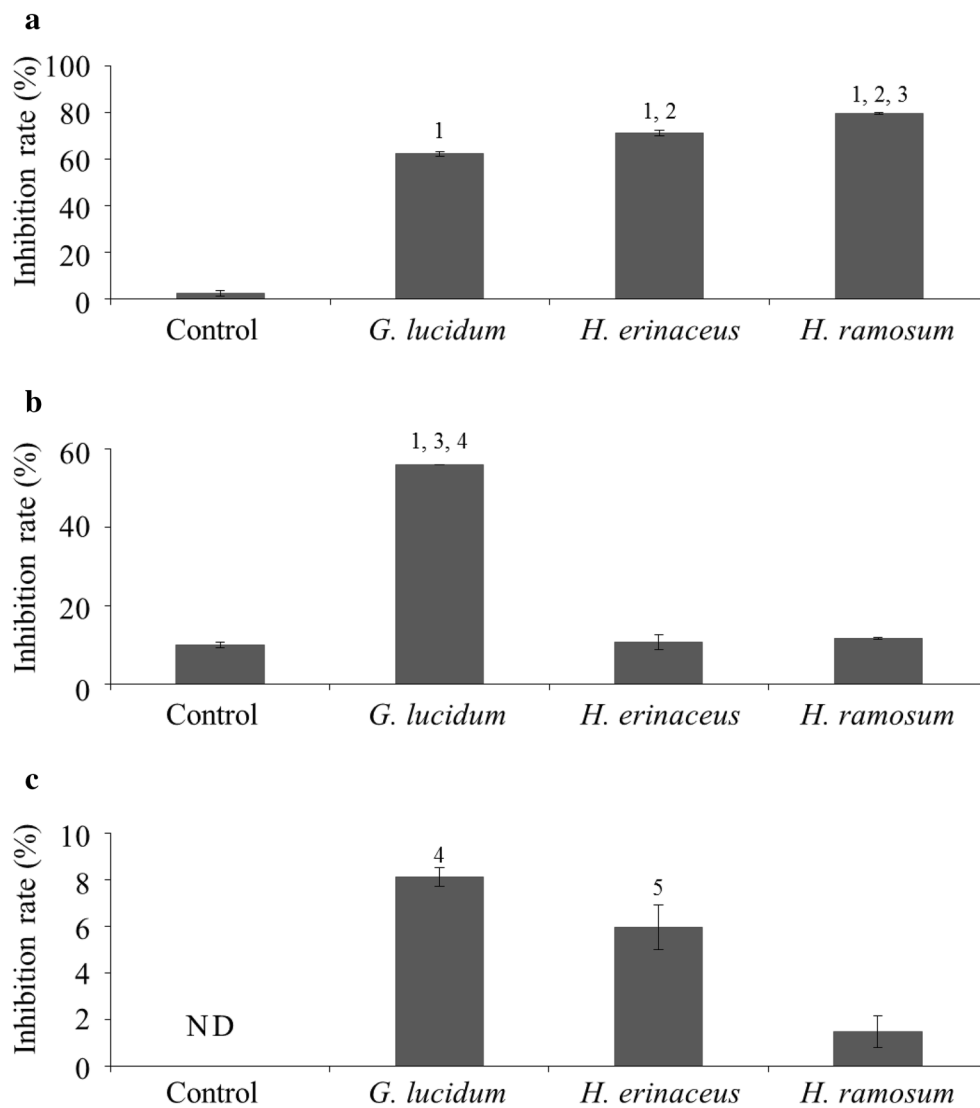


Fig. 2 Alpha-glucosidase inhibitory effect of fermented soybean by mushroom mycelia **a** alpha-glucosidase from yeast, pNP-glucoside as substrate; **b** alpha-glucosidase from rat small intestinal, maltose as substrate; **c** alpha-glucosidase from rat small intestinal, sucrose as substrate. Results are mean \pm SD ($n=3$). ND not detectable. 1: $p < 0.01$ vs Control, 2: $p < 0.01$ vs *G. lucidum*, 3: $p < 0.01$ vs *H. erinaceus*, 4: $p < 0.01$ vs *H. ramosum*, 5: $p < 0.05$ vs *H. ramosum*

were suggested based on retention time and MS data (Table 3).

Discussion

Edible mushroom fruiting bodies and their mycelia have become highly preferred functional foods because they are less toxic and contain bioactive compounds like polysaccharide β -glucans, polysaccharide–protein complexes, peptides, and phenolic derivatives. The extracts of mushroom fruiting bodies and their mycelia have great

therapeutic applications in human health, and they show effective functions like antioxidant, antitumor, anti-obesity, antidiabetic, immunomodulatory, and hypocholesterolemic effects [27]. Mushrooms were used as drugs in ancient times [28, 29]. There are some modern commercial nutraceuticals that use mushrooms, and they are utilised in the form of capsules or tablets as dietary supplements [27]. In addition, mushroom mycelia have been investigated for their potential food applications, and they are used to create fermented foods like soybeans,

Table 2 Isoflavone concentrations in soybeans fermented using mushroom mycelia

Isoflavones (µg/mg)	Control	<i>G. lucidum</i>	<i>H. erinaceus</i>	<i>H. ramosum</i>
Daidzin	0.957 ± 0.071	0.172 ± 0.058	0.126 ± 0.053	0.121 ± 0.067
Glycitin	0.129 ± 0.014	0.047 ± 0.008	0.040 ± 0.011	0.044 ± 0.010
Genistin	1.580 ± 0.046	0.732 ± 0.033	0.260 ± 0.009	0.302 ± 0.014
Daidzein	0.038 ± 0.008	0.323 ± 0.011	0.756 ± 0.011	0.721 ± 0.014
Glycitein	ND	0.045 ± 0.011	0.056 ± 0.010	0.058 ± 0.011
Genistein	0.084 ± 0.025	0.493 ± 0.025	1.465 ± 0.033	1.410 ± 0.043

Results are expressed as mean ± SD (n = 3)

ND not detectable

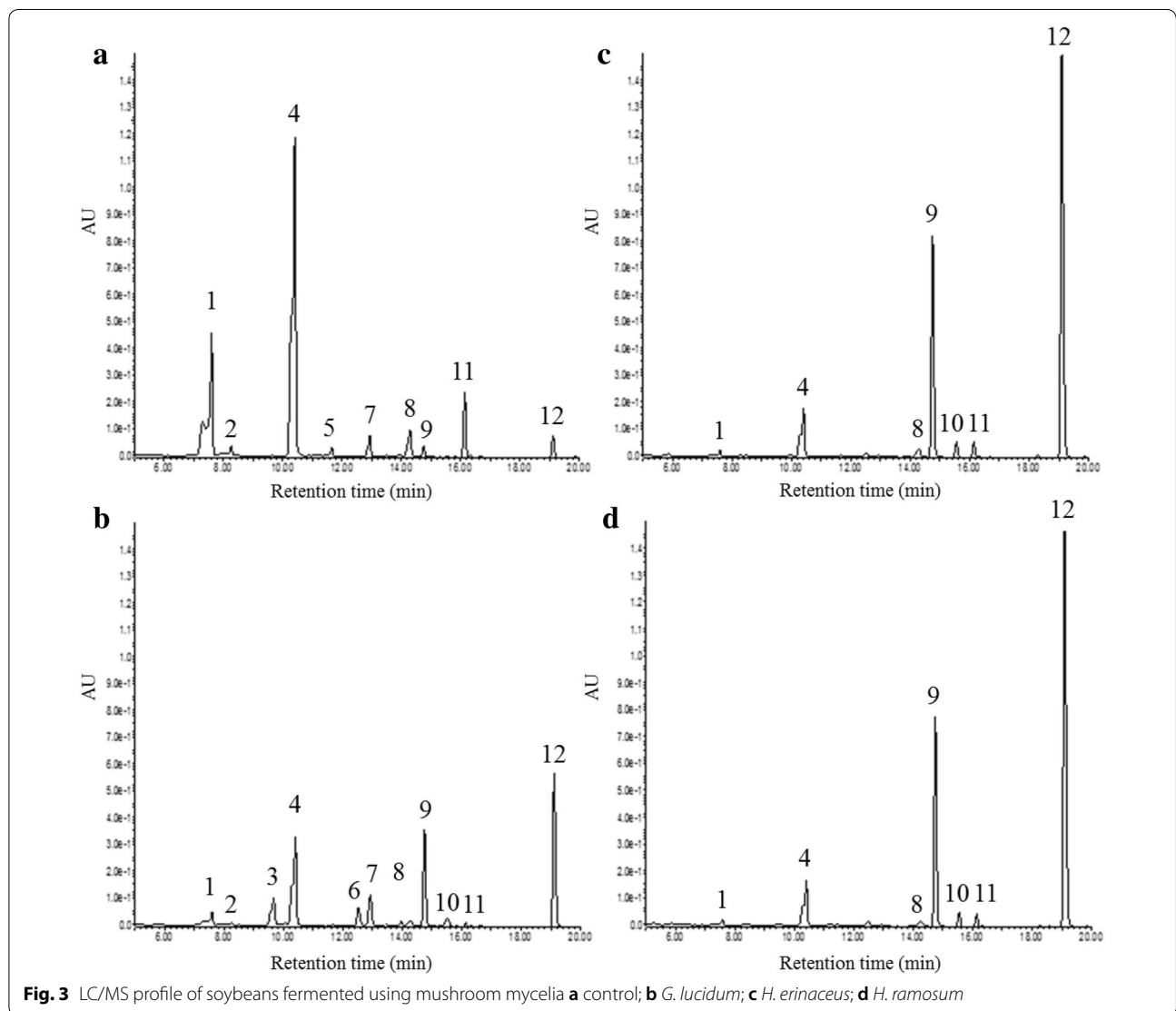


Fig. 3 LC/MS profile of soybeans fermented using mushroom mycelia **a** control; **b** *G. lucidum*; **c** *H. erinaceus*; **d** *H. ramosum*

bread, cheese, and alcoholic drinks [18]. However, there are no experimental data on the ORAC, alpha-glucosidase inhibitory activity, and LC/MS chemical profiles of soybeans fermented by mushroom mycelia. Here, we

prepared fermented soybeans using *G. lucidum*, *H. erinaceus*, and *H. ramosum* mycelia, and the antioxidant and alpha-glucosidase inhibitory activity and LC/MS chemical profiles of these products were analysed to obtain

Table 3 LC/MS data of soybeans fermented using mushroom mycelia

Peak no.	Retention time (min)	MS (m/z)	Molecular formula	Suggested compounds
1	7.65	417.12	C ₂₁ H ₂₀ O ₉	Daidzin
2	8.31	447.13	C ₂₂ H ₂₂ O ₁₀	Glycitin
3	9.67	271.05	C ₁₅ H ₁₀ O ₅	8-Hydroxydaidzein
4	10.45	433.11	C ₂₁ H ₂₀ O ₁₀	Genistin
5	11.71	503.12	C ₂₄ H ₂₂ O ₁₂	6''-O-Malonyldaidzin
6	12.53	271.05	C ₁₅ H ₁₀ O ₅	Unidentified
7	12.98	459.13	C ₂₃ H ₂₂ O ₁₀	6''-O-Acetyldaidzin
8	14.34	519.11	C ₂₄ H ₂₂ O ₁₃	6''-O-Malonylgenistin
9	14.81	255.07	C ₁₅ H ₁₀ O ₄	Daidzein
10	15.60	285.08	C ₁₆ H ₁₂ O ₅	Glycitein
11	16.19	475.12	C ₂₃ H ₂₂ O ₁₁	6''-O-Acetylgenistin
12	19.10	271.06	C ₁₅ H ₁₀ O ₅	Genistein

new information on the effect of fermented soybean on general health.

DPPH radical scavenging activity and the ORAC of fermented soybean mycelia were more potent than the control group (non-fermented soybean). In our previous report, the extract of *H. ramosum* mycelia had the highest DPPH radical scavenging activity compared to 19 other mushroom mycelia, including *G. lucidum* and *H. erinaceus* [19]. The ORAC of *H. ramosum* mycelia fermented soybean was higher than soybeans fermented using *G. lucidum* and *H. erinaceus* mycelia, and these results agree with those obtained in our previous report [19]. Interestingly, the DPPH radical scavenging activity of *G. lucidum* mycelia fermented soybean was higher than soybeans fermented using *H. erinaceus* and *H. ramosum* mycelia in this study. The total phenolic content of soybeans fermented using *G. lucidum* mycelia was slightly higher than soybeans fermented using *H. erinaceus* and *H. ramosum* mycelia. LC/MS analysis showed that soybeans fermented using *G. lucidum* mycelia contained 8-hydroxydaidzein (peak No. 3 in Fig. 3b and Table 3) and one unidentified compound (peak No. 6). We are working to identify compounds in peak No. 6; however, we assumed that this compound was 6-hydroxydaidzein or 3'-hydroxydaidzein from MS (m/z) data and molecular formula in LC/MS analysis. Esaki et al. reported that a potent antioxidative 6-hydroxydaidzein was isolated from soybean koji fermented using *Aspergillus oryzae* [30]. It has been reported that 8-hydroxydaidzein showed stronger antioxidant activity than daidzein [31]. These reports suggest that the high antioxidant effects of fermented soybean by *G. lucidum* mycelia may be related to the phenolic content levels and two kinds of hydroxydaidzeins. Many people have recently been interested in antioxidant foods. Antioxidant foods deserve considerable

practical concern because they prevent human cellular damage by reactive oxygen species and free radicals [32]. Oxidative stress has been linked to the generation of various serious diseases like neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation [33]. In previous studies, we prepared a fermented soy residue ("okara") with *Rhizopus oligosporus*, which showed high antioxidant activity [34–36]. Here, soybeans fermented using mushroom mycelia, especially *G. lucidum* mycelia, could be useful as a novel antioxidant food material or nutritional supplement.

The alpha-glucosidase inhibitory activities of fermented soybean mycelia were significantly more potent than the control group. The inhibitory activities against yeast alpha-glucosidase of fermented soybean by *H. erinaceus* and *H. ramosum* mycelia using the substrate *p*NP-glucoside were more potent than soybeans fermented using *G. lucidum* mycelia. Lee et al. investigated the alpha-glucosidase inhibitory activities of commercial genistein, a soy isoflavone. They reported that genistein showed high inhibitory activities against yeast alpha-glucosidase using the substrate *p*NP-glucoside [37]. In this HPLC analysis, the genistein was contained in fermented soybean by *H. erinaceus* (1.465 ± 0.033 µg/mg dried fermented soybean) and *H. ramosum* (1.410 ± 0.043 µg/mg dried fermented soybean) mycelia, respectively. These results suggest that the high inhibitory effects of fermented soybean by *H. erinaceus* and *H. ramosum* mycelia may be related to genistein. Although yeast alpha-glucosidase has been widely used for screening anti-diabetic agents using *p*NP-glucoside as the substrate, it is not a biologically relevant substrate for mammalian alpha-glucosidase. Therefore, we investigated the inhibitory activity of fermented soybean using mushroom mycelia against rat small intestinal alpha-glucosidase with maltose and sucrose as substrates.

The inhibitory activity against rat small intestinal alpha-glucosidase using the substrate maltose of soybeans fermented using *G. lucidum* mycelia was significantly higher than soybeans fermented using *H. erinaceus* and *H. ramosum* mycelia. Moreover, the inhibitory activity against small intestinal alpha-glucosidase using the substrate sucrose of soybeans fermented using *G. lucidum* and *H. erinaceus* mycelia was significantly higher than soybeans fermented using *H. ramosum* mycelia. Based on these results, hydroxydaidzein in soybeans fermented using *G. lucidum* mycelia might be one of the active compounds against rat small intestinal alpha-glucosidase using maltose and sucrose as substrates in addition to genistein. The underlying exact active compounds in soybeans fermented using mushroom mycelia remain unclear. However, these fermented soybeans could be used as edible compounds or nutritional supplements for diabetes treatment.

Isoflavones are a subgroup of flavonoids, and major isoflavones in soybean are genistein, daidzein, and glycitein. They are found in nature in glycosylated forms like genistin, daidzin, and glycitin. In general, they cannot be easily absorbed in the intestines, and they undergo hydrolysis catalysed by beta-glucosidase from intestinal microflora. Here, the levels of aglycon isoflavone forms daidzein, glycitein, and genistein in soybeans fermented using mushroom mycelia were significantly higher than the control group. In particular, the soybeans fermented using *H. erinaceus* and *H. ramosum* mycelia contained a higher volume of aglycon-form isoflavone than the control group and soybeans fermented using *G. lucidum* mycelia. Approximately 80% of daidzin was hydrolysed to daidzein, and approximately 90% of genistin was hydrolysed to genistein. Beta-glucosidase (EC 3.2.1.21) catalyses the hydrolysis of the *O*-glycosyl linkage of terminal nonreducing β -D-glucosyl residues [38]. Several bacterial species like *Aspergillus niger* [39], *Aspergillus oryzae* [40], *Penicillium brasilianum* [41], and *Phanerochaete chrysosporium* [42] are utilised to ferment products using beta-glucosidase catalysis. Miura et al. characterised soybeans fermented using *G. lucidum* mycelia, and they reported that beta-glucosidase produced by *G. lucidum* mycelia changed the isoflavone glycosides into aglycons [13]. To our knowledge, there is little experimental data on beta-glucosidase activity in soybeans fermented using *H. erinaceus* and *H. ramosum* mycelia. However, the amount of beta-glucosidase produced by *H. erinaceus* and *H. ramosum* mycelia may be greater than in soybeans fermented using *G. lucidum* mycelia. In the present study, we found that intake of soybeans fermented using *H. erinaceus* and *H. ramosum* mycelia provide aglycon-form isoflavone.

On the other hand, even though the levels of isoflavone aglycon from soybeans fermented using *G. lucidum* mycelia are lower than soybeans fermented using *H. erinaceus* and *H. ramosum* mycelia, they contained 8-hydroxydaidzein and one unidentified compound (peak No. 6). We presumed that this compound at peak No. 6 was 6-hydroxydaidzein or 3'-hydroxydaidzein based on MS (m/z) data and the molecular formula in LC/MS analysis. 8-Hydroxydaidzein, an *ortho*-hydroxylation derivative of daidzein, was isolated from the fermentation broth of *Streptomyces* sp. [43]. Chiang et al. successfully produced 6-hydroxydaidzein and 3'-hydroxydaidzein in addition to 8-hydroxydaidzein using *Aspergillus oryzae* and recombinant *Pichia pastoris* [44]. The 8-hydroxydaidzein has some bioactivities like anti-cellular proliferation [45], tyrosinase inhibition [46], aldose reductase inhibition [47], anti-inflammation [48], and antioxidant activity [31]. Therefore, soybeans fermented using *G. lucidum* mycelia may have these bioactivities, including anti-cellular proliferation, tyrosinase inhibition, aldose reductase inhibition, and anti-inflammation because they may contain hydroxydaidzein. In general, hydroxydaidzein was transformed from daidzin using two reactions. First, daidzin was hydrolysis-catalysed to daidzein using beta-glucosidase. Second, daidzein was hydroxylated to hydroxydaidzein [49]. Hydroxydaidzein can be produced using chemical synthesis, but this method has some critical problems like byproduct formation, low product yield, and many reaction steps [50]. Consequently, some researchers have investigated mass production for hydroxydaidzein using fungal fermentation, including *Aspergillus oryzae* [49, 51]. On the basis of our results, we assumed that soybeans fermented using *G. lucidum* mycelia could be useful as a novel food material or nutritional supplement with some bioactivities. Fermentation using *G. lucidum* mycelia might be an excellent method for hydroxydaidzein production.

Conclusions

In this study, we prepared soybeans fermented using *G. lucidum*, *H. erinaceus*, and *H. ramosum* mycelia to obtain new information on soybeans fermented using mushroom mycelia. The volume and type of isoflavones significantly differed between soybean fermentation by *G. lucidum*, *H. erinaceus*, and *H. ramosum* mycelia after 4 weeks of fermentation. The soybeans fermented using *H. erinaceus* and *H. ramosum* mycelia contained a higher volume of aglycon-form isoflavones (daidzein, glycitein, and genistein) than soybeans fermented using *G. lucidum* mycelia. The aglycon-form isoflavone levels in soybeans fermented using *G. lucidum* mycelia were lower than those in soybeans fermented using *H. erinaceus* and *H. ramosum* mycelia, and they contained

two hydroxydaidzeins. Therefore, we suggest two ways of consuming soybeans fermented using mushroom mycelia. Higher intake of soybeans fermented using *H. erinaceus* or *H. ramosum* mycelia provides isoflavone aglycon-form isoflavone that is easily absorbed in the intestines. Soybeans fermented using *G. lucidum* mycelia enhance bioactivities, including antioxidant and alpha-glucosidase inhibitory activity.

Abbreviations

G. lucidum: *Ganoderma lucidum*; *H. ramosum*: *Hericium ramosum*; *H. erinaceus*: *Hericium erinaceus*; ORAC: Oxygen radical absorbance capacity; LC/MS: Liquid chromatography/mass spectrometry; PDA: Potato dextrose agar; MES: 2-Morpholinoethanesulfonic acid monohydrate; DPPH: 1,1-Diphenyl-2-picrylhydrazyl; Trolox: 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; pNP-glucoside: *p*-Nitrophenyl α -D-glucopyranoside; HPLC: High-performance liquid chromatography; SD: Standard deviation.

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Authors' contributions

Experimental design, KS. Separation and culturing mushroom mycelia, KS. Preparation of fermented soybean, KS, TT. Analysis of antioxidant, alpha-glucosidase inhibition, HPLC and LC/MS, KK. Writing manuscript and editing, KS, KK. All authors read and approved the final manuscript.

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Not applicable.

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Competing interests

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