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Metabolomic profiling reveals enrichment of cordycepin in senescence process of *Cordyceps militaris* fruit bodies[§]

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***Cordyceps militaris* is a species of *Cordyceps* that is classified in the Cordycipitaceae family and is well known in East Asia as a traditional medicinal mushroom. Its artificial fruit body has been widely cultivated for commercial use in cosmetics, functional food, and medicine. To explore the metabolites associated with fruit body development, we conducted gas chromatography mass spectrometry (GC-MS) analyses based on developmental stage, which was divided into the growth period (stage 1, stage 2, and stage 3) and aging period (stage 4). We detected 39 biochemical metabolites associated with nucleotide, carbohydrate, and amino acid metabolism. Cordycepin, one of the representative bioactive compounds in *C. militaris*, was significantly enriched in stage 4 of aging period and is associated with glucose accumulation. The accumulation of cordycepin in stage 4 of aging period also seems to be related to the glutamine and glutamic acid pathway. Our results also showed enrichment of other bioactive compounds such as mannitol and xylitol in stage 4 of aging period. Our metabolomic profiling based on the developmental stages of *C. militaris* is useful for exploring bioactive compounds (e.g., cordycepin, mannitol, GABA, and xylitol) that are enriched in stage 4 of aging period and understanding the biosynthetic mechanisms associated with cordycepin production. Through optimization of fruit body cultivation by selecting stage 4 of aging period as a harvesting time, our findings can be utilized in food and medical applications of *C. militaris* in future.**

Keywords: *Cordyceps militaris*, GC-MS profile, cordycepin, metabolic pathway, medicinal mushroom

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Introduction

Species of *Cordyceps sensu lato* are characterized as parasites of insects and have been used in traditional Chinese medicine. This group of fungi consists of over 400 species and is currently classified into three families called Cordycipitaceae, Clavicipitaceae, and Ophiocordycipitaceae (Sung *et al*., 2007; Shrestha *et al*., 2012, 2014). One of the most well-known species in traditional Chinese medicine is *Ophiocordyceps sinensis* that belongs to Ophiocordycipitaceae, due to its value in promoting health and curing diseases in East Asia (Paterson, 2008; Shrestha *et al*., 2012). The second known species of *Cordyceps sensu lato* in traditional Chinese medicine is *Cordyceps militaris* which is a member of Cordycipitaceae and the type species of the genus *Cordyceps*. *C. militaris* has been broadly utilized for several hundreds of years as a folk tonic food in East Asia and is well-known as a functional food material in industry because of its immunomodulation property with anti-cancer, anti-diabetes and anti-viral activities (Yoo *et al*., 2004; Won and Park, 2005; Yu *et al*., 2006). The bioactive components of *C. militaris* include adenosine, cordycepin, mannitol, polysaccharide, and uridine (Ng and Wang, 2005; Jung *et al*., 2007; Paterson, 2008).

Cordycepin, (3′-deoxyadenosine), is a natural and main active compound of *C. militaris* and has been intensively studied and linked to the biological function of *C. militaris* (Su *et al*., 2017). It is a nucleoside analogue and its structure is similar to that of adenosine because it does not contain 3′-hydroxyl group of adenosine. It exhibits a variety of biological and pharmaceutical functions in health benefits including anti-cancer, anti-oxidant, anti-inflammatory, anti-platelet aggregation, and immunomodulatory activities (Kodama *et al*., 2000; Zhou *et al*., 2002; Su *et al*., 2017). Mechanisms underlying its anti-cancer effects have been intensively studied and shown to include apoptosis, cell cycle arrest, and inhibition of cancer cell proliferation and growth in cell lines of various cancer types (Tuli *et al*., 2013). The anti-cancer mechanisms of cordycepin are known to induce apoptosis by regulating intracellular purine biosynthesis inhibition and DNA/RNA biosynthesis. It is also reported that cordycepin inhibits transcriptional factor nuclear factor kappa B (NF-κB) activity and mammalian target of rapamycin (mTOR) signaling through activating adenosine monophosphate-activated protein kinase (AMPK) that plays an important role in the transmission of signals in cells and suppressing the proliferation of cancer cells.

With the importance in the biological property of cordycepin for health food and medicinal applications, several attempts have been made to increase cordycepin content in either culture or fruit body production of *C. militaris* (Kang

et al., 2014, 2017; Tuli *et al*., 2015; Zhang *et al*., 2016). Kang *et al*. (2014) optimized the large-scale culture conditions to increase cordycepin content of *C. militaris* by demonstrating the effects of carbon sources, organic or inorganic compounds, nucleoside analogues, and amino acids in cordycepin production with static liquid culture. Cultural optimization was also conducted with mutants of *C. militaris* to increase cordycepin content using ion beam irradiation and selecting strains based on its cordycepin production (Das *et al*., 2010). The bioactive metabolites such as exopolysaccharide and metal additives (Fe^{2+}, NH_4^+) were added to induce cordycepin production in liquid culture and fermentation (Park *et al*., 2001; Shih *et al*., 2007; Zhang *et al*., 2016).

In the artificial fruit body production, cordycepin content of *C. militaris* increased by utilizing the selected strains that produce higher levels of cordycepin during fruit body cultivation and mating the opposite mating types which were selected for cordycepin production (Kang *et al*., 2017). The fruit body formation of *C. militaris* occurs during the process of sexual reproduction and its reproducing propagules called perithecia were produced on the stromata that can be largely defined as fruit bodies (Shrestha *et al*., 2012). *C. militaris* reproduces sexually by producing ascospores that represent two mating types in the perithecia and mating with the opposite mating types. Heterothallic two mating types genetically differs only in mating type loci that represent idiomorphs called MAT1-1 and MAT1-2 (Shrestha *et al*., 2012). Kang *et al*. (2017) reported that cordycepin production of *C. militaris* differed in mating the combinations of mating types and strains of mating types were selected for increasing cordycepin content of the fruit body of *C. militaris*. However, the metabolic changes of cordycepin content in the fruit body were not addressed according to its developmental stages.

Metabolomics is quantitative and qualitative high-throughput chemical profiling of biological resources. It merges the analysis of multivariate statistics data with nuclear magnetic resonance spectrometry (NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). Metabolomics is one of the latest studies in the omics transformation of biological sciences (Saito and Matsuda, 2010). In an omics approach for cordycepin production based on the complete genome information of *C. militaris*, metabolomics has been employed to understand the biosynthetic mechanism of cordycepin in *C. militaris* and the metabolic regulation of cordycepin production by investigating its global metabolic responses on carbon sources (Xia *et al*., 2017; Raethong *et al*., 2018). However, the cordycepin production of *C. militaris* has not been documented with metabolomic approach for its development of fruit body. In *Cordyceps*, the developmental stages

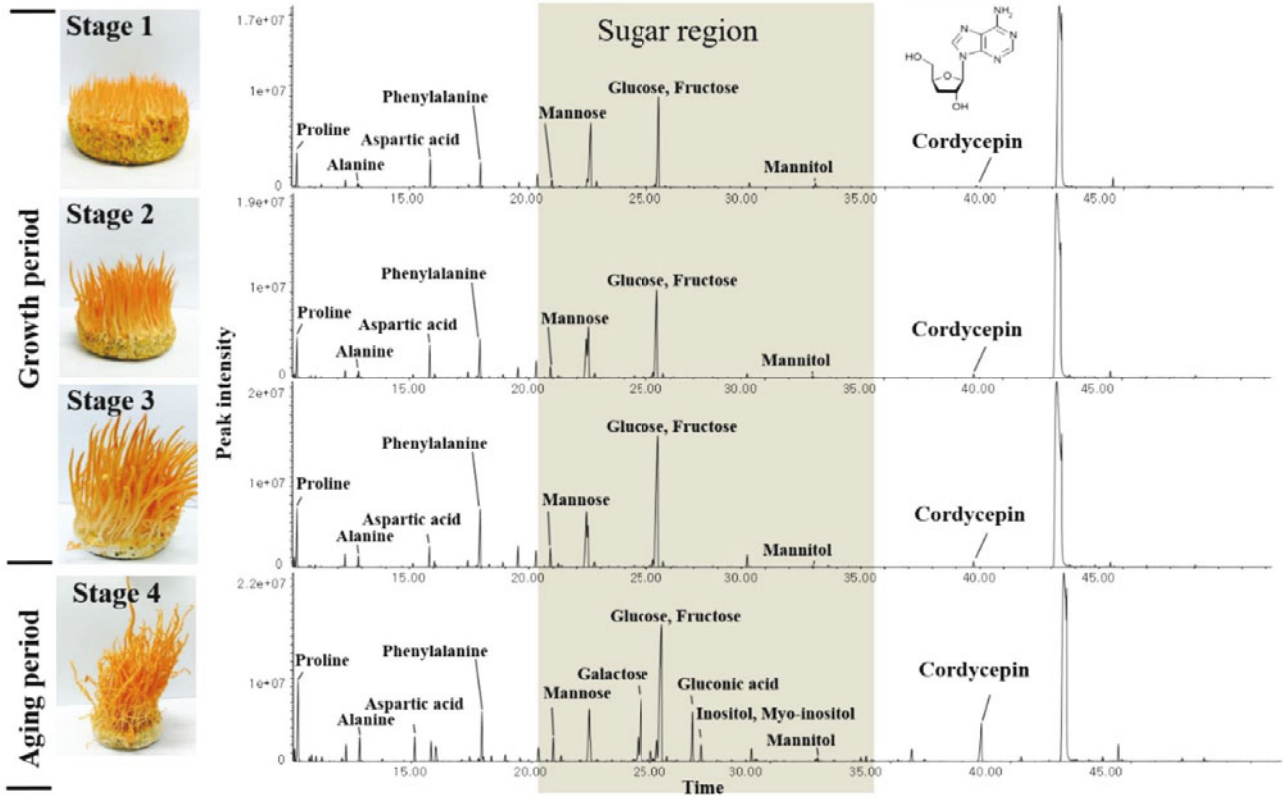

Fig. 1. GC-MS chromatogram of developmental stages and periods of *Cordyceps militaris* fruit bodies. GC-MS chromatogram were generated based on the development of sexual propagules called perithecia. Growth period with immature perithecia were divided into three stages. Stage 1 (2 weeks after inoculation) is characterized by the early stage of fruit body development and perithecia of stage 2 (4 weeks after inoculation) are formed on fruit bodies. While stage 3 (6 weeks after inoculation) initiates perithecium formation on fruit bodies, stage 4 of aging period (10 weeks after inoculation) can be defined as a senescence stage of *C. militaris* because ascospores are fully discharged from its perithiecia.

of *Cordyceps bassiana* were only investigated using GC-MS with relevance to its free radical scavenge activity (Hyun *et al.*, 2013).

In this study, we conducted metabolic profiling of the fruit body of *C. militaris* according to its developmental stages to understand metabolic changes during fruit body development. The development of artificially cultivated fruit bodies was initiated with inoculating the opposite mating types, matured with the formation of perithecia on the stromata of fruit body, and aged with release of ascospores from perithecia (Fig. 1). We used GC-MS spectroscopy in metabolomic analyses of methanol extracts from the fruit body of *C. militaris* with combining spectrometric methods and multivariate statistical analyses of principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) (Eriksson *et al.*, 2006). Because the artificial fruit body of *C. militaris* is currently massively produced and utilized in the health-related industry, this study is useful in providing the metabolic profiles of the bioactive compounds according to the developmental stages of *C. militaris*, identifying the metabolites associated with cordycepin production and obtaining the optimal cultivation and harvesting strategy for the usage in health industry.

Materials and Methods

Fruit body formation and developmental stages of *C. militaris*

The fungal strain of *C. militaris* used in this study for artificial fruit body production was deposited in Korean Agricultural Culture Collection (Accession No. KACC 43316). The fruit body formation was initiated with inoculation of fungal liquid spawn on brown rice medium in a 1,000 ml polypropylene bottle with incubating at 25°C under 1,000 lux of continuous white fluorescent light and 90% humidity in Mushtech Co. Ltd. Four stages were designated for sampling in metabolic analyses to investigate the differences in fungal components according to the development stages of fruit body formation of *C. militaris* (Fig. 1). The stromata of stage 1 (2 weeks after inoculation) is in the early stage of development and stage 2 (4 weeks after inoculation) is defined as no sexual propagules called perithecia formed on stroma that is moderately developed compared with stage 1. Stage 3 (6 weeks after inoculation) is characterized as initiating the formation of perithecia or forming irregular immature perithecia on the developed stroma. In the perithecia of stage 4 (10 weeks after inoculation) which is defined as an aged stage, the stromata are deformed with the development of secondary branches with no ascospore found in perithecia. The fruit bodies including brown rice medium were harvested from 100 bottles for each development stage to prepare the pooled sample for reducing the environmental factors associated with the fruit body development. The pooled harvested samples were immediately freeze-dried for 24 h and powdered. Samples were stored at -70°C for before analysis.

Extraction and preparation for comprehensive metabolite analysis

For each sample of development stages, 30 mg of powder was transferred into Eppendorf tube and extracted with 1 ml of 70% methanol with vortexing for 30 sec and sonicating for 10 min. The sample tube containing the extracts was centrifuged at 2,000 rpm for 10 min. After collecting supernatant from each sample, the supernatant was filtrated with a PTFE 0.45 μm pore size syringe membrane filter (Whatman) and 100 μl of filtrated sample was transferred into GC vials (Agilent Technologies). Samples were stored at room temperature in a nitrogen purge vacuum concentrator for 15 min. For derivatization, 30 μl of methoxylamine hydrochloride (20,000 μg/ml) (Sigma-Aldrich) in pyridine was added and then we added 50 μl of N, O-Bis (trimethylsilyl) trifluoroacetamide (Alfa Aesar) containing 1% trimethyl chlorosilane. In addition, 2-chloronaphthalene (Tokyo Chemical Industry) was obtained and 10 μl of 2-chloronaphthalene (250 μg/ml in pyridine) was added as an internal standard mixture. The derivatized samples were incubated at 65°C for 60 min, after transfer into GC vials suitable for GC-MS analyses.

GC-MS spectrometry analysis

GC-MS spectrometry was performed on a 7890A Agilent GC (Agilent Technologies) interfaced with a 5975C MSD detector (Agilent Technologies). A fused silica capillary column of 5% phenyl-95% dimethylpolysiloxane (DB5-MS, Agilent Technologies) with 30 m × 0.25 mm × 0.25 μm of film thickness dimensions was used for the analyses. Electron Ionization (EI) mode was used for ionization. The column auxiliary, MS source and MS quadrupole temperature were set at 280°C, 230°C, and 150°C, respectively. The initial oven temperature for polar metabolites was at 70°C programmed to 175°C (6°C/min) then to 185°C (5°C/min; hold 3 min) then at 250°C (5°C/min; hold 3 min) then to 320°C (9°C/min; hold 5 min). Helium was used as the carrier gas in constant-flow mode of 1 ml/min. A Split/Splitless injection module (1 μl) with a split ratio of 1:10 was applied.

Mass spectral data processing and biochemical identification

We used the Gene data Expressionist MSX software (Genedata, Version 2013) to process GC-MS data. The Raw Agilent+ data files (.d) were imported into the Gene data Expressionist MSX software. It was initially used for deconvolution, which eliminated target peaks from overlapping and chemical noise peaks. The identification of all metabolites were conducted using mass spectral database (NIST08) and Wiley W8N08 library (John Wiley and Sons, Inc.) with Human Metabolome Database (HMDB, http://www.hmdb.ca/) and Golm Metabolome Database (GMD, http://gmd.mpimp-golm.mpg.de/). The information of biological function and pathways of the relevant compounds was obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/) to map detected metabolites to the relevant biological pathways.

Data analysis and multivariate statistical analysis

After mass spectral data were processed, the relative intensities of integrated peak area were summed and considered as a single chemical compound. The internal standard peak area deviation was performed in the peak intensity across the entire set of measurement (Shurubor *et al.*, 2005; Parsons

Table 1. Biochemical compounds detected in GC-MS spectrometry for development stages or periods of *Cordyceps militaris* fruit bodies

The relative intensity was calculated by dividing the percentage area of each compound by the percentage area of internal standard and its standard deviation (SD) for each biochemical compound was obtained with biological quintuplicate (n = 5). Significance was determined by Tukey's test ($P < 0.05$) with one-way analysis of variance (ANOVA) and denoted with the letters (e.g., a, b, c, ab and bc) in superscript for each biochemical compound. ND: not detected, RT: retention time, TMS: trimethylsilylation, KEGG: Kyoto encyclopedia of genes and genomes, HMDB: human metabolome database, MPIMP: Max Planck institute of molecular plant physiology.

Class	Biochemical compound	RT (min)	m/z	KEGG	HMDB	MPIMP	TMS	Relative quantity			
								Growth period		Aging period	
								Stage 1	Stage 2	Stage 3	Stage 4
Amino acid	Isoleucine	10.76	158	C00407	HMDB00172	A132002	2 TMS	1.573 ± 0.209^{a}	0.942 ± 0.079^{b}	1.671 ± 0.139^{a}	6.038 ± 0.197^{c}
Amino acid	Proline	10.86	142	C00148	HMDB00162	A132003	2 TMS	0.071 ± 0.008^{a}	0.027 ± 0.003^{a}	0.548 ± 0.027^{b}	2.752 ± 0.137^{c}
Amino acid	Glycine	11.04	174	C00037	HMDB00123	A133001	3 TMS	0.027 ± 0.003^{a}	0.036 ± 0.005^{a}	0.645 ± 0.044^{b}	1.909 ± 0.071^{c}
Amino acid	Serine	12.29	204	C00065	HMDB00187	A138001	3 TMS	7.746 ± 0.614^{a}	6.901 ± 0.392^{a}	10.025 ± 0.660^{b}	16.250 ± 0.628^{c}
Amino acid	Threonine	12.83	100	C00188	HMDB00167	A140001	3 TMS	5.107 ± 0.388^{a}	3.615 ± 0.134^{b}	5.184 ± 0.276^{a}	13.237 ± 0.754^{c}
Amino acid	Asparagine	18.91	218	C00152	HMDB00168	A168001	3 TMS	1.093 ± 0.123^{a}	1.648 ± 0.123^{b}	2.352 ± 0.155^{c}	3.632 ± 0.176^{d}
Amino acid	α-Aminoadipic acid	19.85	117	C00956	ND	A172006	3 TMS	0.431 ± 0.043^{ab}	0.391 ± 0.033^{a}	0.445 ± 0.024^{ab}	0.470 ± 0.028^{b}
Amino acid	Tyrosine	25.71	218	C00082	HMDB00158	A194002	3 TMS	2.939 ± 0.265^{a}	6.895 ± 0.269^{b}	8.703 ± 0.394^{c}	8.079 ± 0.434^{c}
Amino acid	Spermidine	32.72	144	C00315	HMDB01257	A226002	5 TMS	0.242 ± 0.059^{a}	0.119 ± 0.010^{b}	0.090 ± 0.011^{b}	ND
Amino acid	γ-Aminobutyric acid (GABA)	32.93	174	C00334	HMDB00112	A153003	3 TMS	ND	0.006 ± 0.001^{a}	0.007 ± 0.001^{a}	0.062 ± 0.001^{b}
Amino acid	Alanine	13.78, 13.8	116	C00041	HMDB00161	A138002	3 TMS	0.035 ± 0.005^{a}	0.063 ± 0.010^{a}	0.447 ± 0.028^{b}	2.308 ± 0.073^{c}
Amino acid	Aspartic acid	15.82, 15.84	232	C00049	HMDB00191	A152002	3 TMS	36.789 ± 3.445^{a}	34.742 ± 1.453^{a}	17.469 ± 1.157^{b}	21.666 ± 1.467^{c}
Amino acid	Glutamic acid	17.93, 17.96	246	C00025	HMDB00148	A163001	3 TMS	32.391 ± 2.213^{a}	42.137 ± 1.469^{b}	60.116 ± 3.324^{c}	57.371 ± 3.939^{c}
Amino acid	Phenylalanine	17.99, 18.02	192	C00079	HMDB00159	A164001	2 TMS	0.945 ± 0.086^{a}	0.558 ± 0.015^{b}	0.439 ± 0.019^{b}	2.825 ± 0.241^{c}
Amino acid	Lysine	19.71, 25.28	84	C00047	HMDB00182	A186002	3 TMS	0.851 ± 1.085^{a}	2.659 ± 0.154^{b}	3.494 ± 0.194^{b}	10.796 ± 0.639^{c}
Amino acid	Putrescine	20.26, 20.29	376	C00134	HMDB01414	A175002	4 TMS	24.416 ± 2.918^{a}	25.238 ± 1.182^{a}	22.968 ± 1.265^{a}	21.774 ± 1.967^{a}
Amino acid	Ornithine	20.64, 22.38, 22.40	142	C00077	HMDB00214	A176006	3 TMS	29.423 ± 2.416^{a}	22.826 ± 0.789^{b}	41.587 ± 3.189^{c}	46.638 ± 2.669^{d}
Amino acid	Glutamine	21.21, 21.23	156	C00064	HMDB00641	A178001	3 TMS	1.389 ± 0.185^{a}	1.214 ± 0.075^{a}	2.651 ± 0.105^{b}	4.611 ± 0.395^{c}
Amino acid	Histidine	25.14, 25.16	154	C00135	HMDB00177	A192006	3 TMS	1.037 ± 0.233^{a}	0.838 ± 0.131^{a}	1.598 ± 0.209^{b}	3.280 ± 0.173^{c}
Amino acid	Tryptophan	31.91, 31.94	202	C00078	HMDB00929	A223001	3 TMS	1.540 ± 0.214^{a}	0.396 ± 0.034^{b}	0.501 ± 0.044^{b}	3.039 ± 0.172^{c}
Nucleoside	Adenosine	40.45	230	C00212	HMDB00050	A273001		0.025 ± 0.004^{a}	0.014 ± 0.001^{a}	0.011 ± 0.001^{a}	1.729 ± 0.109^{b}
Nucleoside	Cordycepin	41.62	236	C08431	ND	ND		0.009 ± 0.001^{a}	0.015 ± 0.002^{a}	0.017 ± 0.002^{a}	0.553 ± 0.062^{b}
Nucleoside	Uridine	35.48, 36.44	217	C00299	HMDB00296	A247002	3 TMS	0.032 ± 0.010^{a}	0.023 ± 0.003^{a}	0.033 ± 0.004^{a}	0.755 ± 0.063^{b}
Organic acid	Fumaric acid	12.12	245	C00122	HMDB00134	A137001	2 TMS	4.206 ± 0.396^{a}	3.583 ± 0.131^{b}	1.509 ± 0.161^{c}	5.726 ± 0.186^{d}
Organic acid	Isocitric acid	22.51	217	C00311	HMDB00193	A182003	4 TMS	0.361 ± 0.043^{a}	0.154 ± 0.012^{b}	0.167 ± 0.014^{b}	ND
Organic acid	Succinic acid	11.28, 13.98	147	C00042	HMDB00254	A134001	2 TMS	0.038 ± 0.006^{a}	0.052 ± 0.004^{a}	0.904 ± 0.091^{b}	5.946 ± 0.309^{c}
Organic acid	Malic acid	15.14, 15.17	233	C00711	HMDB00744	A149001	2 TMS	23.107 ± 2.051^{a}	9.281 ± 0.413^{c}	6.111 ± 0.383^{b}	24.595 ± 1.492^{a}
Sugar	Xylitol	19.89	103	C00379	HMDB02917	A171001	5 TMS	0.110 ± 0.009^{a}	0.073 ± 0.007^{c}	0.038 ± 0.006^{b}	0.252 ± 0.014^{d}
Sugar	Xylose	23.85	73	C00181	HMDB00098	A165001	4 TMS	0.181 ± 0.024^{a}	0.065 ± 0.007^{c}	0.028 ± 0.007^{b}	0.193 ± 0.023^{a}
Sugar	Fructose	24.13	103	C02336	HMDB00660	ND	6 TMS	0.014 ± 0.004^{a}	0.002 ± 0.002^{b}	0.003 ± 0.001^{b}	0.102 ± 0.009^{c}
Sugar	Galactose	24.44	204	C00984	HMDB00143	A191002	5 TMS	1.198 ± 2.252^{a}	4.240 ± 0.194^{b}	5.898 ± 0.473^{b}	21.573 ± 2.152^{c}
Sugar	Gluconic acid	27.13	103	C00257	HMDB00625	A200001	6 TMS	0.146 ± 0.019^{a}	0.025 ± 0.003^{a}	0.011 ± 0.001^{a}	7.167 ± 0.564^{b}
Sugar	Myo-inositol 1-phosphate	35.72	318	C04006	HMDB00213	M000724	7 TMS	0.224 ± 0.030^{a}	0.101 ± 0.013^{bc}	0.074 ± 0.003^{c}	0.126 ± 0.009^{b}
Sugar	Xylonic acid	43.83	284	C05411	HMDB60256	ND		0.198 ± 0.051^{a}	0.389 ± 0.030^{ab}	0.850 ± 0.077^{b}	2.567 ± 0.634^{c}
Sugar	Mannose	21.99, 22.00, 25.03	217	C00159	HMDB00169	A188202	5 TMS	4.602 ± 0.326^{a}	2.886 ± 0.077^{b}	2.314 ± 0.146^{b}	6.419 ± 0.734^{c}
Sugar	Glucose	24.24, 24.30, 24.43, 24.56, 24.73, 26.76, 26.77	319	C00031	HMDB00122	A193007	5 TMS	2.700 ± 0.363^{a}	1.875 ± 0.096^{a}	1.682 ± 0.087^{a}	87.929 ± 7.908^{b}
Sugar	Mannitol	25.45, 32.06, 38.78	218	C00392	HMDB00765	A193002	6 TMS	70.794 ± 4.809^{ab}	60.097 ± 1.995^{a}	81.668 ± 6.703^{b}	109.697 ± 9.308^{c}
Sugar	Inositol	27.79, 27.81, 27.83	243	C00137	HMDB02256	A189011	6 TMS	0.908 ± 0.092^{a}	0.476 ± 0.034^{b}	0.355 ± 0.086^{b}	0.668 ± 0.064^{c}
Sugar	Myo-inositol	29.23, 29.26	305	C00137	HMDB02256	R000105	6 TMS	2.538 ± 0.204^{a}	1.796 ± 0.072^{b}	4.971 ± 0.286^{c}	6.219 ± 0.330^{d}

et al., 2009). Detected compound variables were considered alone in turn by one-way ANOVA test and significant different level were observed in all compounds among the developmental stages of fruiting bodies using SPSS Statistics 22 software (IBM). Significance was determined by Tukey's significant difference test with a *P*-value threshold (< 0.05). The principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and statistical tests of significance were performed like one-way ANOVA test for GC-MS peak of relative intensity with the normalization methods applied to the data (Hyun *et al.*, 2013; Oh *et al.*, 2014). Mean centering and Pareto scaling were applied for PCA, PLS-DA, heatmap, and hierarchical clustering analysis (HCA) by MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) (Xia *et al.*, 2015). HCA was conducted with clustering the replicates of relative intensities of each developmental stage using Ward's method based on the Euclidean distance matrix.

Results and Discussion

GC-MS metabolic characterization of developmental stages of *C. militaris*

In metabolic profiling of developmental stages of fruiting body of *C. militaris*, four different developmental stages were designated and characterized by GC-MS analyses (Fig. 1). The biochemical compounds attributed to each development stage were identified using HMDB and GMD databases. As listed in Table 1 and Fig. 1, 39 metabolites including 20 amino acid, 3 nucleosides, 4 organic acids, and 12 sugars were detected from 70% methanol extracts of *C. militaris* fruiting bodies. In categorizing the developmental stages of *C. militaris* in Fig. 1 into two periods, stage 1, 2, and 3 can be categorized as growth period because sexual propagules termed perithecia were not fully developed and only stage 4 can be classified as aging period since ascospores were completely discharged from perithecia in stromata. The relative intensities of most metabolites in amino acid class were significantly different between growth period (stage 1, 2, and 3) and aging period (stage 4). We detected a total of 20 amino acids: alanine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, histidine, lysine, ornithine, proline, putrescine, serine, isoleucine, threonine, phenylalanine, spermidine, tryptophan, tyrosine, γ-aminobutytic acid (GABA), and α-aminoadipic acid.

In nucleoside class, three compounds of cordycepin, uridine, and adenosine were detected. The relative intensities of all of these compounds were dramatically increased in stage 4 of aging period (Fig. 1). Cordycepin, which is considered as one of the most important bioactive compounds in *C. militairs*, was dramatically increased in stage 4 of aging period (Table 1). The amount of cordycepin included in stage 4 of aging period was approximately 61.4 times higher than that of stage 1 in growth period. Since the stage definition in this study is closely related with the cultivation time, this result indicates that the longer time of cultivation (10 weeks after inoculation) is efficient in enriching cordycepin by selecting the harvest time as stage 4 of aging period of fruiting body development for the potential medicinal use in controlling human diseases and promote human healthcare.

Multivariate statistical analyses, hierarchical clustering analyses, and heatmap visualization

To compare the metabolic profiles of four developmental stages and address the difference between growth period (stage 1, 2, and 3) and aging period (stage 4), GC-MS data

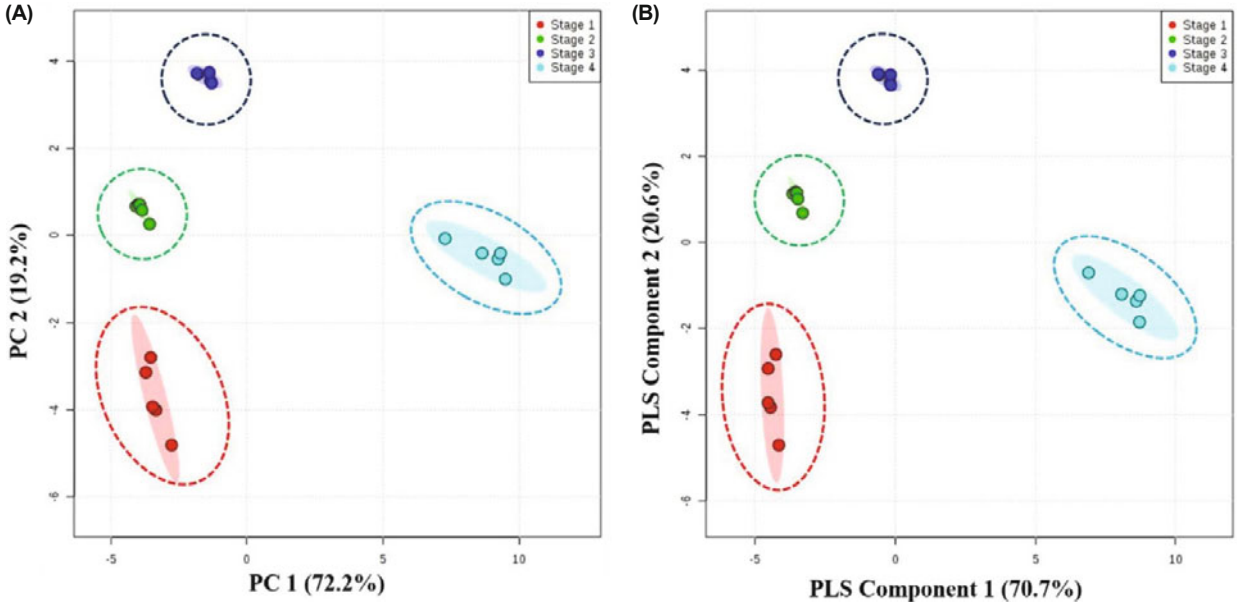

Fig. 2. PCA and PLS-DA plots of multivariate statistical analyses to differentiate developmental stages and periods of *Cordyceps militaris* fruit bodies. (A) PCA score plot based on two principal components (PC1 72.2% and PC2 19.3%) to separate developmental stages or periods of *C. militaris* fruit bodies. (B) PLS-DA score plot based on two PLS components (PLS1 component 1 70.7% and PLS2 component 2 20.6%) to separate developmental stages or periods of *C. militaris* fruit bodies. ($R^2Y = 0.994$, $Q^2Y = 0.990$).

matrix was subjected to multivariate analyses of PCA and PLS-DA. In PCA analyses, the percentages of variances were calculated as PC1 72.2% and PC2 19.2% after data normalization of five replicates of each developmental stage (Fig. 2A). Aging period (stage 4) was clearly separated from stages of growth period by principal component 1 with 72.2% of the variance. Further analyses of PLS-DA with different developmental stages showed the similar result as shown in PCA analyses and the percentages of variances were calculated as PLS Component 1 70.7% and PLS component 2 20.6% with the R^2Y and Q^2Y parameters of 0.994 and 0.990 (Fig. 2B). We calculated R^2Y and Q^2Y parameters to compare them with those of reordered models to determine the fitness and prediction level of PLS-DA model. Generally, R^2Y parameter close to 1.0 in PLS-DA model is usually considered as good fitness. PLS-DA model with Q^2Y parameter of 0.5–0.9 and 0.9–1.0 were considered to be good and excellent in prediction level, respectively. The specific information of metabolites which were responsible for the separation on the PLS-DA score plot was presented in Supplementary data Fig. S1. In PLS-DA analyses, stage 4 of aging period was also clearly separated from growth period (stage 1, 2, and 3) by PLS component 1 with 70.7% of the variance.

In hierarchical clustering analysis (HCA), GC-MS data were used for calculating the distances among developmental stages using Ward linkage method. Similar to PCA and PLS-DA analyses, the resulting dendrogram presented in Supplementary data Fig. S2 showed descriptively similar results to those

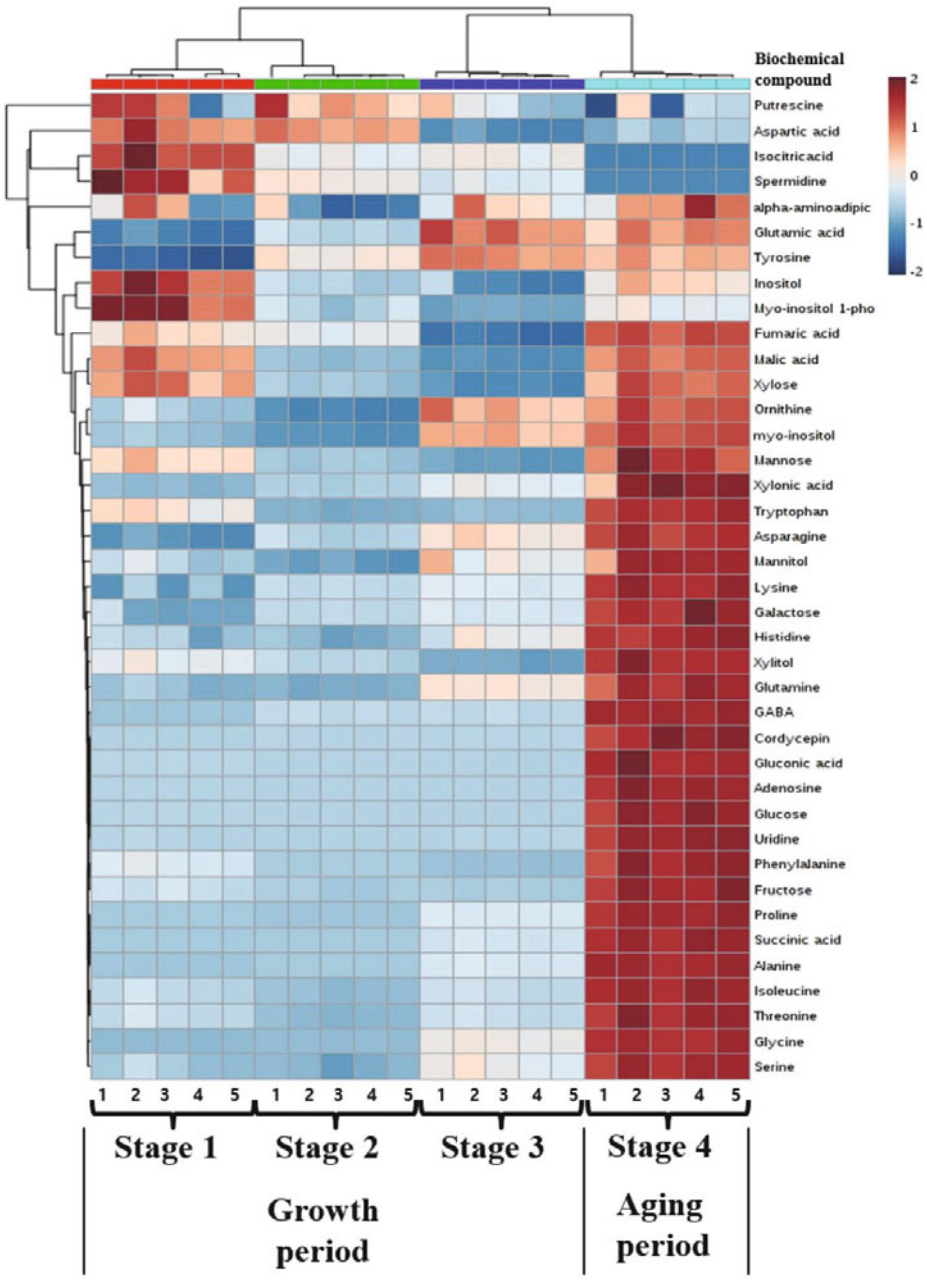

Fig. 3. Heatmap of biochemical compounds detected in GC-MS metabolic profiling based on developmental stages and periods of *Cordyceps militaris* fruit bodies. A red-blue color representation is used in presenting the relative abundance of each biochemical compound. While red color (+2) is the most abundant for each biochemical compound, blue color (-2) is the least abundant for each biochemical compound.

of PCA. In clustering of samples, stage 1 and 2 in growth period were closely related and grouped with stage 3 in growth period. Because all samples in growth period were separated from stage 4 in aging period, the trend associated with the differences among stages are clearly separated in relation with the cultivation time. The significant changes of metabolites including amino acids and sugars in stage 4 of aging period were visualized with heatmap analyses in Fig. 3. With a particular interest to the increased amount of cordycepin in stage 4 of aging period, we also detected other bioactive compounds which showed the higher amounts in aging period of stage 4. For example, GABA was known as one of major inhibitory neurotransmitters in the mammalian central nervous system and it was enriched in stage 4 of aging period with the relative intensity of 0.062 (Fig. 1).

Metabolic profiling and biological pathway

To better understand and detect the metabolites associated with the increased cordycepin production in stage 4 of aging period, we obtained the information of the biological functions and pathways of the relevant compounds from KEGG database by mapping each of the relative intensities of detected metabolites to the biological pathways as presented in Fig. 4. The metabolic pathway of cordycepin in stage 4 was turned out to be associated with the complex metabolic processes such as nucleotide, carbohydrate and amino acid metabolisms (Fig. 4). In nucleotide metabolism, three nucleosides (e.g., adenosine, cordycepin, and uridine) were detected in our GC-MS spectrometry and assumed to be correlated due to their high proportions only in stage 4 of aging period. The genetic and metabolomic mechanism of cordycepin production in *C. militaris* were recently revealed with omics approach using the complete genome information (Zheng *et al*., 2011; Xia *et al*., 2017). The biosynthetic genes of cordycepin in *C. militaris* turned out to be present in a gene cluster which produces cordycepin in the process of hydroxyl phosphorylation, dephosphorylation, and reduction from adenosine. In addition to cordycepin, Xia *et al*. (2017) reported *C. militaris* produces pentostatin which is known as a chemotherapeutic drug for hairy cell leukemia. Its biosynthetic mechanism of pentostatin from its precursor, adenosine, is mediated with the deamination of cordycepin. The *cns3* in the gene cluster is known to be involved in the production of both cordycepin and pentostatin with its dual gene function for the regulation of overproduced cordycepin through its detoxification in *C. militaris* (Xia *et al*., 2017). In our results, both adenosine and cordycepin were enriched at stage 4 of aging period. Because pentostatin was not quantified in our study, it is required to pursue a further study to understand the interrelation between cordycepin and pentostatin productions of *C. militaris* in stage 4 of aging period. We also detected uridine in our metabolic profiling to compare the developmental stages of the fruit body. The amount of uridine in stage 4 of aging period was approximately 23.5 times higher than that of stage 1 in growth period and it is one of the most effective nootropic supplements (Table 1 and Fig. 4). Due to the enrichment of bioactive compounds of nucleosides (e.g., adenosine, cordycepin, and uridine) in stage 4 of aging period, it is expected that stage 4 of aging period is optimal in producing fruiting body of *C. militaris* for the healthcare industry.

We detected a total of 12 sugar compounds in carbohydrate metabolism based on GC-MS spectrometry and our results indicated that stage 4 of aging period of fruit body is optimal in enriching not only cordycepin, but also sugar compounds such as glucose, mannitol and xylitol (Fig. 4). In the previous experiment to investigate the effects of carbon sources to cordycepin production in submerged culture, glucose is the most favorable carbon source for cordycepin production (Raethong *et al*., 2018). It is consistent with our results in the increased amount of glucose in stage 4 of aging period (Fig. 4). The amount of glucose in stage 4 is approximately 52.2 times higher than that of stage 3 in growth period (Table 1). It is assumed that the accumulation of cordycepin in stage 4 is tightly linked to the aging mechanism of the fruit body development, which may be initiated with glucose accumulation. In addition to glucose, sugar metabolites of galactose, fructose and xylose, which can be carbon sources in submerged culture, were also accumulated in stage 4 of aging period. It is assumed to result from the accumulation of glucose based on the metabolic pathway of Fig. 4. Mao *et al*. (2005) showed that these metabolites are not favorable carbon sources compared with glucose for cordycepin production in submerged culture. Therefore, the overall accumulation of sugar compounds in stage 4 of aging period is not associated with cordycepin production except for glucose metabolism in the metabolic pathway of *C. militaris* (Fig. 4).

In the perspective of bioactivity of sugar compounds detected in our GC-MS spectrometry, mannitol and xylitol can be considered as bioactive compounds and they were detected in higher amount of stage 4 in aging period. Mannitol is an isomer of quinic acid with a structure of 1,3,4,5-tetrahydroxycyclohexanoic acid. It was first isolated from *O. sinensis* in 1957 with a name of cordycepic acid (Chatterjee *et al*., 1957). It is considered as one of the most common fungal metabolites and it has been generally found in most of medicinal mushrooms. Mannitol is known to play an important role in the liver fibrosis treatment and reducing the diuretic osmotic pressures such as intracranial and intraocular pressures (Ouyang *et al*., 2013; Nomani *et al*., 2014). Xylitol is also found to be significantly richer in stage 4 of aging period than growth period (Fig. 4). It is commonly used as an alternative to sugar and functional food additives as sweetener. With its anti-cancer effect, Wada *et al*. (2017) showed the synergistic effect of cordycepin and xylitol on human malignant melanoma. Because stage 4 of aging period is rich in both cordycepin and xylitol, the anti-cancer activity with the use of the fruit body from stage 4 of aging period is expected to be enhanced with the synergism of cordycepin and xylitol.

In amino acid metabolism, a total of 20 amino acids were detected in our metabolic profiling for the fruit body development of *C. militaris* and the majority of amino acids including glutamine, phenylalanine and tryptophan were present in the higher amounts at stage 4 of aging period than growth period (Fig. 4). The glutamine and glutamic acid pathway is interested due to its relevance in cordycepin production. In the mycelial culture, the production of cordycepin is affected by the additives of glutamine or glutamic acid (Leung and Wu, 2007). With the dose-dependent input of

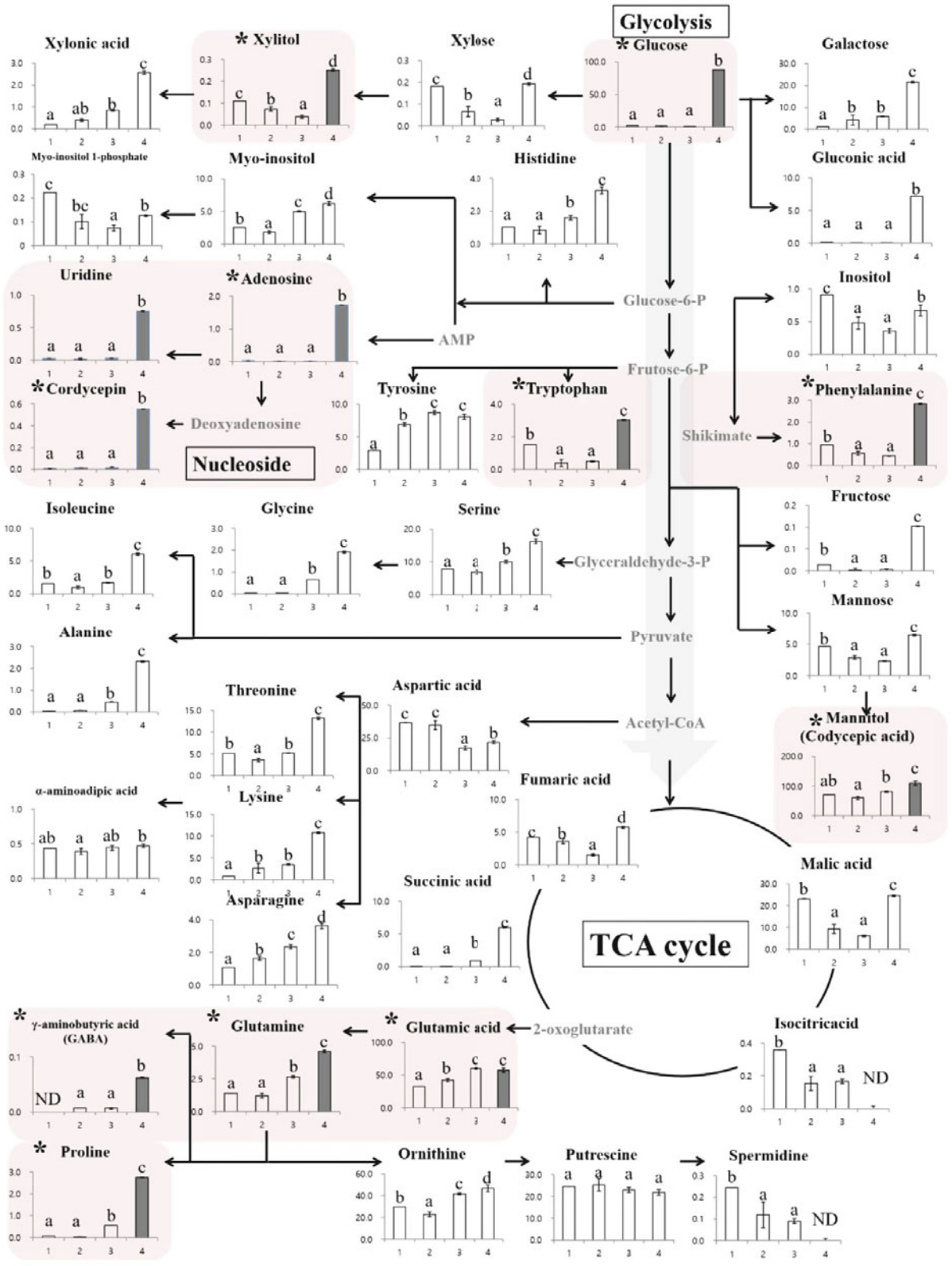

Fig. 4. Schematic diagram of metabolic pathway associated with detected biochemical compounds in GC-MS spectrometry. The relative intensity of each compound in Table 1 is graphically represented on its relevant metabolic pathway for the comparison of developmental stages of *C. militaris* fruit bodies. The metabolic pathway was obtained and modified from KEGG database. The important biochemical compounds (e.g., cordycepin, mannitol, and xylitol) enriched in stage 4 of aging period were shaded in the perspective of their bioactive properties. Significance was determined by Tukey's test ($P < 0.05$) with one-way analysis of variance (ANOVA) and denoted with the letters (e.g., a, b, c, ab, and bc) in superscript for each biochemical compound. The "ND" means "not detected".

glutamine or glutamic acid in culture, cordycepin were produced in dose-dependent manner, indicating that cordycepin production is related with glutamine and glutamic acid pathway. In our experiment, glutamine was enriched at stage 4 of aging period and glutamic acid were enriched at stage 3 and 4. Glutamine is a product of the condensation of glutamic acid and ammonia by glutamine synthase in the process of ammonia assimilation (Horton *et al*., 2002; Leung and Wu, 2007). Because glutamine is a nitrogen donor for nucleotide metabolism, the overproduction of cordycepin in stage 4 of aging period might be tightly related with the increased amount of glutamine in stage 4. GABA is known as one of important neurotransmitters in central nervous system and synthesized through glutamine and glutamic acid pathway (Erecińska and Silver, 1990; Struzyńska and Sulkowski, 2004). In nervous system, GABA, glutamine and glutamic acid are key elements in brain metabolism and neuron uses glutamine from glial cells as a main substrate for the syntheses of GABA and glutamic acid.

Generally, secondary metabolites with benzene ring structure plays a vital role as free radical scavenger with their anti-oxidant effect (Lü *et al*., 2010). Amino acids (e.g., phenylalanine and tryptophan) with a common benzene ring are important in secondary metabolism due to its usage as a precursor substrate of shikimic acid and phenylpropanoid biosynthetic pathway (Park and Lee, 2010). For example, the addition of tryptophan in the mycelial culture of *Ganoderma neo-japonicum*, significantly increased the amount of phenolic compounds. In the present study, phenylalanine, tryptophan and proline were enriched at stage 4 of aging period (Fig. 4). It is expected to generate secondary metabolites by producing phenolic compounds such as polyphenol compounds. Further study on characterizing secondary metabolism of *C. militairs* is required to explore the secondary metabolites associated with shikimic acid and phenylpropanoid pathway to verify the medicinal value of stage 4 of aging period for the use in healthcare industry.

Conclusion

We found that the developmental stage 4 of aging period in fruit body development of *C. militaris* was enriched for bioactive compounds that were associated with nucleotide, carbohydrate and amino acid metabolisms. Cordycepin coupled with its precursor, adenosine, was enriched possibly due to the aging mechanism of its development with the enrichment of glucose. In carbohydrate metabolism, mannitol and xylitol were two bioactive compounds which have a synergistic bioactive property with cordycepin in stage 4 of aging period for the use of healthcare industry. The biosynthetic production of cordycepin may be regulated by glutamine and glutamic acid pathway based on the patterns of our metabolic analyses in amino acid metabolism. We also found that stage 4 of aging period was enriched with amino acids that possess benzene ring, which may be associated with the production of secondary metabolites such as polyphenols. Therefore, our metabolomic analyses for the development of *C. militaris* are useful in exploring bioactive compounds in stage 4 of aging period and understanding the biosynthetic mechanism related with cordycepin production. Because stage 4 of aging period was enriched with various bioactive compounds (e.g., cordycepin, mannitol, and GABA), it can be utilized for function food and medical application in future.

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Conflict of Interest

The authors have no conflict of interest to declare.

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