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Fermentation of soybeans with *Pleurotus cornucopiae* and *Pleurotus ostreatus* increases isoflavone aglycones, total polyphenol content and antioxidant activity

Yuta Sawada[a], Toshitsugu Sato[a*], Ryosuke Fukushi[a], Yoshihito Kohari[a], Yuuki Takahashi[a], Sayaka Tomii[a], Lifeng Yang[a], Takashi Yamagishi[a], Hirofumi Arai[a]

[a] National University Corporation Hokkaido Higher Education and Research System, Kitami Institute of Technology, 165 Koen-cho, Kitami, Hokkaido 090-8507, Japan.

ABSTRACT

Edible basidiomycetes are highly active in the oxidative decomposition and polymerisation of polyphenols, and soybeans contain large amounts of isoflavones, which are polyphenol glycosides. Isoflavone aglycones exhibit weak estrogenic activities. In this study, we investigated the isoflavone content, polyphenol production, antioxidant activity and ergothioneine (EGT) content of soybeans fermented by *Pleurotus cornucopiae* and *Pleurotus ostreatus*. Isoflavone glycosides, which were abundant in unfermented soybeans, decreased, and aglycones increased on day 10 of culture in both edible basidiomycete-fermented soybeans. The total maximum polyphenol content in soybeans fermented by both mushrooms were approximately 4 times higher on day 30 to 40 of culture, than that of unfermented soybeans. *P. cornucopiae*-fermented soybeans showed maximum antioxidant activity on day 20 of culture, and this was approximately 6.1 times higher than that of unfermented soybeans. EGT was not detected in unfermented soybeans, whereas both fermented soybeans showed a maximum EGT content on day 20 of culture, which was especially high in *P. cornucopiae*-fermented soybeans. The antioxidant activity and EGT of *P. cornucopiae*-fermented soybeans were higher than those of *P. ostreatus*, suggesting that EGT was responsible for the increase in the antioxidant activity of *P. cornucopiae*-fermented soybeans.

Keywords: 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, ergothioneine (EGT), functional food, HPLC analysis, mushroom

1. Introduction

Edible mushrooms belonging to basidiomycetes are used not only as food, but also as raw materials for pharmaceuticals, especially polysaccharides, as anticancer agents (Mizuno & Nishitani, 2013). Among them, mushrooms belonging to the wood decay fungi can degrade lignin, which is a constituent of wood with high molecular weight polyphenols, and can decompose polyphenols through oxidation or polymerisation (Lundell et al., 2010; Janusz et al., 2013). In addition, mushrooms are rich in the antioxidant compound ergothioneine (EGT). EGT has been suggested to have important biological activities such as cytoprotection against oxidative stress (Paul & Snyder, 2010; Kushairi et al., 2020) and anti-apoptosis (Ko et al., 2021; Salama et al., 2021). EGT is an essential component in humans, and its blood levels have been shown to decrease with age (Cheah et al., 2016). In some countries, EGT in-

take appears to be positively correlated with longevity, especially in relation to mortality from neurological diseases (Beelman et al., 2020). Therefore, various age-related diseases can be suppressed by actively consuming EGT. Soybeans are widely used as a raw material for various foods such as natto, miso, soy sauce, and tofu. They are an agricultural product containing large amount of isoflavones, a type of polyphenol, as a functional ingredient (Rizzo & Baroni, 2018; Cao et al., 2019). Isoflavones exhibit estrogen-like activity by binding to the estrogen receptor β (Rizzo & Baroni, 2018; Krizova et al., 2019). They are expected to be effective against symptoms associated with decreased estrogen secretion, such as menopause and osteoporosis (Tham et al., 1998; Krizova et al., 2019). Daidzein, an isoflavone present in soybeans, is converted into equol by specific intestinal bacteria (Sathyamoorthy & Wang, 1997; Bowey et al., 2003; Jackson et al., 2011; Kawada et al., 2018). Sathyamoorthy and Wang (1997) reported that equol was 100-fold more potent than daidzein in stimulating estrogenic responses.

Some studies have reported that the fermentation of soybeans or soybean extracts by basidiomycetes leads to the conversion of isoflavone glycosides into aglycones (Miura et al., 2002; Fukuda et al.,

2007; Sameshima et al., 2019). Beans fermented with mushrooms also exhibit increased antioxidant activity and polyphenol content (Espinosa-Paez et al., 2017; Xu et al., 2018). Furthermore, soybeans fermented with mushrooms exhibit thrombosis-preventive, fibrinolytic, and antiangiogenic activities (Miura et al., 2002; Okamura-Matsui et al., 2003; Fukuda et al, 2007).

However, few reports have confirmed changes in isoflavone and equol content, polyphenol content, and antioxidant activity after soybean fermentation with edible basidiomycetes over an extended period. Therefore, in this study, we investigated changes in isoflavone components and equol, total polyphenol content, antioxidant activity and EGT in soybeans fermented with *Pleurotus cornucopiae* and *Pleurotus ostreatus*.

2. Materials and Methods

2.1. Organisms and culture conditions

Wild-type dikaryotic strains of *Pleurotus cornucopiae var. citrinopileatus* Pc98-3 (*P. cornucopiae*) (Harada et al., 2008) and *Pleurotus ostreatus* ATCC66376 (*P. ostreatus*) were used in this study. *P. cornucopiae* was provided by the Hokkaido Research Organization, Forest Research Department, Forest Products Research Institute (Hokkaido, Asahikawa, Japan). A commercially available variety of soybean, Yukipirika, was used as fermentation material. Yukipirika was purchased from ISOPP Agri System Co., Ltd. (Hokkaido, Kitami, Japan). *Pleurotus cornucopiae* and *P. ostreatus* were cultured on SMYP (2% soluble starch, 1% malt extract, 0.1% yeast extract, 0.1% peptone) agar and $0.25 \times$ MYPG agar medium (Nagai et al., 2002), respectively. The soybean medium was prepared by adding distilled water to soybeans to a water content of 65% and autoclaving at 121 °C for 30 min. *Pleurotus cornucopiae* and *P. ostreatus* were cultured at 25 °C and 70% relative humidity. Fermentation of soybeans with *P. cornucopiae* or *P. ostreatus* was performed for periods increasing by 10 d from 10 to 80 d. Fermented soybeans cultivated for each period were freeze-dried and stored at −30 °C.

2.2. Extraction of isoflavones in fermented soybeans

Isoflavone extraction was performed according to the Association of Official Analytical Chemists (AOAC) method (Klump et al., 2001). The freeze-dried fermented soybeans were powdered using a mortar and pestle. Isoflavone extraction was performed by adding 40 mL of 80% methanol to 2.5 g soybean powder, followed by shaking at 95 rpm and 65 °C for 2 h. After cooling to 25 °C, 3 mL of 2 M NaOH was added and shaken at 95 rpm and 25 °C for 10 min. Then, 1 mL of glacial acetic acid was added, and the mixture was transferred to a 50-mL measuring flask. Subsequently, 100% methanol was added to the flask to make up the volume to 50 mL, and the mixture was mixed vigorously, and left overnight at 4 °C to serve as the isoflavone extract. Then, 1 mL of the supernatant of the isoflavone extract was transferred to a 1.5-mL microtube and centrifuged at $18,800 \times g$ and 20 °C for 10 min, and 10 μL of the supernatant was subjected to high-performance liquid chromatography (HPLC).

2.3. Isoflavone analysis using HPLC

Isoflavone extracts were analysed using an HPLC system equipped with a System Controller SCL-10A, Liquid Chromatograph LC-10AT, UV-VIS Detector SPD-10A, and Column Oven CTO-10A (Shimadzu Corporation, Kyoto, Japan) using a reversed-phase C_{18} column, Inertsil ODS-4 (250×4.6 mm, 5 μm) (GL Sciences Inc., Tokyo, Japan). The analysis was performed at a col-

umn temperature of 40 °C, detection wavelength of 260 nm, and a flow rate of 1 mL/min. As the mobile phase, eluent A was a solution in which ultrapure (MQ) water, methanol, and acetic acid were mixed at 44:5:1, and eluent B was a solution in which methanol and acetic acid were mixed at 49:1. First, the column was equilibrated with a mobile phase containing 90% of eluent A and 10% of eluent B. The analysis was performed by changing eluent B from 0 to 30 min with a linear concentration gradient of 10% to 60%, and from 30 to 33 min, eluent B was further changed to a linear concentration gradient of 60% to 100%; then, eluent B was held at 100% for 2 min, then changed to a linear gradient of 100% to 10% from 35 to 37 min, finally returning to eluent A 90% and eluent B 10% and returning to equilibrium with the initial condition from 37 to 50 min. To calculate the content of each isoflavone in fermented soybeans, a calibration curve was prepared for each of the six isoflavone components (daidzin, daidzein, glycitin, glycitein, genistin, and genistein) and equol. The concentration of each isoflavone in the sample was determined from a calibration curve.

2.4. Analysis of equol-like substance using liquid chromatography/mass spectrometry (LC/MS)

A powder (200g) of soybeans fermented with *P. cornucopiae* for 60 d was extracted. Forty bottles, each containing 5.0 g of soybean powder, were prepared, and 40 mL of 80% methanol was added to each bottle, followed by shaking at 95 rpm and 65 °C for 2 h. The mixture was centrifuged at $1,500 \times g$ for 10 min. The extracts were evaporated under reduced pressure and freeze-dried to yield 93.5 g. The freeze-dried extract (30 g) was dissolved in 100 mL of MQ water and applied to a 200×50 mm i.d. column of Diaion HP20 (Mitsubishi Chemical Co., Ltd., Tokyo, Japan). The sample was eluted with 200 mL of MQ water containing 0, 10, 20, 30, 40, 50, 60, 70, 80 and 90% methanol (fractions 1–10), with the methanol concentration increasing stepwise from low to high. Finally, further elution was performed with 1000 mL of 100% methanol solution, and 200 mL of the eluate was collected (fractions 11–15). Fractions 13 and 14 containing an equol-like substance, were combined, and the solvent was evaporated and freeze-dried. The resulting extract (1.22 g) was dissolved in 10 mL of 60% methanol and applied to 300×14 mm i.d. column of Diaion HP20. The sample was eluted with 20 mL of 60, 80, 85, 90, and 95% methanol, with the concentration increased stepwise from low to high (collecting 10 ml of each fraction 1–10). Finally, further elution was performed with 70 ml of 100% methanol solution, and 10 ml of the eluate was collected (fractions 11–17). Fractions 7–14, containing equol-like substances, were combined, and the solvent was evaporated and freeze-dried. The resulting extract (112.34 mg) was dissolved in methanol (3 mL). Further separation was conducting using Wakosil-II5C$_{18}$HG (20×250mm, 5μm, Wako, Osaka, Japan) at 40 °C, with a flow rate of 7.0 mL/min, detected at 260 nm, and the detected peak was collected, 100 μL was injected, and it was repeated 30 times. A dual pump KP-12 (From J Co., Tokyo, Japan) and UV Detector S-3120 (Soma Optics., Tokyo, Japan) was used. The solvent was evaporated and freeze-dried, and the purified product (1mg) was recovered and subjected to LC/MS analysis.

An LC/MS system consisting of a System Controller CBM-20A, Liquid Chromatograph LC-20ADXR, UV-VIS Detector SPD-20A, Column Oven CTO-20A, and Mass Spectrometer LCMS-2020 (Shimadzu Corporation, Kyoto, Japan) was used. The column used was a reverse-phase C_{18} column, Inertsil ODS-4 (150×4.6 mm, 5 μm) (GL Sciences Inc., Tokyo, Japan), and 50% methanol containing 2% acetic acid was used as the mobile phase. The resulting purified product (1 mg) was dissolved in 1 mL of 100% methanol to prepare

the sample for analysis. Equol (1 mg/mL) was used as the standard. The sample injection volume was 10 μL, the column temperature was 40 °C, the detection wavelength was 260 nm, the flow rate was 0.2 mL/min, and the mass scan was performed in the range of m/z 10–1000.

2.5. Extraction of polyphenols from fermented soybeans

Fermented soybeans were powdered with a mortar and pestle. The soybean powder (2.5 g) was transferred to a 50-mL conical flask, and 20 mL of 70% ethanol was added to the flask. After ultrasonic treatment of the flask for 30 min, the mixture was left overnight at 4 °C. The supernatant was transferred into a 50 mL glass centrifuge tube and centrifuged at $1,500 \times g$ at 4 °C for 20 min. The supernatant was filtered into a 50-mL measuring flask using No. 2 filter paper. Then, 20 mL of 70% ethanol was added to the conical flask containing the residue, and the procedure described above was repeated. The resulting polyphenol re-extract was transferred to the 50-mL measuring flask containing the initial extract, and 70% ethanol was added to obtain a final volume of 50 mL. After mixing well, the combined extract was transferred to a 50-mL vial and stored at 4 °C. This extract was used as a polyphenol extract.

2.6. Measurement of polyphenols using the Folin-Denis method

Polyphenol content was measured using the Folin-Denis method (Folin & Denis, 1915). MQ water was added to an appropriate amount of polyphenol extract to make a volume of 500 μL and mixed, and then 500 μL of Folin-Ciocalteu's phenol reagent (Kanto Chemical Co., Inc., Tokyo, Japan) was added. After the mixture was maintained at 25 °C for 3 min, 500 μL of 10% Na_2CO_3 solution was added, and the mixture was maintained at 30 °C for 30 min. Absorbance was measured at 760 nm using distilled water as a blank. The total phenolic content was expressed as gallic acid equivalents (mmol GAE/100 g dry weight) using a calibration curve of gallic acid.

2.7. Measurement of antioxidant activity using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

Antioxidant activity was measured according to the method described by Blois (DPPH radical scavenging activity) (Blois, 1958), with some modifications. The antioxidant activity of the polyphenol extract was measured using DPPH' radical scavenging ability. The polyphenol extract (100 μL) and a 100% ethanol mixture were added to a 96-well plate, followed by 50 μL of MQ water and 50 μL of 400 μM DPPH ethanol solution. After stirring, the reaction mixture was left undisturbed in the dark at room temperature (approximately 25 °C) for 20 min. The absorbance of the reaction mixture was measured at 517 nm (sample A) using a Multiskan GO plate reader (Thermo Fisher Scientific, Inc., MA, USA). The control was prepared by adding 50 μL of 100% ethanol instead of the above 400 μM DPPH solution, and the same operation was performed except for the standing time in the dark (control A). In addition, for both reaction mixtures, sample-free reaction solutions (blanks), in which 100 μL of 100% ethanol was added instead of the extract, were prepared, and the same steps were performed. Trolox was used as the standard, and the results were expressed as Trolox equivalents (μM TE/100 g dry weight).

2.8. EGT analysis using HPLC

EGT extraction was performed by adding 5 mL of 500 mM trichloroacetic acid to 500 mg soybean powder, followed by shaking at 120 rpm and 40 °C for 30 min. The mixture was centrifuged at $1,500 \times g$ for 10 min. Subsequently, 1 mL of the supernatant was filtered through a Sep-Pak C_{18} Plus column equilibrated with MQ water and eluted with 4 mL MQ water, which was used as the HPLC injection sample solution. The EGT extracts were analysed using an HPLC system equipped with a System Liquid Chromatograph PU-980, UV-VIS Detector UV-2075 Plus, and Column Oven CO-965 (JASCO Corporation., Tokyo, Japan) using a reversed-phase C_{30} column, Develosil C_{30}-UG-5 (250 × 4.6 mm, 5 μm) (Nomura Chemical Co., Ltd., Aichi, Japan). The analysis was performed at column temperature of 40 °C, detection wavelength of 260 nm, and a flow rate of 1 mL/min. Gradient elution was performed using 0.1% acetic acid (v/v) in water (eluent A) and 0.1% acetic acid (v/v) in methanol (eluent B). First, the column was equilibrated using a mobile phase containing 100% of eluent A. The analysis was performed by changing eluent B from 5 to 10 min with a linear concentration gradient of 0% to 100%, eluent B was held at 100% for 15 min, then changed to a linear gradient of 100% to 0% from 25 to 27 min, finally returning to eluent A 100%, and equilibrating with the initial condition from 27 to 40 min. To calculate the EGT content in the fermented soybeans, a calibration curve was prepared. The EGT concentrations in the samples were determined using a calibration curve.

3. Results

3.1. Changes in isoflavone content during soybean fermentation with P. cornucopiae and P. ostreatus

The isoflavone content in the soybeans fermented with *P. cornucopiae* and *P. ostreatus* was analysed every 10 d until day 80. Most of the isoflavones in the unfermented soybeans were glycosides, and the amount of daidzin and genistin was 421.75 and 460.51 (μmol/100 g dry weight), respectively (Supplementary Table S1). The total amount of isoflavones in unfermented soybeans was 961.30 (μmol/100 g dry weight) (Supplementary Table S1), and the proportions of daidzin and genistin was 43.8% and 48.0%, respectively, resulting in a total isoflavone content of 91.8% (Fig. 1A).

In contrast, all glycosides (daidzin, genistin, and glycitin) in soybeans fermented with *P. cornucopiae* and *P. ostreatus* decreased significantly on day 10 of culture, whereas aglycones (daidzein, genistein, and glycitein) increased. Isoflavone levels remained constant until day 30, before they began to decrease on day 40 and had almost disappeared by day 80 (Fig. 1B, C). The amount of aglycones in the soybeans fermented with *P. cornucopiae* reached a maximum of 704.11 (μmol/100 g dry weight] on day 20, when they contained 367.64 and 317.16 (μmol/100 g dry weight) of daidzein and genistein, respectively (Supplementary Table S2). The two aglycones made up 94.6% of the total isoflavone content (723.84 [μmol/100 g dry weight]). The amount of aglycones in soybeans fermented with *P. ostreatus* reached a maximum of 774 (μmol/100 g dry weight) on day 10 with 381.66 and 371.43 (μmol/100 g dry weight) of daidzein and genistein, respectively (Supplementary Table S3). The two aglycones made up 86.3% of the total isoflavone content (873.66 [μmol/100 g dry weight]).

Equol-like substance was detected as a large peak at 29.2 min in soybeans fermented for 50 d with *P. cornucopiae* (Fig. 2C) and *P. ostreatus* (Fig. 2D), which was consistent with the retention time of
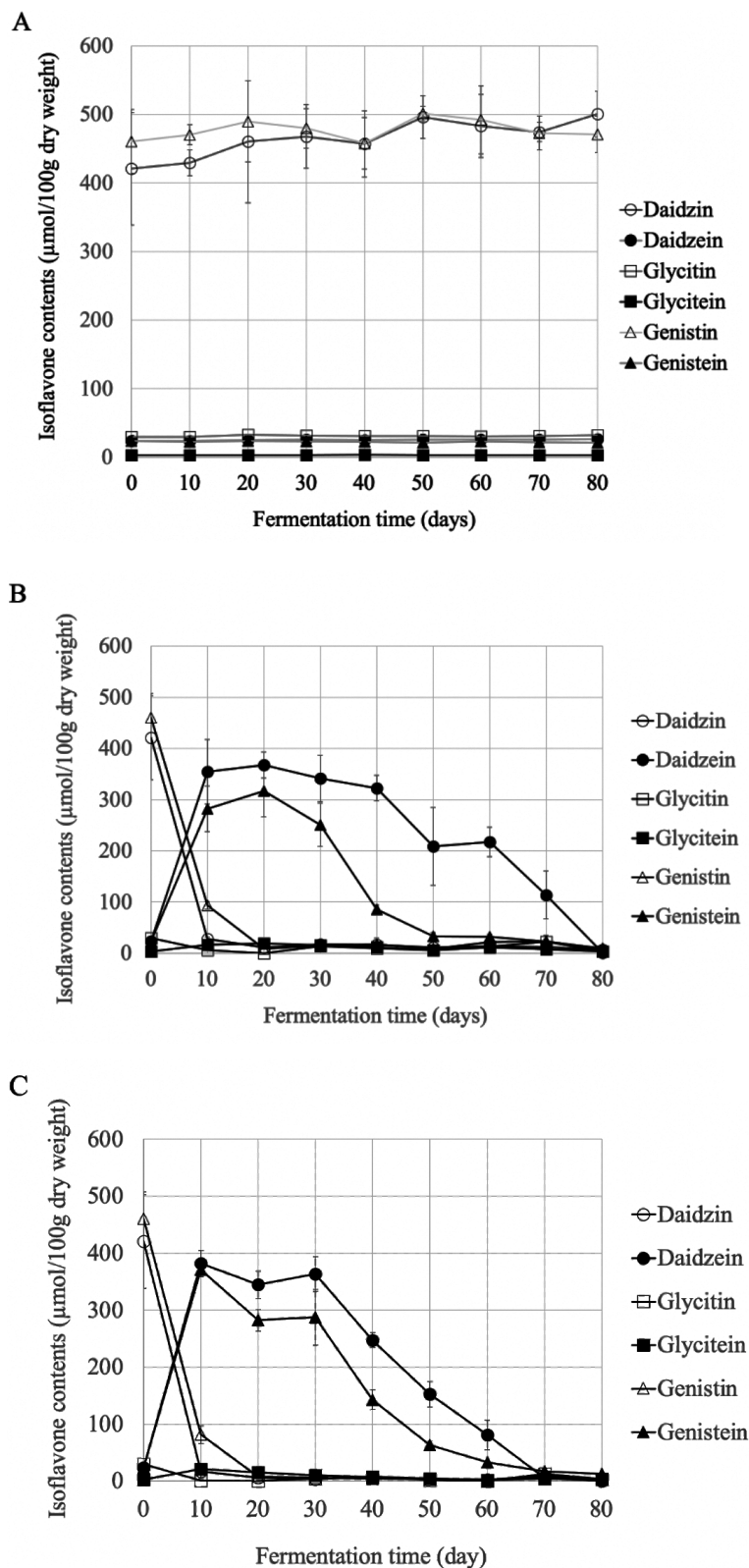
Fig. 1 — Changes in isoflavone contents during fermentation. Soybeans were fermented with *Pleurotus cornucopiae* and *Pleurotus* ostreatus for up to 80 d. Fermented soybeans were sampled and freeze-dried every 10 d. The extracts from these samples were analysed by HPLC. A: Unfermented soybeans (control). B: Soybeans fermented with *P. cornucopiae*. C: Soybeans fermented with *P. ostreatus*. Error bars represent standard deviation.

the equol standard (Fig. 2A), whereas this peak was not detected in unfermented soybeans (Fig. 2B). When the equol-like substance

content was calculated from the calibration curve of the standard equol, soybeans fermented for 50 d with *P. cornucopiae* and *P. ost-*

reatus showed maximum values of 7.28 and 2.13 (mmol/100 g dry weight), respectively (Table 1).

3.2. Analysis of equol-like substance using LC/MS

Equol standard were detected at peaks 243.05 and 281.05 in the mass spectrum (MS) using LC/MS analysis (Fig. 3A). Since the molecular weight of equol is 242.27 g/mol, $[M+H]^+$ was detected at 243.05 and $[M+K]^+$ at 281.05. In contrast, the MS of equol-like substances purified from soybeans fermented for 60 d with *P. cornucopiae* detected peaks at 325.10, 355.00, 283.05, 125.10, 235.05, and 181.05 in descending order of peak intensity (Fig. 3B). Therefore, the purified equol-like substance is considered different from equol. The MS peaks of equol-like substances were retrieved from two databases: MassBank (www.massbank.jp, 2023, 6) and SDBSWeb: https://sdbs.db.aist.go.jp (National Institute of Advanced Industrial Science and Technology, 2023, 6); however, no matching substances were found and no substances could be identified. Therefore, the equol-like substance may be a novel substance.

3.3. Changes in total polyphenol content during soybean fermentation with P. cornucopiae and P. ostreatus

The total amount of polyphenols equivalent to gallic acid in soybeans fermented with *P. cornucopiae* and *P. ostreatus* was determined. The total amount of polyphenols in the unfermented soybeans (culture day 0) was 1.61 (mmol GAE/100 g dry weight) (Table 2). The total amount of polyphenols in soybeans fermented with *P. cornucopiae* reached a maximum of 6.31 (mmol GAE/100 g

dry weight) on day 40 of culture, which was approximately 4.3 times higher than that of unfermented soybeans (Table 2). The total amount of polyphenols in soybeans fermented with *P. ostreatus* reached a maximum value of 5.69 (mmol GAE/100 g dry weight) on day 30 of culture, which was approximately 3.9 times higher than that of unfermented soybeans (Table 2). The polyphenol contents of soybeans fermented with *P. cornucopiae* and *P. ostreatus* gradually decreased after reaching the maximum (Table 2).

3.4. Changes in antioxidant activity during soybean fermentation with P. cornucopiae and P. ostreatus

The time course of the antioxidant activity of *P. cornucopiae*- and *P. ostreatus*-fermented soybeans was investigated. The DPPH radical scavenging activity of the unfermented soybeans was approximately 156.0 (μmol TE/100 g dry weight) (Table 3). In soybeans fermented with *P. cornucopiae*, DPPH radical scavenging activity increased sharply and reached a maximum of 953.7 (μmol TE/100 g dry weight) on day 30, which was approximately 6.1 times that of the unfermented soybean. Subsequently, the antioxidant activity decreased sharply on day 40, and continued to decline until it reached the same level as that of the unfermented soybean by day 50 (Table 3). In contrast, in soybeans fermented with *P. ostreatus*, the DPPH radical scavenging activity reached a maximum value of 296.5 (μmol TE/100 g dry weight) on day 20, which was approximately twice that of the unfermented soybean. It then decreased on day 40 and dropped to the same level as the control after day 40 (137.4 [μmol TE/100 g dry weight]) (Table 3).
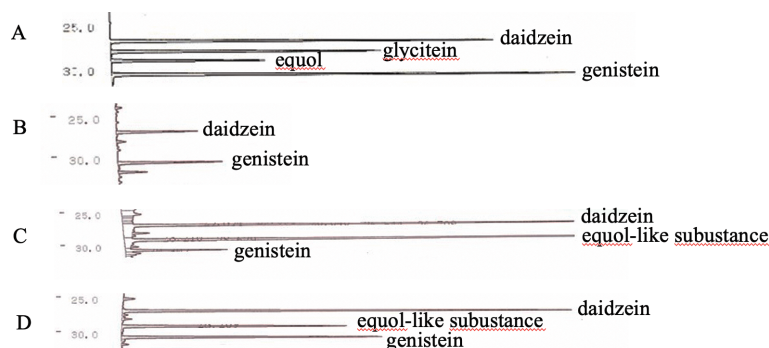

Fig. 2 — HPLC chromatograms of isoflavone and equol standards and the extracts of fermented soybeans. A: Isoflavone and equol standards, B: unfermented soybeans for 50 d incubation, C: soybeans fermented with *Pleurotus cornucopiae* for 50 d, D: soybeans fermented with *P. ostreatus* for 50 d. Retention time at approximately 27.0 min, 28.2 min, 29.2 min, 30.8 min show daidzein, glycitein, equol, and genistein, respectively.

Table 1. Changes in equol-like substance in fermented soybean extracts during fermentation.

Fermentation time (d)	Unfermented soybeans (control)	Fermented soybeans with *Pleurotus cornucopiae*	Fermented soybeans with *Pleurouts ostreatus*
0	0	0	0
10	0	0	0
20	0	0	0
30	0	0.84 ± 0.99	0
40	0	5.95 ± 0.72	0.38 ± 0.30
50	0	7.28 ± 1.44	2.13 ± 0.18
60	0	6.86 ± 0.18	1.99 ± 0.29
70	0	5.33 ± 0.69	0.84 ± 0.62
80	0	2.24 ± 0.38	0.72 ± 0.29

Soybeans were fermented with *P. cornucopiae* and *P. ostreatus* for up to 80 d. Fermented soybeans were sampled and freeze-dried every 10 d. Equol-like substance content was measured by HPLC. The total equol-like substance content is expressed as equol equivalents (mmol of equol/100 g dry weight) ± SD.
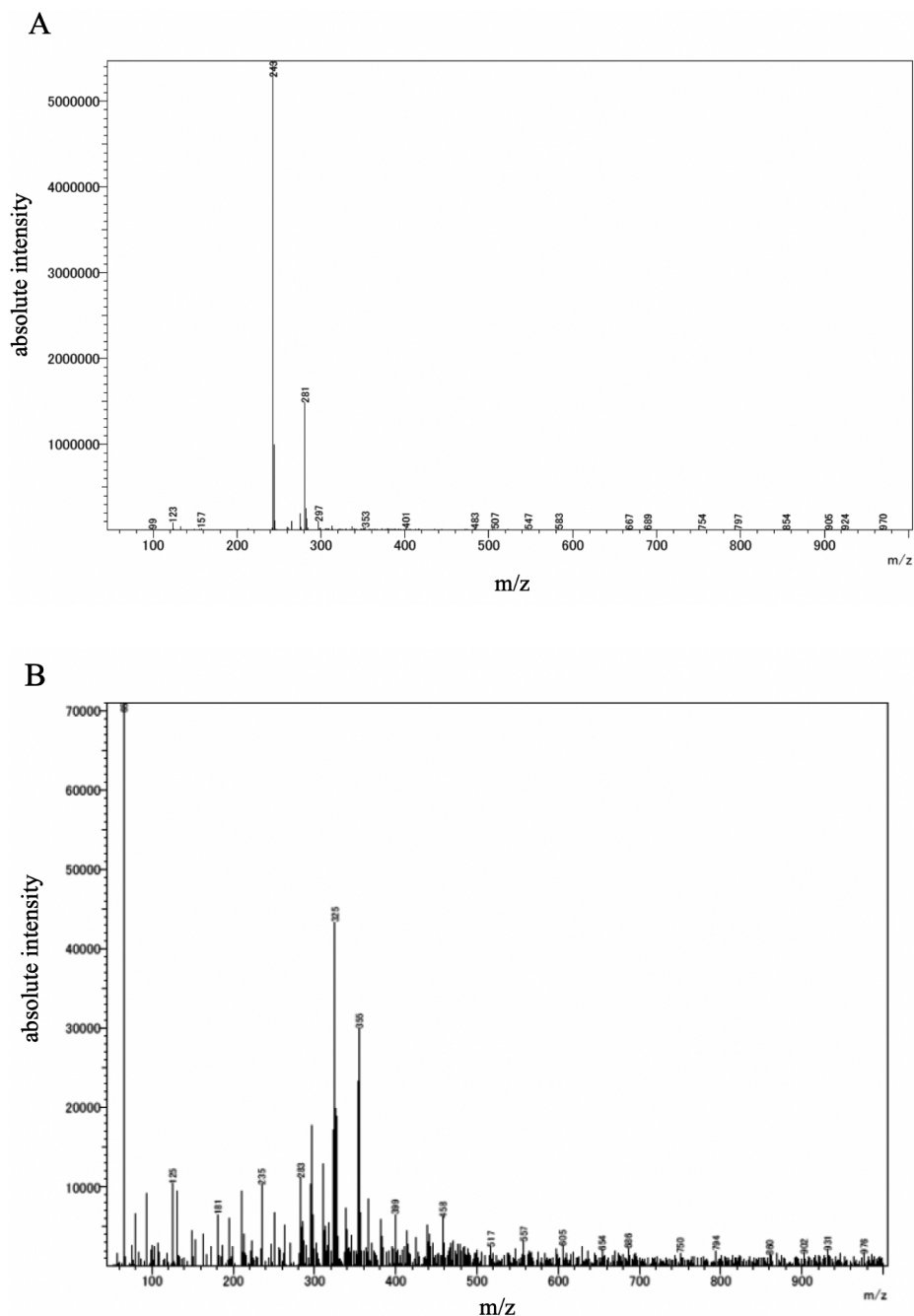
A

B

Fig. 3 — Liquid chromatography–mass spectrometry spectrum. A: Equol, B: Equol-like substance. A: 243.05 and 281.05 in descending order of peak intensity, B: 325.10, 355.00, 283.05, 125.10, 235.05, and 181.05 in descending order of peak intensity.

3.5. Changes in EGT during soybean fermentation with *P. cornucopiae* and *P. ostreatus*

EGT was not detected in the unfermented soybeans (culture day 0). EGT in soybeans fermented with *P. cornucopiae* was detected from days 10 to 80, and in soybeans fermented with *P. ostreatus*, EGT was detected from days 10 to 50. EGT content in soybeans fermented for 40 d showed a maximum value of 79.70 (mg/100g dry weight) for *P. cornucopiae* and 16.34 (mg/100g dry weight) for the *P. ostreatus* before declining (Table 4). *Pleurotus cornucopiae*-fermented soybeans were 4.9 times higher than *P. ostreatus*-fermented soybeans.

4. Discussion

In this study, we analysed changes in isoflavone content in soybeans fermented with *P. cornucopiae* and *P. ostreatus*. Although the total amount of isoflavones (961.30 [μmol/100 g dry weight]) gradually decreased (Fig. 1B, C), the major soybean isoflavone glycosides daidzin (420.75 [μmol/100 g dry weight]) and genistin (460.51 [μmol/100 g dry weight]) (Supplementary Table S1), were mostly converted to aglycones (daidzein and genistein) in the early stage of fermentation. The total amount of isoflavones in soybeans fermented with *P. cornucopiae* and *P. ostreatus* decreased to 70.0% and 63.4%, respectively, compared with that in unfermented soybeans on day 20, whereas the proportions of aglycones reached a maxi-

Table 2. Changes in total polyphenol content during fermentation.

Fermentation time (d)	Unfermented soybeans (control)	Fermented soybeans with *Pleurotus cornucopiae*	Fermented soybeans with *Pleurotus ostreatus*
0	1.61 ± 0.01	1.61 ± 0.01	1.61 ± 0.01
10	1.63 ± 0.03	2.59 ± 0.11	2.48 ± 0.18
20	1.56 ± 0.01	4.46 ± 0.25	4.21 ± 0.31
30	1.45 ± 0.02	6.00 ± 0.56	5.69 ± 0.36
40	1.45 ± 0.03	6.31 ± 0.14	5.57 ± 0.16
50	1.54 ± 0.05	5.31 ± 0.60	4.73 ± 0.27
60	1.47 ± 0.05	4.09 ± 0.29	2.40 ± 0.20
70	1.35 ± 0.03	3.34 ± 0.73	2.09 ± 0.03
80	1.33 ± 0.05	2.50 ± 0.01	1.28 ± 0.02

Soybeans were fermented with *P. cornucopiae* and *P. ostreatus* for up to 80 d. Fermented soybeans were sampled and freeze-dried every 10 d. Total polyphenol content was measured using the Folin-Denis method. Total phenolic content is expressed as gallic acid equivalents (mmol GAE/100 g dry weight) ± SD.

Table 3. Changes is antioxidant activity of fermented soybean extracts during fermentation.

Fermentation time (d)	Unfermented soybeans (control)	Fermented soybeans with *Pleurotus cornucopiae*	Fermented soybeans with *Pleurotus ostreatus*
0	155.9 ± 26.6	155.9 ± 26.6	155.9 ± 26.6
10	134.2 ± 18.4	424.3 ± 204.1	104.7 ± 12.0
20	147.5 ± 28.2	857.4 ± 145.2	187.0 ± 28.1
30	144.8 ± 18.4	953.7 ± 213.4	296.5 ± 36.6
40	159.8 ± 20.2	341.7 ± 125.4	137.4 ± 16.5
50	173.9 ± 33.9	142.6 ± 21.4	75.8 ± 6.4
60	178.6 ± 26.5	112.3 ± 1.5	76.2 ± 4.1
70	171.3 ± 30.3	107.0 ± 23.4	103.4 ± 2.0
80	172.7 ± 19.2	121.5 ± 9.2	109.6 ± 5.6

Soybeans were fermented with *P. cornucopiae* and *P. ostreatus* for up to 80 d. Fermented soybeans were sampled and freeze-dried every 10 d. The antioxidant activity of the extracts was analysed by DPPH radical scavenging activity. Trolox was used as a standard and the results were expressed as Trolox equivalents (μmol TE/100 g dry weight) ± SD.

Table 4. Changes is ergothioneine of fermented soybean extracts during fermentation.

Fermentation time (days)	Unfermented soybeans (Control)	Fermented soybeans with *Pleurotus cornucopiae*	Fermented soybeans with *Pleurotus ostreatus*
0	0	0	0
10	0	2.64 ± 0.23	3.20 ± 2.77
20	0	23.71 ± 11.04	7.62 ± 0.53
30	0	79.70 ± 29.97	16.34 ± 3.18
40	0	4.57 ± 0.98	7.14 ± 0.63
50	0	4.99 ± 0.42	3.18 ± 2.75
60	0	2.59 ± 0.18	0
70	0	3.15 ± 1.18	0
80	0	3.31 ± 0.31	0

Soybeans were fermented with *P. cornucopiae* and *P. ostreatus* for up to 80 d. Fermented soybeans were sampled and freeze-dried every 10 d. Ergothioneine content was measured by HPLC (mg of ergothioneine /100 g dry weight) ± SD.

mum of 97.3% and 97.9%, respectively. These results indicate that mushroom-fermented soybeans on day 20 using both *P. cornucopiae* and *P. ostreatus* can be considered a source of aglycones.

Isoflavone analysis of soybeans fermented directly with mushrooms has been reported by two research groups. Fukuda et al. (2007) reported that in soybeans fermented with *P. cornucopiae* for 14 d, all glycosides disappeared, daidzin was converted to almost 100% daidzein, and genistin was converted to approximately 88% genistein. However, the amount of isoflavone extracted was approximately one-eighth to one-tenth of the amount extracted in this study, which could be partly due to the different extraction methods and varieties of soybeans and *P. cornucopiae* used. Sameshima et al. (2019) reported that 83.75 (mg/100 g dry weight] of unfermented black soybean glycosides disappeared after 28 d of fermentation with *Schizophyllum commune*, resulting in 64.7 (mg/100 g dry weight) of aglycones. In this study, the total amount of isoflavones in unfermented soybeans was 400.85 (mg/100 g dry weight) (= 961.30 [μmol/100 g dry weight]), and on day 20 of fermentation, the amount of aglycones was 184.66 (mg/100 g dry

weight) (total isoflavone:193.01 mg = 723.84 [μmol/100 g dry weight]) for *P. cornucopiae* and 168.29 (mg/100 g dry weight) (total isoflavone:174 mg = 656.13 [μmol/100 g dry weight]) for *P. ostreatus* (Fig. 1, Supplementary Table 1~3). Therefore, the results showed 2.5 times more aglycones than reported by Sameshima et al. (2019). In addition, Sameshima et al. (2019) showed that all the aglycones of black soybeans fermented with *S. commune* were daidzein, and genistein was not detected.

Conversely, Lee et al. (2019) reported that the maximum conversion rate of glycosides to aglycones in germinated soybeans fermented with *Tricholoma matsutake* was 88% on day 9, which was 500 (μmol/100 g dry weight). In addition, Miura et al. (2002) reported that the conversion rate from glycosides to aglycones was approximately 70.7% and that from genistin to genistein was 81.6% in soybeans fermented for 15 d with *Ganoderma lucidum*. Because the experimental conditions were different, a direct comparison could not be made, and the conversion rate of glucosides to aglycones was higher in this study. As described above, all reports support a high conversion rate of isoflavone glycosides to aglycones by basid-

iomycetes. This study is the first to analyse each isoflavone component in detail and demonstrated that most of the glycosides are converted to aglycones in soybeans fermented for 10–30 d with *P. cornucopiae* and *P. ostreatus*. As the conversion rate from glycosides to aglycones was greater than 97%, soybeans fermented with *P. cornucopiae* and *P. ostreatus* had a higher aglycone production efficiency than those fermented with other basidiomycetes.

Equol, which is not originally present in soybeans, is metabolised and produced from daidzein by the gut flora (Sathyamoorthy & Wang, 1997; Jackson et al., 2011). Equol-like substances were detected as large peaks in soybeans fermented with *P. cornucopiae* after 30 d of culture and those fermented with *P. ostreatus* after 40 d of culture (Table 1). Daidzein was added to the liquid medium to examine the equol-producing ability of *P. cornucopiae* and *P. ostreatus*. However, although daidzein levels decreased, equol levels could not be confirmed (data not shown). On LC/MS analysis of the equol-like substance purified from soybeans fermented with *P. cornucopiae*, it was found that the molecular weight was greater than that of equol and it was not equivalent (Fig.3A, B). This result indicates that an equol-like substance was detected in fermented soybeans at approximately the same elution time as that of equol. In the future, it will be necessary to purify the equol-like substance and identify it using accurate mass spectrometry and NMR.

Soybeans fermented with *P. cornucopiae* and *P. ostreatus* for 30–40 d had increased amounts of polyphenols approximately four times that of unfermented soybeans, and then the polyphenol content decreased (Table 2). The total amount of isoflavones (961.30 [μmol/100 g dry weight]) in the unfermented soybeans accounted for 60.1% of the total amount of polyphenols (1.61 [mmol GAE/100 g dry weight]) (Table 2). It has been reported that the total amount of polyphenols equivalent to gallic acid in soybeans is 1.17 to 1.53 (mmol GAE/100 g dry weight) (Xu & Chang, 2007, 2011), which is similar to our results. Nevertheless, the total polyphenol content of soybeans fermented with *P. cornucopiae* and *P. ostreatus* increased by approximately 3.5-to 3.9-fold (5.57 to 6.31 [mmol GAE/100 g dry weight]) on days 30 and 40 of fermentation and then decreased (Table 2), whereas the total isoflavone content of fermented soybeans gradually decreased during fermentation (Fig. 1). This result indicated that in soybeans fermented with *P. cornucopiae* and *P. ostreatus*, the substance that caused the increase in the total polyphenol content was the polyphenol component newly produced by mushroom fermentation rather than isoflavones. The epidermis of soybeans contains other polyphenol components in addition to isoflavones, specifically high molecular weight lignin and proanthocyanidins (Xu & Chang, 2009; Senda et al., 2017). Since edible basidiomycetes have high lignin-degrading activity (Lundell et al., 2010), *P. cornucopiae* and *P. ostreatus* can decompose these high-molecular-weight polyphenols. As a result, it is possible that *P. cornucopiae* and *P. ostreatus* degrade the high-molecular-weight polyphenols contained in soybeans, increasing the amounts of polyphenols. Additionally, edible basidiomycetes not only oxidatively degrade polyphenols but also polymerise them (Rodriguez Couto & Toca Herrera, 2006). Therefore, it is possible that the absolute amount of the polyphenol component decreased after fermentation for 40 to 50 d due to the repolymerization of each low-molecular-weight polyphenol component by *P. cornucopiae* and *P. ostreatus*. Alternatively, polyphenols may be metabolized and degraded into smaller molecules. Espinosa-Páez et al. (Espinosa-Paez et al., 2017) reported that black beans fermented with *P. ostreatus* for 14 d resulted in a 1.26-fold increase in total polyphenol content (unfermented black bean: 148 [mg GAE/100 g dry weight] in comparison to black beans fermented with *P. ostreatus*: 187 [mg GAE/100 g dry weight]). Xu et al. (2018) reported that soybeans

fermented with *Agaricus bisporus* for 35 d showed a 1.2-fold increase in total polyphenols from 50 (mg GAE/100 g dry weight) to 60 (mg GAE/100 g dry weight). In this study, the total amount of polyphenols increased from 274.35 (mg GAE/100 g dry weight) (= 1.61 [mmol GAE/100 g dry weight]) in unfermented soybeans to 1.67 times (421.77 [mg GAE/100 g dry weight] = 2.48 [mmol GAE/100 g dry weight]) by day 10 of fermentation with *P. ostreatus* and 2.83 times (716.11 [mg GAE/100 g dry weight] = 4.21 [mmol GAE/100 g dry weight]) by day 20 (Table 2). In the above reports, although the amounts of polyphenols were lower than those in our study, the tendency for total polyphenols to increase due to mushroom fermentation was the same. In addition, this study revealed that the total amount of polyphenols in mushroom fermentation of soybeans for approximately 40 d increased up to approximately four times, reaching a maximum of 1,074.13 (mg GAE/100 g dry weight] (= 6.31 [mmol GAE/100 g dry weight]) for *P. cornucopiae* and 967.56 (mg GAE/100 g dry weight) (= 5.57 [mmol GAE/100 g dry weight]) for *P. ostreatus*. This study established for the first time that fermenting soybeans with *P. cornucopia* increased polyphenol content. In the future, the polyphenol components of fermented soybeans with the highest polyphenol content should be analysed.

Analysis of the antioxidant activity of fermented soybeans by DPPH radical scavenging ability on day 20 showed that the radical scavenging ability was approximately twice as high in *P. ostreatus* fermentation and approximately nine-fold higher in *P. cornucopiae* fermentation than in unfermented soybeans. These results revealed that the antioxidant activity of *P. cornucopiae*- fermented soybeans was much higher than that of *P. ostreatus*-fermented soybeans (Table 3). Espinosa-Páez et al. (2017) reported that the DPPH radical-capturing capacity of black beans fermented with oyster mushrooms for 14 d increased by approximately 1.4-fold compared to that of unfermented beans. In this study, both mushrooms had higher antioxidant activities than the above reported results, and especially in the case of *P. cornucopiae*, the antioxidant activity was more than four times higher than that of *P. ostreatus* (Table 3). This study demonstrated for the first time that fermenting soybeans with *P. cornucopiae* notably increases antioxidant activity. Xu et al. (2018) reported that soybeans fermented for 35 d with three basidiomycetes, *A. bisporus*, showed increased DPPH radical scavenging activity.

EGT, which has antioxidant activity and is abundant in mushrooms, has been reported to be more abundant in the fruiting body than in the mycelia (Kalaras et al., 2017; Krakowska et al., 2020). It is known to be particularly abundant in the fruiting bodies of *P. cornucopiae*. EGT was newly synthesized in soybeans fermented with both mushrooms and showed a maximum value on day 30 of fermentation, 79.70 (mg/100 g dry weight) in *P. cornucopiae*, which was approximately five times higher than that in *P. ostreatus*. It has been reported that *P. cornucopiae* fruiting bodies contain 394 (mg/100 g dry weight) of EGT (Kalaras et al., 2017), and the present results correspond to one-fifth of this value. DPPH radical scavenging ability (Table 3) was high on fermentation days with a large amount of EGT (Table 4). Since EGT is known to be a strong scavenger of DPPH *in vitro* (Fukuda et al., 2012; Katsube et al., 2022), DPPH radical scavenging activity was measured using standard EGT, yielding 4,698 (μmol TE/g). The contribution rate of EGT to the DPPH radical scavenging ability was estimated to be 40% in *P. cornucopiae* and 25% in *P. ostreatus* after 30 d of fermentation, which showed the maximum antioxidant activity and EGT amount. Park et al. (2010) reported that the DPPH and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activities of *Gastrodia elata* rhizome extracts was correlated with the EGT content of the extract, and Bhattacharya et al. (2014) reported that the

DPPH scavenging activity of *P. ostreatus* extracts was correlated with EGT content. In the comparison between *P. cornucopiae* and *P. ostreatus* fermented soybeans, differences were observed only in the DPPH radical scavenging ability and the amount of EGT, and both values were higher in *P. cornucopiae*. These results suggest that EGT contributes to the DPPH radical scavenging ability, similar to the above studies.

Moreover, the amount of polyphenols on the day 30 (6.00 [mmol GAE/100 g dry weight]), when the antioxidant activity was at maximum (953.7 [µmol TE/100 g dry weight]), was the same as on the day 40 of fermentation (6.31 [mmol GAE/100 g dry weight]), when the amounts of polyphenols was at maximum, whereas the antioxidant activity on the day 40 (341.7 [µmol TE/100 g dry weight]) was significantly lower than on the day 30 (Tables 2, 3). This indicates that the amount of polyphenols with antioxidant activity was high on the day 30, and that large amounts of polyphenols did not necessarily result in high antioxidant activity. The antioxidant activity of isoflavone aglycones is reportedly higher than that of their glycosides (Arora et al., 1998). In this study, the amount of aglycones on days 10 and 20 was almost at its maximum (Fig. 1B, C); however, the antioxidant activity on day 10 was less than half that on day 20 (Table 3), indicating that the contribution of aglycone to the antioxidant activity was low. Lee et al. (2019), Sameshima et al. (2019) reported that the increase in antioxidant activity may be due to an increase in the amount of isoflavone aglycones. In this study, it was suggested that the increase in antioxidant activity of soybeans fermented with mushrooms was influenced by the production of the antioxidant-active substance EGT. It was estimated that the contribution rate of EGT to the antioxidant activity was approximately 40% at the maximum in the soybeans on day 30 of *P. cornucopiae* fermentation, which had high antioxidant activity compared to the DPPH radical scavenging ability of the EGT standard. This suggests that the remaining antioxidant activity in mushroom-fermented soybeans involves the production of substances other than EGT, which exhibit multiple antioxidant activities. Because isoflavone aglycones and EGT have high antioxidant activity and are expected to be used as various physiologically functional materials, 30-day *P. cornucopiae*-fermented soybeans containing both substances will be expected as a functional food. In the future, it will be necessary to analyse the components of each fermented soybean and their physiological functionality.

Disclosure

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed.

Acknowledgements

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