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Metabolomics Revealed Novel Isoflavones and Optimal Cultivation Time of *Cordyceps militaris* Fermentation

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Germinated soybean (GS) cultivated with *Cordyceps militaris* (GSC) might be a promising efficacious source of novel bioactive compounds. In this study, the metabolome changes between GS and GSC were investigated by liquid chromatography−mass spectrometry (LC-MS) and gas chromatography−mass spectrometry (GC-MS) analysis coupled with a multivariate data set. Principal component analysis (PCA) and orthogonal projection to latent structures discriminate analysis (OPLS-DA) of GSC clearly showed higher levels of soyasaponin Bd, soyasaponin Bc(II), daidzein, genistein, four isoflavones (compounds **1**−**4**), glycerol, proline, glutamine, pentitol, fructose, inositol, octadecanoic acid, and sucrose together with lower levels of pyroglutamic acid, citric acid, histidine, and palmitic acid in GSC than in GS. The structures of compounds **1**−**4** were analyzed by mass and NMR spectroscopy and were determined to be novel isoflavone methylglycosides (daidzein 7-*O*-*β*-D-glucoside 4″-*O*-methylate (**1**), glycitein 7-*O*-*β*-D-glucoside 4″-*O*-methylate (**2**), genistein 7-*O*-*β*-D-glucoside 4″-*O*-methylate (**3**), and genistein 4′-*O*-*β*-D-glucoside 4″-*O*-methylate (**4**)). Multivariate statistical models showed that metabolic changes of GSC were maximal within 1 week after the *C. militaris* inoculation, consistent with the strongest antioxidant activity of GSC cultivated for 1 week. This metabolomics study provides valuable information in regard to optimizing the cultivation process for bioactive compound production and describes an efficient way to screen for novel bioactive compounds from GSC.

KEYWORDS: ***Cordyceps militaris*; soybean; LC-ESI-IT-MS; GC-EI-IT-MS; metabolomics**

INTRODUCTION

Fungal metabolites have been traditionally used in eastern Asia for health care in the prevention and treatment of human diseases (*1*, *2*). *Cordyceps*, which is one of the most well-known fungal traditional Chinese medicines (FTCM), has multiple pharmacological activities, and it is believed to cure various diseases (*2*). Previous research on *Cordyceps* has provided convincing experimental evidence that it possesses significant pharmacological activities (*3*). In addition, one of the most renowned medicinal fungi, *Cordyceps militaris*, has been reported to have a multitude of pharmacological properties (*4*, *5*).

C. militaris has been reported to display various biological activities such as anti-inflammatory (*5*), antifibrotic (*6*), and anticancer activities (*7*). Subsequently, several other studies have been conducted to examine the biological activities of *Cordyceps*. As a result of these additional studies, many other biologically active components have been reported such as polysaccharides, cyclic peptides, dipicolinic acidm and 10-membered macrolides (*8*−*11*). Although these components were biologically effective, obtaining large amounts of nutrients from *C. militaris* is not easy because of its high production cost and long incubation time. However, recent progress in the investigation of submerged fermentation of *C. militaris* in mixed natural culture has been made by growing *C. militaris* on germinated soybeans known to be rich in nutrients and biologically active compounds (*8*, *12*).

Recently, there has been a worldwide increase in the consumption of soybeans due to the beneficial properties of its components such as phenolic acid, flavonoids, and lignins (*13*, *14*). Many research groups have reported that these nutritional components in soybean were associated with human health benefits such as decreased risks of various cancers, heart disease, cardiovascular disease, and increased antioxidative effects (*15*−*17*). In this regard, germinated soybeans cultivated with *C. militaris* (GSC) might provide a source of novel nutraceutical compounds that reflect the biologically effective components of the two materials.

However, metabolomic studies of GSCs have not been reported to date. Metabolomics is an emerging and rapidly developing science and technology that includes a comprehensive experimental analysis of metabolite profiles, either as targeted compounds or as global metabolites (*18*). Mass spectrometry (MS) has been frequently used in earlier studies to detect and quantify the metabolome (*19*). Among the different mass spectrometry methods, LC-MS has been shown to be a powerful tool for

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metabolomic study not only because it gives information on analytes but also because it detects a broad group of metabolites. However, it is hard to detect primary or highly polar metabolites such as sugars, sugar-phosphates, sugar-alcohols, organic acids, and amino acids (*19*). Therefore, to obtain more information on metabolites, LC- and GC-MS analyses has been used to investigate metabolic changes in GSC caused by the cultivation time of *C. militaris*. Typically, in metabolomic studies, the data sets were hard to summarize and visualize without multivariate statistical analysis tools (*20*). In the present study, principal component analysis (PCA) and orthogonal partial least-squares to latent structure discriminant analysis (OPLS-DA) models were employed to identify metabolites that had significantly changed as a function of GSC.

In this paper, we determined the changes in antioxidant activity and metabolites as a function of GSC cultivation time, using LC-ESI-IT and GC-EI-IT-MS coupled with multivariate analyses. The information provided in this study will prove to be valuable in regard to optimizing the cultivation process of GSC for bioactive compound production of GSC. In addition, several novel isoflavone methyl-glucosides were detected in GSC, and those structures were identified by 1D and 2D NMR spectroscopy.

MATERIALS AND METHODS

Chemicals. HPLC-grade acetonitrile and water were supplied by Burdick and Jackson (Muskegon, MI), and analytical grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO). NaOH, $AlCl_3$, sodium carbonate, gallic acid, Folin−Ciocalteu's phenol reagent, potassium persulfate, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2′-azinobis(2-methyl-propionamidine) dihydrochloride (AAPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-*s*-triazine (TPTZ), $FeCl_3 \cdot 6H_2O$, $C_2H_3NaO_2 \cdot 3H_2O$, dimethyl sulfoxide (DMSO), pyridine, methoxyamine hydrochloride, and *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) were also obtained from Sigma-Aldrich.

Preparation of GSC Extracts. *C. militaris* was grown on GSCs as previously described (*8*). Briefly, the mycelium of *C. militaris* was inoculated on germinated soybeans and cultured at 20−25 °C for 8 weeks, and samples were collected once each week. Each cultured material (5 g) and soybean were ground and extracted with 80% MeOH for 48 h under reflux. The collected extracts were filtered, and the residue was extracted in the same manner. After three extractions, all of the combined extracts were concentrated to dryness in a rotary evaporator at 30 °C.

Determination of Total Polyphenol Contents (TPC) and Total Flavonoid Contents (TFC). TPC was measured using the Folin−Ciocalteu method (*21*). Briefly, 10 μL of each GSC crude extract was added to 0.2 N Folin−Ciocalteu phenol reagent (160 μL) in 96 wells. After 3 min, 30 μL of a saturated sodium carbonate solution was added to the mixture and then incubated at room temperature for 1 h. The resulting mixture's absorbance was measured at 750 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT). TPC was calculated on the basis of a standard curve with gallic acid. The standard solution concentrations ranged from 12.5 to 400 μg/mL. Results were expressed in milligrams of gallic acid equivalents (GAE) per gram of 80% methanol extracts.

TFC was measured using a colorimetric assay described previously (*22*) with a slight modification. Twenty microliters of the samples or standard solutions of narigin was added to each well on a 96-well plate. The standard solution concentrations ranged from 100 to 800 μg/mL. Distilled water (40 μL) and 6 μL of 5% (w/v) sodium nitrite were added to each well. After 5 min, 12 μL of 10% (w/v) $AlCl_3$ was added, and after 6 min, 40 μL of 1 M NaOH was also added to the mixture, followed by the addition of 42 μL of distilled water. Absorbance was measured at 515 nm, and the flavonoid content was expressed as milligrams of naringin equivalents per gram of 80% methanol extracts. Experiments were carried out in triplicate.

Determination of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Free Radical Scavenging Activity. The free radical scavenging activity of GSC crude extracts was measured using a previously described protocol (*23*). Decoloration of the solution indicated the scavenging efficiency of the added samples. For each crude extract, 20 μL of a 10 mg/mL solution in 80% methanol was added to 180 μL of a DPPH solution (200 μM) and incubated at 37 °C for 30 min. The absorbance was then measured at 517 nm on a microplate reader (Bio-Tek Instruments), and the percent inhibition was determined by comparison with the 80% methanol treated control groups. Results were expressed in milligrams of vitamin C equivalent concentration per gram of 80% methanolic extracts from GSCs. The concentration of the standard solutions ranged from 0.156 to 2.5 mM. Experiments were carried out in triplicate.

Determination of Total Antioxidant Capacity by Stable 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) Diammonium Salt (ABTS) Radical Caution. The antioxidant capacity was represented as the vitamin C equivalent antioxidant capacity (VCEAC) (*24*). AAPH (1.0 mM) was mixed with 2.5 mM ABTS as a diammonium salt in a phosphate-buffered saline solution (0.1 M potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl) and heated at 70 °C for 45 min. The concentration of the resulting blue-green ABTS radical solution was calibrated to an absorbance of 0.650 ± 0.020 (mean ± SD) at 734 nm. After 20 μL of each crude extract solution (10 mg/mL dissolved in 80% methanol) had been added to 180 μL of the ABTS radical solution, the samples were incubated in darkness at 37 °C for 20 min, and the decrease in absorbance at 734 nm was measured using a microplate reader (Bio-Tek Instruments). Twenty microliters of 80% methanol and 180 μL of the ABTS solution were used as the control. The ABTS radical scavenging capacities of GSCs were presented on an extract weight basis as milligrams of vitamin C equivalent antioxidant capacity per 100 g (VCEAC). The vitamin C standard curves at 1.6, 3.1, 6.25, 12.5, 25, and 50 μM concentrations of L-ascorbic acid were obtained using the ABTS solution.

Ferric Reducing Ability of Plasma (FRAP) Assay. The antioxidant capacity of the crude extracts from GSCs was determined using a modified version of the FRAP assay developed by Benzie and Strain (*25*). Briefly, the FRAP reagent was prepared from 300 mmol/L acetate buffer, pH 3.6, 20 mmol/L ferric chloride, and 10 mmol/L 2,4,6-tripyridyl-*s*-triazine made up in 40 mmol/L hydrochloric acid. Afterward, 300 μL of the FRAP reagent heated to 37 °C was mixed with 10 μL of test sample (dissolved in 80% methanol), and the 80% methanol solution was used as the reagent blank. The absorbance was measured at 593 nm after the sample was allowed to react at room temperature for 6 min. Results were expressed in milligrams of vitamin C equivalents concentration per gram of 80% methanolic extracts from GSCs.

Liquid Chromatography Coupled with Electrospray Ionization and Ion Trap Tandem Mass Spectrometry (LC-ESI-IT-MS/MS) Analysis. The dried extracts were dissolved in MeOH (Burdick and Jackson) and analyzed by LC-ESI-IT-MS/MS. The analyzed data were obtained via either direct infusion or liquid chromatographic introduction into a Varian 500-MS equipment (Varian Inc., Palo Alto, CA), which consisted of a 212-LC binary gradient solvent delivery pump, a ProStar 335 photodiode array detector, a ProStar 410 autosampler, and a 500-ion trap mass spectrometer. Metabolite profiling of the various extracts from GSCs was achieved using a Chromosep SS C18 column [150 × 2.0 mm i.d., 5 μm (Varian)] at a flow rate of 0.2 mL/min. Mobile phases A and B were high-purity water and acetonitrile, respectively, both containing 0.1% formic acid. Gradient elution was conducted as follows: 0−50 min for 5−100% B with a linear gradient, followed by 50−55 min of 100% B. Afterward, 5% B was flowed for 55−60 min. A photodiode array was used to continuously record the absorbance from 200 to 600 nm for metabolite identification. The chromatographic mass spectrometry determination was performed with an electrospray source in positive and negative ionization modes over the range of m/z 50−1000. The needle voltage was set to 4000 V and −4000 V, the spray shield voltage was set to 600 V and −600 V, the capillary voltage was set to 80 V, and the spray chamber temperature was set at 50 °C. High-purity nitrogen (N_2) was used as the drying gas, and its pressure and temperature were set to 20 psi and 350 °C, respectively. High-purity helium (He) was used as the damping gas, and its flow rate was set to 1 mL/min. High-purity nitrogen (N_2) was also used as the nebulizer gas in the positive ion mode, but a mixed gas of nitrogen and air at a pressure of 40 psi was used in the negative ion mode. MS^n was analyzed using Turbo DDS, data-dependent scanning for the 500-MS system, under the same conditions used for full metabolite scanning.

Sample Derivatization for GC-MS Analysis. The soybean and GSC extracts were dried in a freeze-dryer until the extract was completely dry (for 24 h). The lyophilized extracts were derivatized in two steps to

Table 1. Effect of Cultivation Time on Antioxidant Activity and Total Flavonoid and Total Phenolic Contents of GSCs[a]

cultivation time (weeks)	ABTS (mg of VCE/g)	DPPH (mg of VCE/g)	FRAP (mg of VCE/g)	TFC (mg of NAE/g)	TPC (mg of GAE/g)
soybean	1.32 ± 0.31a	0.05 ± 0.01 a	4.14 ± 0.30 a	3.21 ± 0.01 b	10.34 ± 0.28 a
1	3.46 ± 0.21 b	0.58 ± 0.02 bcd	8.64 ± 0.44 cd	4.36 ± 0.01 a	24.25 ± 0.63 bcd
2	2.30 ± 0.28 c	0.54 ± 0.02 b	9.79 ± 0.58 d	2.67 ± 0.01 c	23.92 ± 0.60 bc
3	2.09 ± 0.18 bc	0.61 ± 0.02 de	8.04 ± 0.56 bc	1.96 ± 0.01 d	23.38 ± 0.82 b
4	2.12 ± 0.39 bc	0.64 ± 0.03 e	9.16 ± 0.81 de	1.61 ± 0.01 fg	23.92 ± 0.34 bc
5	1.95 ± 0.45 b	0.63 ± 0.03 de	8.57 ± 0.65 cd	1.52 ± 0.01 h	23.68 ± 0.47 bc
6	1.88 ± 0.31 b	0.59 ± 0.03 cd	9.43 ± 0.67 d	1.63 ± 0.01 f	23.89 ± 0.24 bc
7	1.80 ± 0.43 b	0.62 ± 0.03 de	7.91 ± 1.00 bc	1.68 ± 0.01 e	24.68 ± 0.75 cd
8	1.83 ± 0.24 b	0.55 ± 0.01 bc	7.30 ± 0.84 b	1.63 ± 0.01 g	25.03 ± 0.72 d

[a] Data are expressed as means of triplicate experiments on a dry weight basis. Values marked by the same letters within each sample in each column are not significantly different ($p < 0.05$).

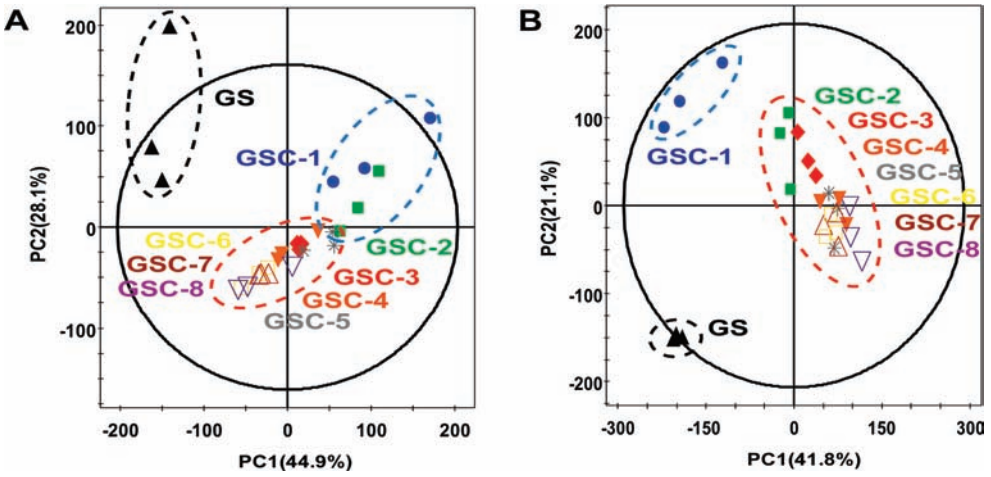

Figure 1. Principal component analysis (PCA) score plots derived from LC-MS (**A**) and GC-MS (**B**) data sets of germinated soybean (GS) and *Cordyceps militaris* grown on germinated soybeans (GSCs) extracted with 80% MeOH. These plots show that the metabolomes were clearly different and depended on the cultivation time of *C. militaris*. The numbers indicate the cultivation time in weeks.

protect carbonyl function. First, dried samples were dissolved in 100 μL of 20 mg/mL solution of methoxyamine hydrochloride in pyridine (Sigma, St. Louis, MO) and reacted at 60 °C for 60 min. Afterward, to increase the volatility of the polar compounds, acidic protons were exchanged against the trimethysilyl group using 100 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA, Sigma) at 70 °C for 60 min.

Gas Chromatography Coupled with Electron Impact and Ion Trap Tandem Mass Spectrometry (GC-EI-IT-MS) Analysis. Each 1 μL of sample was analyzed via GC-EI-IT-MS, which consisted of a CP-3800 gas chromatograph coupled to a 4000 ion trap mass spectrometer equipped with a CP-8400 autosampler (Varian). The samples were vaporized at 250 °C in standard split mode (1:25) and separated on a 30 m × 0.25 mm VF-1MS capillary column coated with a 0.25 μm low bleed, FactorFour column (Varian). The inlet temperature and transfer line temperature were set to 250 and 280 °C, respectively. The oven temperature was set to 100 °C for 2 min, then increased to 300 at 10 °C/min, and held at 300 °C for 10 min. Helium carrier gas (purity >99.999%) flow rate was adjusted to 1 mL/min. The interface and ion source temperatures were set at 200 °C, and electron impact ionization (70 eV) was utilized. The scan average was set to 3 microscans and full scanning with a range of m/z 50−1000. Metabolites were identified by comparison to the NIST 2005 database (version 2.0, FairCom Co., Columbia, MO).

Data Processing. Data preprocessing was performed with Varian MS Workstation 6.9 software. The LC-MS raw data files were converted into netCDF (*.cdf) format with Vx Capture (version 2.1, Laporte, MN) for further analysis. After conversion, automatic peak detection and alignment were performed by XCMS. R-program version 2.9.0 (The R project for statistical computing (www.r-project.org) and XCMS version 1.16.3 were used). The XCMS parameters for the R language were performed by simple commands as XCMS's default settings with the following: http://masspec.scripps.edu/xcms/documentation.php. Also, GC-MS data files were converted into netCDF (*.cdf) format for further analysis. For automated baseline correction, mass spectra extraction, and subsequent spectral data alignment, GC-MS data sets were processed simultaneously using the dedicated MetAlign software package (http://www.metalign.nl). With mass/retention time pairs, the corresponding peaks were confirmed in the original chromatogram and compared to the NIST mass spectral database (National Institute of Standards and Technology, FairCom Co.).

Multivariate Statistical Analysis. Statistical analyses were performed on all continuous variables using the SIMCA-P+ (version 12.0, Umetrics, Umeå, Sweden). Univariate statistics were performed by breakdown and one-way ANOVA using SPSS (version 15.0, SPSS Inc., Chicago, IL) and Statistica (version 7.0, StatSoft Inc., Tulsa, OK). Unsupervised principal component analysis (PCA) was run to obtain a general overview of the variance of metabolites, and supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) was performed to obtain information on differences in the metabolite composition of the samples. The ellipse that was shown in the PCA score plots of the models defines the 95% confidence interval of the modeled variations (*26*). The quality of the fitting model can be explained by R^2x and Q^2 values. R^2x displays the variance explained in the model and indicates the goodness of fit. Q^2 displays the variance in the data predictable by the model and indicates the predictability (*26*). Biomarkers for differences between soybean and GSCs were subsequently identified by analyzing the loading S-plot of OPLS-DA, which was declared with the covariance (p) and correlation ($pcorr$). All variables were mean centered and scaled to Pareto for both PCA and OPLS-DA of the metabolites from LC and GC-MS in a column-wise manner. The correlation coefficient at different cultivation times was determined using Statistica.

Isolation and Structural Analysis of New Isoflavone Methylglycosides. The mycelium of GSC cultivated for 1 week (50 g) was ground and extracted with 80% MeOH. The methanol solution was evaporated to dryness and suspended in high-purity distilled water (500 mL) and then extracted using EtOAc (500 mL × 3). The extracts

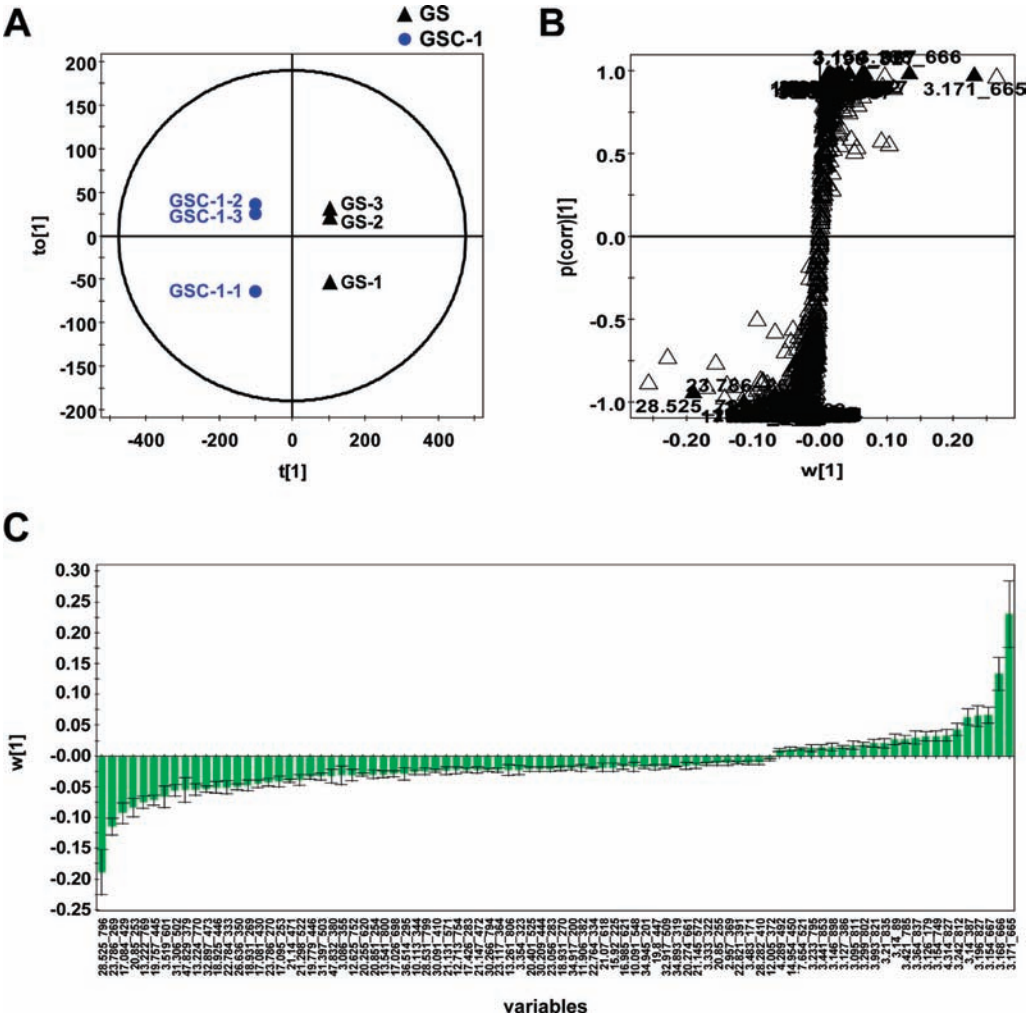

Figure 2. OPLS-DA score plots (**A**) and loading S-plots (**B**) derived from LC-MS data set of GS and GSC1 extracted with 80% MeOH. The S-plot shows the covariance *w* against the correlation *pcorr* of the variables of the discriminating component of the OPLS-DA model. A loading plot with jack-knifed confidence intervals (**C**) was used for metabolites that were significantly different between GS and GSC1. A cutoff value of $p < |0.01|$ was used. The selected variables are highlighted in the S-plot with retention time and *m*/*z* (▲). The last numbers indicate the sample numbers.

Table 2. Significantly Different Metabolites between GS and GSCs Identified by LC-MS

no.	t_R (min)	$[M - H]^-$ (*m*/*z*)	MS^n fragment ions (*m*/*z*)	tentative identification	ref
1	17.08	429	429, 252 ≫ 223191, 169	compound **1**	
2	17.42	459	459, 283 ≫ 249, 175	compound **2**	
3	18.92	445	445, 268 ≫ 240, 223, 211, 195, 179, 167	compound **3**	
4	19.76	445	445, 269 ≫ 240, 223, 211, 195, 179, 167	compound **4**	
5	20.85	253	253 ≫ 223, 209, 197, 167, 135	daidzein	(*29*)[a]−(*32*)
6	21.14	473	473, 268 ≫ 240, 223, 211, 195, 179	genistein *O*-hexoside acetylated	(*29*)[a]−(*32*)
7	22.82	957	957 ≫ 895, 795, 733, 597, 525, 457	soyasaponin Bd	standard[b]
8	23.78	269	269 ≫ 225, 213, 201, 181, 169, 133, 107	genistein	(*29*)[a]−(*32*)
9	28.53	912	912 ≫ 849, 703, 615, 525, 457	soyasaponin Bc(II)	standard

[a] Identified by an in-house library. [b] Identified by direct comparison with standard.

appeared as brown syrup (20 g) upon concentration. The sample was then fractionated using silica gel column chromatography (50 × 4.3 cm i.d.) in a stepwise gradient solvent system comprising 800 mL each of chloroform/methanol mixtures (99:1, 98:2, 96:4, 93:7, 86:14, 76:24, 60:40 v/v). Fraction 5 was concentrated and loaded onto a Sephadex LH-20 column (120 × 3.3 cm i.d.) in a solution that contained a methanol/distilled water ratio of 8.5:1.5. Ten milliliter fractions were collected separately, and the final purification of compounds **1**−**4** was accomplished via HPLC (YMC pack-pro C_{18} column) using an isocratic aqueous acetonitrile solvent system. Each purified compound was dissolved in CD_3OD and analyzed by 1D NMR (^1H (500 MHz) and ^{13}C (125 MHz) NMR, Varian Unity-500) and 2D NMR (HMBC, Varian Unity-500) techniques.

RESULTS AND DISCUSSION

Antioxidant Activities and Total Polyphenolic and Flavonoid Contents of GSCs. To determine the differences in the metabolite composition between soybean and cultivated GSCs, the TFC and TPC were investigated. As summarized in **Table 1**, a significant difference ($p < 0.05$) in TFC and TPC was observed in all of the germinated soybeans cultivated with *C. militaris* (GSC). In particular, the TFC content in GSC, which was cultivated for 1 week, was higher than that of soybean germinated only (GS), and it decreased significantly with cultivation time. The amounts

Table 3. NMR and MS Data of Compounds **1**−**4**

(**A**) ***m/z***

mode	1	2	3	4
HRFAB (positive)	431.1406 $(M + H)^+$	461.1483 $(M + H)^+$	447.1324 $(M + H)^+$	447.1291 $(M + H)^+$
ESI (positive)	431 $(M + H)^+$, 453 $(M + Na)^+$	461 $(M + H)^+$, 483 $(M + Na)^+$	447 $(M + H)^+$, 469 $(M + Na)^+$	447 $(M + H)^+$, 469 $(M + Na)^+$
ESI (negative)	429 $(M - H)^-$	459 $(M - H)^-$	445 $(M - H)^-$	445 $(M - H)^-$

(**B**) **^{1}H and ^{13}C NMR Data**[a]

	^{1}H				^{13}C				DEPT				HMBC			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
2	8.19 s	8.18 s	8.07 s	8.07 s	155.1	155	155.4	153.5	CH	CH	CH	CH	C_3, C_4, C_9, $C_{1'}$	C_3, C_4, $C_{1'}$	C_3, C_4, C_9, $C_{1'}$	C_3, C_4, C_9, $C_{1'}$
3					124.2	124.3	123.2	122.7	C	C	C	C				
4					178.2	177.9	182.6	180.4	C	C	C	C				
5	8.14 d, J = 7.5 Hz	7.59 s			128.4	106.2	163.7	162.3	CH	CH	C	C	C_4, C_7	C_4, C_6, C_7, C_9		
6	7.20 dd, J = 9.0, 2.5 Hz		6.50 d, J = 2.5 Hz	6.19 d, J = 2.5 Hz	117.1	149.5	101.2	99.3	CH	C	CH	CH	C_7, C_8, C_{10}		C_7, C_8, C_{10}	C_8, C_{10}
7					163.5	153.6	164.8	166.2	C	C	C	C				
8	6.31 d, J = 2.5 Hz	7.29 s	6.68 dd, J = 2.5 Hz	6.31 dd, J = 2.0 Hz	105.0	105.3	95.9	93.8	CH	CH	CH	CH	C_6, C_7, C_9, C_{10}	C_6, C_7, C_9, C_{10}	C_6, C_7, C_9, C_{10}	C_6, C_7, C_9, C_{10}
9					159.3	153.5	159.3	158.4	C	C	C	C				
10					120.3	119.8	108.1	104.4	C	C	C	C				
1′					126.3	125.7	125.1	125	C	C	C	C				
2′	7.38 dd, J = 8.0, 2.0 Hz	7.39 dd, J = 9.0, 2.0 Hz	7.39 dd, J = 8.5, 2.0 Hz	7.48 dd, J = 8.0, 2.0 Hz	131.5	131.5	131.5	129.8	CH	CH	CH	CH	$C_{1'}$, $C_{4'}$	$C_{1'}$, $C_{4'}$	$C_{1'}$, $C_{4'}$	C_3, $C_{4'}$
3′	6.86 dd, J = 7.5, 2.0 Hz	6.86 dd, J = 8.5, 2.0 Hz	6.86 dd, J = 8.5, 2.0 Hz	7.15 dd, J = 7.5, 2.0 Hz	116.4	116.4	116.4	116.2	CH	CH	CH	CH	$C_{1'}$, $C_{4'}$	C_3	$C_{1'}$, $C_{4'}$	$C_{1'}$, $C_{4'}$
4′					158.9	158.8	159	157.6	C	C	C	C				
5′	6.86 dd, J = 7.5, 2.0 Hz	6.86 dd, J = 8.5, 2.0 Hz	6.86 dd, J = 8.5, 2.0 Hz	7.15 dd, J = 7.5, 2.0 Hz	116.4	116.4	116.4	116.2	CH	CH	CH	CH	$C_{1'}$, $C_{4'}$	C_3	$C_{1'}$, $C_{4'}$	$C_{1'}$, $C_{4'}$
6′	7.38 dd, J = 8.0, 2.0 Hz	7.39 dd, J = 9.0, 2.0 Hz	7.39 dd, J = 8.5, 2.0 Hz	7.48 dd, J = 8.0, 2.0 Hz	131.5	131.5	131.5	129.8	CH	CH	CH	CH	$C_{1'}$, $C_{4'}$	$C_{1'}$, $C_{4'}$	$C_{1'}$, $C_{4'}$	C_3, $C_{4'}$
1″	5.09 d, J = 7.5 Hz	5.12 d, J = 7.5 Hz	5.03 d, J = 7.5 Hz	4.93 d, J = 7.5 Hz	101.7	101.8	101.8	100.6	CH	CH	CH	CH	C_7	C_7	C_7	$C_{4'}$
2″	3.54 t	3.54 t	3.48 t	3.48 t	75	74.8	74.8	73.6	CH	CH	CH	CH	$C_{3''}$, $C_{1''}$	$C_{3''}$, $C_{1''}$	$C_{3''}$, $C_{1''}$	$C_{3''}$, $C_{1''}$
3″					78	77.9	78	76.5	CH	CH	CH	CH	$C_{7'}$	$C_{7'}$	$C_{7'}$	$C_{7'}$
4″	3.21 t	3.21 t	3.19 t	3.19 t	80.6	80.6	80.6	79.1	CH	CH	CH	CH	$C_{7''}$, $C_{5''}$	$C_{7''}$	$C_{7''}$, $C_{5''}$	$C_{7''}$
5″					77.5	77.5	77.5	75.8	CH	CH	CH	CH				
6″					62.1	62.1	62.1	60.6	CH_2	CH_2	CH_2	CH_2				
CH_3(7″)	3.59 s	3.59 s	3.59 s	3.59 s	61	61	61	61	CH_3	CH_3	CH_3	CH_3	$C_{4''}$	$C_{4''}$	$C_{4''}$	$C_{4''}$
6-OCH_3		3.94 s				56.9				CH_3				C_6		

[a] s, singlet; d, doublet; dd, double-doublet; t, triplet; m, multiplet.

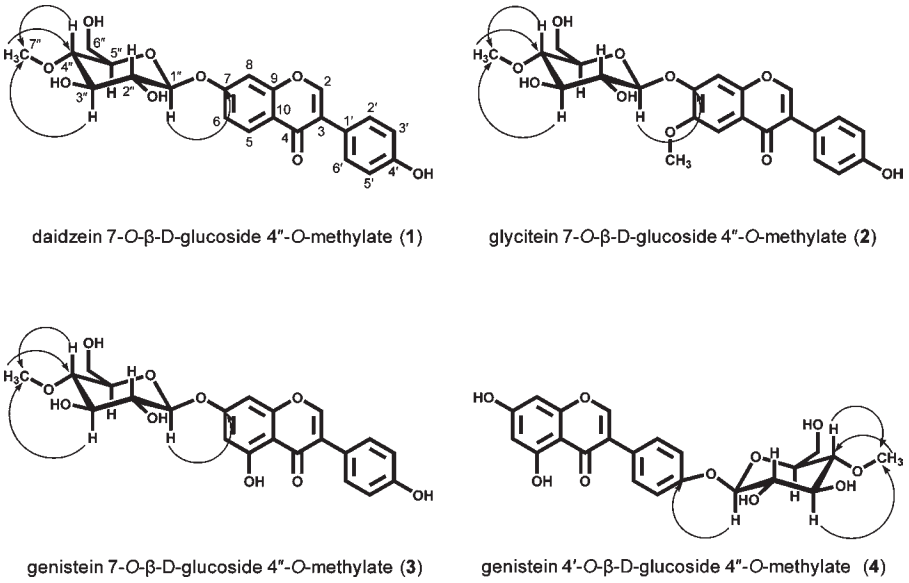

Figure 3. Structures of compounds **1**−**4** and HMBC correlations.

Table 4. Significantly Different Metabolites between Soybean and GSCs Identified by GC-MS

no.	name	t_R (min)	MS fragment ions (*m*/*z*)	derivatized[a]	*p* value
1	glycerol	6.53	218, 205, 147, 117, 103, 73	TMS(×3)	<0.005
2	proline	6.81	244, 216, 142, 73	TMS(×2)	<0.001
3	pyroglutamic acid	9.36	258, 230, 156, 147, 73	TMS(×2)	<0.001
4	glutamine	9.91	348, 320, 246, 147, 128, 73, 56	TMS(×3)	<0.005
5	pentitol	10.78	319, 231, 205, 147, 73, 69	TMS(×4)	<0.005
6	citric acid	12.77	465, 375, 348, 274, 211, 147, 73	TMS(×4)	<0.001
7	fructose	13.53	364, 307, 277, 217, 147, 73	TMS(×5)	<0.001
8	histidine	13.63	356, 254, 217, 154, 73	TMS(×3)	<0.001
9	inositol	14.48	612, 507, 318, 266, 218, 147, 73	TMS(×6)	<0.001
10	palmitic acid	14.78	328, 313, 145, 129, 117, 75	TMS(×1)	<0.001
11	octadecanoic acid	16.52	357, 341, 201, 129, 117, 73	TMS(×1)	<0.001
12	sucrose	21.80	361, 305, 217, 204, 147, 73	TMS(×8)	<0.005

[a] Number of hydrogen atoms derivatized.

of TPC in the GSC extracts were found to be similar, but were higher than the amount of TPC in GS. The differences in antioxidant activities of GS and GSCs were also investigated using the ABTS, DPPH, and FRAP assays. On the basis of the results of these three different assays, the antioxidant activities of GSCs were significantly higher than those of soybean except for the DPPH radical scavenging activity. However, the antioxidant activities of all samples decreased at longer cultivation times (**Table 1**). The antioxidant activities in raw soybeans have recently been reported, and it was shown that TFC and TPC are the most important factors affecting the antioxidant activity of soybean (*27*). Therefore, amount of TFC in GSC is important to have maximum antioxidant activity.

Multivariate Statistical Analysis of GS and GSCs Derived from LC and GC-MS Data Set. To determine significant differences in metabolite compositions between GS and GSCs and variations in compositions as a function of cultivation time, metabolites were profiled for 27 samples (3 soybean samples and 3 sets of GSCs that were collected for 8 weeks at weekly intervals) using mass spectrometry analysis combined with multivariate statistics. From the 27 samples, 897 peaks were extracted by XCMS from the LC-MS data set and 1153 peaks were extracted by metAlign from the GC-MS data set, respectively. The preliminary differences between GS and GSCs were assessed by the unsupervised PCA method for the entire data set. The PCA score plot helps to determine in what aspect one sample is different from another, and the loading plot shows which variable contributes the most to this difference (*28*). As shown in **Figure 1**, PCA score plots derived from LC- and GC-MS data sets explained 73 and 62.9% of the total variance (R^2), respectively. The predictability (Q^2) of each data set was 55.9 and 47.1%, respectively. According to the score plot derived from the LC-MS data set, GS and GSCs were clearly separated by PC1 (44.9%) and variation among the GSCs as a function of cultivation time was discriminated by PC2 (28.1%) (**Figure 1A**). Also, the score plots of GS and GSCs derived from the GC-MS data set were clustered into different regions and separated by PC2 (21.1%). In addition, GSCs were divided depending on the cultivation time in compliance with PC1 (41.8%) (**Figure 1B**).

As shown in these two score plots, GSCs were separated from GS and divided according to cultivation time. The result of the PCA score plots suggested that the compositions of metabolites in GSCs changed during the early fermentation process and changed slowly from 3 to 8 weeks.

Identification of Significantly Different Metabolites between GS and GSC by LC-MS Analysis. To clearly identify compounds that discriminated GS and GSCs, OPLS-DA models were applied to the LC-MS data set. This model was constructed with data from GS and GSC that had been cultivated for a week. The model showed one orthogonal component that was explained with $R^2 = 0.691$, $Q^2 = 0.996$ (**Figure 2A**). The periodic metabolite changes in *C. militaris* cultivation were subsequently analyzed using

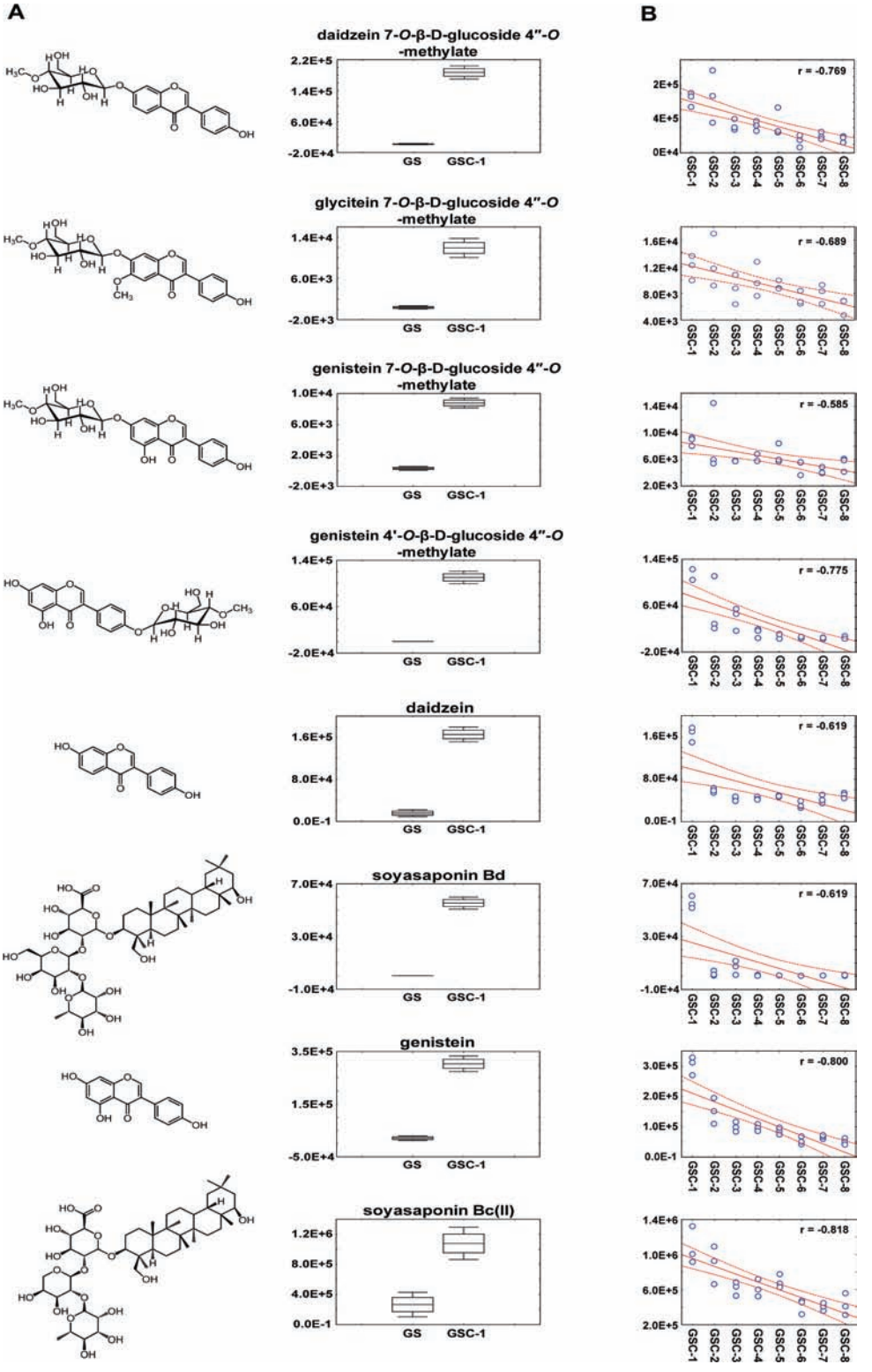

Figure 4. (**A**) Box and whisker plot of compounds that were significantly different between GS and GSCs cultivated for 1 week, which were identified with LC-MS. (**B**) Significant compounds associated with cultivation time.

loading S-plots, which were represented with covariance (*w*) against correlation (*pcorr*) (*20*). The S-plots of the OPLS-DA were used to identify potential biomarkers of group separation (*20*). This can be used to identify variables that most significantly differentiate closely related samples. The indicators in the loading S-plot representing the metabolites with a cutoff value of $p < 0.01$ were used (**Figure 2B**). To avoid overinterpretation of the S-plots, only metabolites having variables showing a jack-knifed confidence interval were investigated (**Figure 2C**). Significantly different metabolites were identified using an in-house database (*29*), cochromatography, and the MS/MS spectral data of the authentic compounds available and/or by comparison with published literature (*30*−*32*).

The most important variables that contributed to this difference are summarized in **Table 2**. Genistein, daidzein, genistein *O*-hexoside acetylated, soyasaponin Bd, soyasaponin Bc(II), and

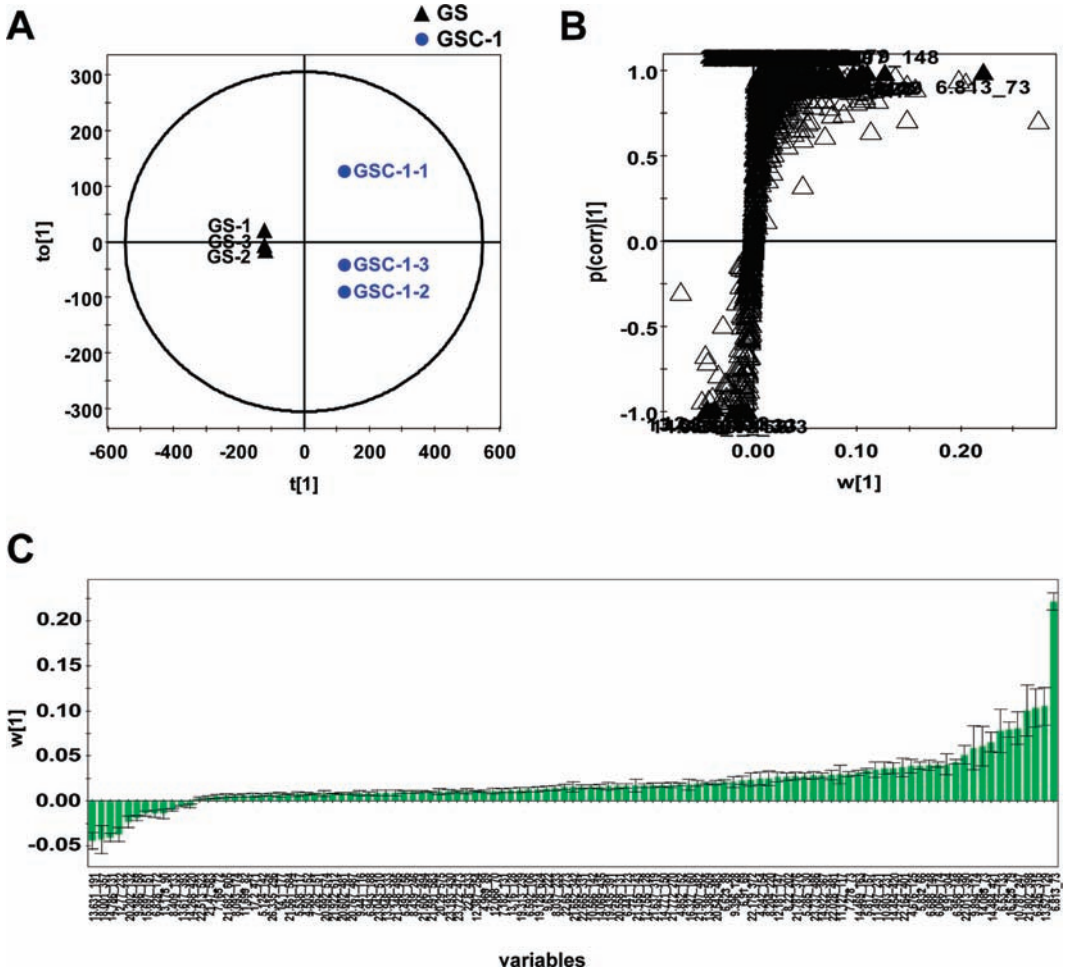

Figure 5. OPLS-DA score plots (**A**) and loading S-plots (**B**) derived from GC-MS data set of GS and GSC1 extracted with 80% MeOH. The S-plot shows the covariance *w* against the correlation *pcorr* of the variables of the discriminating component of the OPLS-DA model. Loading plot with jack-knifed confidence intervals (**C**) was used to select the metabolites that were significantly different between GS and GSC1. A cutoff value of $p < |0.001|$ was used. The variables are highlighted in the S-plot with retention time and *m*/*z* (▲). The last numbers indicate the sample numbers.

several novel compounds **1**−**4** were detected as significant variables between GS and GSC1-M. According to previous studies, the total contents of isoflavone glycosides were decreased, but those of isoflavone aglycones, including daidzein, glycitein, and genistein, showed a dramatic increase during fermentation of soybean (*33*). Thus, it is likely that the different amounts of genistein and daidzein in GSC1-M were due to the cultivation of *C. militaris*.

Structural Identification of Compounds 1−**4.** Compounds **1**−**4** were purified according to the methods described under Materials and Methods. The molecular formula of compounds **1**−**4** were determined as $C_{22}H_{22}O_9$, $C_{23}H_{24}O_{10}$, $C_{22}H_{22}O_{10}$, and $C_{22}H_{22}O_{10}$ from HRFAB-MS data at *m*/*z* 431.1406, 461.1483, 447.1324, and 447.1291, indicating a $[M + H]^+$, and also the ESI-MS negative ion detected at *m*/*z* 429, 459, 445, and 445, respectively (**Table 3B**). As summarized in **Table 3A**, the proton and carbon NMR spectra of compounds **1**−**4** exhibited the same characteristic patterns of daidzin, glycitin, and genistin (*34*−*36*), with a variation only in the glucosyl moiety of compound **4**. In comparison, compounds **1**−**3** differed from compound **4**, where the latter had a glucose molecule bound to the 4′-position of the B ring. The HMBC correlations from H-1″ to C-7 (**1**−**3**) and from H-1″ to C-4′ (**4**) proved that the glucose molecules were connected by 1,7-glycosidic linkages in compounds **1**−**3** and by a 1,4′-glycosidic linkage in compound **4**, respectively. In addition, the anomeric proton H-1″ was coupled to H-2″ (J = 7.5 Hz), indicating that the compounds were β-D-glucopyranosides (*37*). In addition, in all of the compounds one more methylene proton and carbon peak were detected at the same chemical shift, δ_H (CD_3OD) 3.59 (3H, s) and δ_C (CD_3OD) 61 (C-7″). The HMBC correlations from H-3″,-4″ to C-7″ and from H-7″ to C-4″ confirmed the position of the methylene, which was bound at C-4″ through an O-methyl linkage to the glucose molecules.

On the basis of this structural analysis, compounds **1**−**4** were determined to be daidzein 7-*O*-β-D-glucoside 4″-*O*-methylate (**1**), glycitein 7-*O*-β-D-glucoside 4″-*O*-methylate (**2**), genistein 7-*O*-β-D-glucoside 4″-*O*-methylate (**3**), and genistein 4′-*O*-β-D-glucoside 4″-*O*-methylate (**4**) (**Figure 3**), respectively.

Metabolite Difference Dependent on Cultivation Time in LC-MS Analysis. To clearly interpret the cultivation time dependent metabolite change, the mass peak intensities of metabolites were further analyzed using box and whisker plots and correlation coefficients. As displayed in **Figure 4A**, GSC1-M contained significantly higher amounts of daidzein 7-*O*-β-D-glucoside 4″-*O*-methylate, glycitein 7-*O*-β-D-glucoside 4″-*O*-methylate, genistein 7-*O*-β-D-glucoside 4″-*O*-methylate, genistein 4′-*O*-β-D-glucoside 4″-*O*-methylate, genistein, daidzein, soyasaponin Bd, and soyasaponin Bc(II) than GS (t test, $p < 0.01$). In addition, levels of certain compounds, daidzein 7-*O*-β-D-glucoside 4″-*O*-methylate ($r = -0.769$), glycitein 7-*O*-β-D-glucoside 4″-*O*-methylate ($r = -0.689$), genistein 7-*O*-β-D-glucoside 4″-*O*-methylate ($r = -0.585$), genistein 4′-*O*-β-D-glucoside 4″-*O*-methylate ($r = -0.775$),

daidzein ($r = -0.619$), soyasaponin Bd ($r = -0.619$), genistein ($r = -0.800$), and soyasaponin Bc(II) ($r = -0.818$), in GSCs were significantly decreased at increasing cultivation time of GSCs up to 8 weeks (**Figure 4B**). As mentioned above, the amount of TFC and ABTS radical scavenging activity in GSCs also significantly decreased with cultivation time. Therefore, different amounts of the compounds dependent on the cultivation time and between GS and GSCs can explain why GSCs had a high content of total flavonoids and high antioxidant activity. On the basis of these combined results, the optimum cultivation time for maximum bioactive compound production and biological activity of GSCs appears to be 1 week after inoculation.

Identification of Significantly Different Metabolites between GS and GSC by GC-MS Analysis. As shown in the PCA score plot (**Figure 1B**), clear differentiation was observed between GS and GSCs, revealing clear metabolic differences derived by GC-MS. OPLS-DA models were applied to identify the discriminating metabolites of GSC1-M from GS. One orthogonal component was identified by the model and had an $R^2 = 0.657$ and a $Q^2 = 998$, respectively (**Figure 5A**). The variables most relevant to differentiating between GS and GSC1-M were identified in the loading S-plots. Moreover, the metabolites were indicated by retention time m/z and cutoff value of $p < 0.001$ (**Figure 5B**). Also, to not overinterpret the S-plots, only the metabolites having variables that showed a jack-knifed confidence interval were investigated (**Figure 5C**). The metabolites contributing to the differentiations between GS and GSC1-M were identified using a NIST library and are summarized in **Table 4**. The OPLS-DA models clearly showed higher levels of glycerol, proline, glutamine, pentitol, fructose, inositol, octadecanoic acid, and sucrose of GSC1-M (Figure S1 in the Supporting Information) together with lower levels of pyroglutamic acid, citric acid, histidine, and palmitic acid compared to GS (Figure S2 in the Supporting Information).

Soybeans contain functional isoflavone glycosides, and the isoflavone glycosides are converted to aglycone through fermentation with some microbes (*33*). Thus, it is likely that in the present study some of the saccharides in GSCs, such as inositol, fructose, and sucrose, were increased to higher levels than found in GS, and *C. militaris* may be involved in the breakdown of those carbohydrates. According to previous research, *Cordyceps*-fermented rice exhibited higher amounts of metabolites such as amino acids, carbohydrate, lipid, dietary fiber, and vitamin E than raw rice (*38*). Thus, the higher amount of some amino acids such as proline and glutamine in GSCs than in GS would be associated with the growth of *C. militaris*.

Metabolite Difference Dependent on Cultivation Time in GC-MS Analysis. To investigate significant changes in metabolites in regard to the cultivation time of GSCs, correlation coefficients were calculated using Statistica. Significant compounds were identified in the same manner described above (**Table 4**). The concentrations of most of the metabolites decreased significantly when the cultivation time increased, but a few compounds were found to increase, including proline ($r = 0.602$), pentitol ($r = 0.533$), pyroglutamic acid ($r = 0.643$), and citric acid ($r = 0.924$) (Figures S1 and S2 of the Supporting Information).

These results revealed that the concentration of most of the primary metabolites in GSCs decreased during prolonged cultivations, which indicates that the fermentation process should be completed within a week after *C. militaris* inoculation.

In conclusion, this study showed not only the difference in chemical composition between GS and GSCs but also changes in the metabolite composition of GSCs as a function of cultivation time with *C. militaris*. Furthermore, this is the first study in which a metabolomic approach was used to determine compositional differences between GS and GSCs. The metabolic approach with LC- and GC-MS analyses coupled with a multivariate statistical data set was used to identify metabolites dependent on cultivation condition and time. In the metabolomic study, four isoflavone methyl-glycosides (compounds **1**−**4**) were detected as major contributing compounds of the fermentation process, and those structures were identified by 1D and 2D NMR spectroscopy as novel compounds.

Supporting Information Available: Cultivation time dependent metabolite changes of GSCs analyzed by GC-MS (Figures S1 and S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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