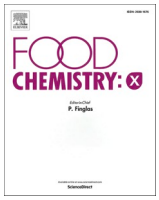
Food Chemistry: X 19 (2023) 100788

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Impacts of selenium enrichment on nutritive value and obesity prevention of *Cordyceps militaris*: A nutritional, secondary metabolite, and network pharmacological analysis

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ARTICLE INFO

Keywords:
Cordyceps militaris
Selenium enrichment
Nutritional ingredient
Obesity
Network pharmacology

ABSTRACT

This study aimed to compare the nutritive value and obesity prevention of ordinary *Cordyceps militaris* (CM) and selenium-enriched CM (SeCM). The results indicated that Se enrichment significantly increased the total carbohydrate and soluble dietary fiber content, while the protein and insoluble dietary fiber content decreased. Although the fat content was not affected, the medium and long-chain fatty acids content significantly changed. Moreover, Se enrichment significantly elevated the secondary metabolites belonging to terpenoids and alkaloids, which are linked with the enhanced biosynthesis of secondary metabolites. Both CM and SeCM reduced body weight, adipose accumulation, impaired glucose tolerance, and lipid levels in high-fat diet (HFD)-fed mice, and there was no significant difference between them. Network pharmacological analysis revealed that dietary CM and SeCM prevented HFD-induced obesity and associated metabolic diseases with multi-ingredients acting on multi-targets. Overall, Se enrichment improved the nutritive value of CM without altering its role in preventing obesity.

1. Introduction

Cordyceps militaris (CM), a precious edible and medicinal mushroom, has attracted much attention recently all over the world (Zhang, Wen, Duan, Zhang, & Ma, 2019). It contains several essential biologically active ingredients, including polysaccharide, protein, fat, amino acid, fatty acid, and kinds of secondary metabolites (Jedrejko, Lazur, & Muszynska, 2021; Zhang, Zhang, Yin, Li, & Kang, 2018). These ingredients have been reported to possess multiple bioactivities such as anti-obesity effects (Huang et al., 2022), anti-inflammatory effects (Phull, Ahmed, & Park, 2022), anti-oxidant properties (Barido, Jang, Pak, Kim, & Lee, 2020), procoagulant effects (Zhang et al., 2018), immune regulation effects (Wang et al., 2012), and liver protection (Wang et al., 2018). Furthermore, as a traditional Herb, whole CM is used as a natural source of raw material in Asian ethnomedicine to alleviate fatigue and enhance immune system function in humans. Due to these versatile characteristics, therefore, CM has been designated as a New Resource Food, verified by the Ministry of Public Health in China (Announcement No. 3, 2009), and possesses significant research and development value.

Selenium (Se) is a nutritionally essential trace element, which acts a crucial role in reducing the risk of certain diseases, including metabolic disorders, in humans (Rayman, 2012). Importantly, the beneficial effects of Se have been shown to be strictly dependent on its chemical form (Wrobel, Power, & Toborek, 2016). Organic Se-containing compounds and inorganic Se compounds represent its chemical forms. Generally,

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https://doi.org/10.1016/j.fochx.2023.100788
Received 20 April 2023; Received in revised form 3 July 2023; Accepted 6 July 2023
Available online 12 July 2023

the organic Se form is usually absorbed more efficiently than its inorganic forms, resulting in better bioavailability (Liu, Zhu, Sun, Gao, & Zhang, 2017). Nowadays, increasing endeavors have been devoted to the conversion of inorganic Se compounds into organic Se compounds through Se-enriched cultivation (Dong, Lei, Ai, & Wang, 2012; Dong, Xiao, & Wu, 2021). As a result, intaking Se-enriched foods like Se-enriched CM (SeCM) has become a promising strategy to supplement organic Se in the body.

Accumulating evidence has suggested that Se enrichment has been found to possess an impact on the nutritive value and biological activities of the original food (Gao, Xu, Ruan, & Yin, 2020; Wang et al., 2018; Wang, Tan, Nima, Sang, Cai, & Xue, 2022). For example, compared to ordinary CM, SeCM contained much higher contents of cordycepin and selenocystine, while the content of reducing sugars did not vary significantly between SeCM and CM (Wang et al., 2018). Likewise, Se-enriched blank tea showed better efficacy than ordinary blank tea in attenuating hyperglycemia and insulin resistance induced by high-fat diet (HFD)-fed Sprague-Dawley rats, but they exhibited similarly in reducing fat accumulation (Gao et al., 2020). Studies in more-depth indicated that the differences in efficacy might be partly due to their different roles in regulating gut microbiota (Wu, Wu, Zhu, Li, Wei, & Wang, 2022). Nevertheless, there is still limited understanding regarding the comprehensive impacts of Se enrichment on the nutritive value and bioactivities of most original food items. Furthermore, existing studies lack a thorough analysis of nutritional components, particularly secondary metabolites, and their impact on bioactivities remains poorly understood. Given that both CM and SeCM with highly valued nutraceuticals have been industrially cultivated at a large scale in many Asian countries (Dong, Ding, Yu, Lei, Zheng, & Wang, 2013; Jedrejko, Lazur, & Muszynska, 2021), these findings prompted us to explore the impact of Se enrichment on the nutritive value of CM. In addition, its impact on preventing obesity was also discussed since the obesity prevention has been demonstrated in various nutritional components from CM and SeCM, especially polysaccharides (Huang et al., 2022; Kim et al., 2014; Li et al., 2022; Yu et al., 2021).

In the present study, the alteration of nutritive value in CM resulting from Se enrichment was systematically analyzed on the basis of nutrients and secondary metabolites. Furthermore, the different effects of CM and SeCM on obesity in HFD-fed mice were thoroughly conducted through network pharmacological analysis. Our study presents new insights into the impacts of Se enrichment on the nutritive value and obesity prevention of CM.

2. Materials and methods

2.1. Materials

The dried fruiting bodies of CM and SeCM were obtained from Guangdong Micro Edible Mushroom Technology Co., Ltd., located in Guangzhou, China (source: https://www.bioyuewei.com/productinfo/1051680.html). These fruiting bodies were produced in strict accordance with GH/T 1240–2019 guidelines. Subsequently, they were ground into powder using a grinder (YB-2000A, YUN BANG, China). Importantly, it should be emphasized that the samples originated from the same batch and bag, and were divided into 3 portions for subsequent analysis of nutrients and secondary metabolites. Methanol, acetonitrile, formic acid, and phenol were bought from National Pharmaceutical Group Co., Ltd., Beijing, China. The food for mouse experiments was obtained from Changzhou SYSE Bio-Tec Co., Ltd., China, and the food ingredients and energy densities are shown in Table S1. Other chemical reagents were obtained from Sangon Biotechnology Co. (Shanghai, China).

2.2. Determining nutrients in CM and SeCM

The Se content was measured by atomic fluorescence spectrometry method regarding the National standard of China (GB 5009.93-2017). The total carbohydrate content was analyzed by phenol–sulfuric acid method according to the National standard of China (GB 15672-2009). The total protein content was determined by Kjeldahl determination method described in National standard of China (GB 5009.5-2016). The total fat content was determined by Soxhlet extraction method described in National standard of China (GB 5009.6-2016). The soluble and insoluble dietary fiber contents were measured by the method regarding the National standard of China (GB 5009.88-2016). The detailed operation processes of the above standard methodologies were described in Supplementary Method 1.

2.3. Analysis of nutrients composition in CM and SeCM

2.3.1. Amino acid composition

An UPLC-Orbitrap-mass spectrometry (MS) system (UPLC, Vanquish; MS, QE) was used to analyze the amino acid content in both CM and SeCM samples. Samples and standards (2 mg) were digested separately with 1 mL of 6 M HCl at 110 °C for 24 h. After centrifugation (15000 *g*, 10 min), the resulting supernatants (200 μL) were neutralized with 535 μL of 2 M NaOH followed by four-fold dilution with AccQ•Tag Ultra Borate buffer. The neutralized samples or standards (10 μL) were then combined with 70 μL of Borate buffer and 20 μL of AccQ•Tag reagent, and subsequently derivatized at room temperature for 1 min and at 55 °C for 10 min for UPLC-MS analysis. The analytical conditions of UPLC were as follows: Waters ACQUITY UPLC BEH C18 column (1.7 μm, 50 × 2.1 mm), column temperature: 55 ℃, flow rate: 0.5 mL min^{-1}, injection: 1 μL, mobile phase A: water (0.1% formic acid), mobile phase B: acetonitrile (0.1% formic acid), gradient program: 95:5 (v/v) at 0 min, 90:10 (v/v) at 5.5 min, 75:25 (v/v) at 7.5 min, 40:60 (v/v) at 8 min, 95:5 (v/v) at 8.5 min, 95:5 (v/v) at 13 min. The parameters of MS equipped with an ESI source (Thermo Fisher Scientific) adopting the Full MS acquisition mode were as follows: spray voltage −3 kV, sheath gas pressure −40 arb, aux gas pressure −10 arb, capillary temperature −320 ℃, and aux gas heater temperature –350 ℃.

2.3.2. Medium and long-chain fatty acid (MLFA) composition

A GC–MS (Agilent Technologies) analysis was used to determine the MLFA content in the CM and SeCM. 0.1–0.5 g of the sample was extracted using 4 mL of chloroform by shaking for 30 s, followed by the addition of a 0.9% NaCl solution, and vortexed for 30 s. After centrifugation (3500 *g*, 15 min), the lower layer was collected and sequentially extracted with dichloromethane, methanol, and *n*-hexane. Subsequently, the solution was dried with nitrogen and then added to an appropriate volume of isooctane based on the sample concentration for GC–MS detection. A GC system (Agilent Technologies 7820A) coupled to an Agilent Technologies 5977B inert MSD quadrupole MS was utilized, with a CP-Sil 88 (0.25 μm, 100 × 0.25 mm) column, and an injection of 1 μL at a flow rate of 1 mL min^{-1} in split mode (1:10). The initial oven temperature was set at 100 °C for five minutes, ramped up to 240 °C at a rate of 4 °C min^{-1}, and held for 15 min. The MS mode used was selected ion monitoring, with a quadrupole temperature of 150 °C, fragmentation voltage of 70 eV, and scan range of *m/z* 30 to 550.

2.4. Analysis of secondary metabolites in CM and SeCM

Samples (100 mg) were mixed with the extracted solution (500 μL, methanol: water = 4:1) containing 10 μg/mL of internal standard (L-2-chlorophenylalanine), and the mixtures were homogenized at 45 Hz for 4 min and sonicated for 1 h in an ice-water bath. After standing at −40 °C for 1 h, the mixtures were centrifuged at 12,000 *g* for 15 min at 4 °C. The supernatant was filtered using a microporous membrane (0.22 μm). The secondary metabolites in the supernatant were analyzed through an ultra-high pressure liquid chromatography (UHPLC) system (Vanquish, Thermo Fisher Scientific) coupled to Q Exactive HFX mass spectrometer in positive and negative ion modes (Orbitrap MS, Thermo

Fisher Scientific) on a BEH Amide column (2.1 mm * 100 mm, 1.7 μm) with a flow of 0.5 mL/min. The MS2 information (MS2 fragments, MS2 adduct, M/Z-med, and RT-med) of secondary metabolites were obtained through the UHPLC-MS after peak alignment, and then the secondary metabolites were authenticated by searching an in-house MS2 database (Shanghai BIOTREE Biological Technology Co., Ltd.). The secondary metabolites were remained when MS2 score >0.6 for subsequent analysis. The initial secondary metabolites were performed by principal component analysis (PCA) and orthometric partial least squares discriminant analysis (OPLS-DA), and screened when variable important in the projection (VIP) > 1 and $p < 0.05$. Significantly differential secondary metabolites were determined as those acquired from volcano plot [$p < 0.01$ and $|\log_2$fold change (FC)$| > 2$] and exhibited by pheatmap package in R software. The KEGG pathway enrichment was performed to analysis the potential pathways in CM changed by the Se enrichment.

2.5. Preventive effects of CM and SeCM on obesity

2.5.1. Animal experiments

After approval by the Animal Ethics Committee of Institute of Microbiology, Guangdong Academy of Sciences (GT-IACUC202008111), 32 male C57BL/6J mice (SPF, 20 ± 1 g) were purchased from Guangdong Medical Laboratory Animal Center (SCXK 2018–0002). The mice were kept under the conditions of 23 ± 2 °C, 55 ± 5% relative humidity, and a 12 h light–dark cycle throughout study. After a one-week acclimatization period, the mice were randomly assigned to four groups, each containing eight mice (with four mice per cage). The first group served as the healthy control and was fed a normal diet (referred to as the NMD group, D12450J). The second group known as the model control and was fed a HFD (referred to as the HFD group, D12492). Both of these groups received an oral administration of 0.1% sodium carboxymethyl cellulose, which served as the solvent control. The remaining two groups were the experimental groups: HFD + CM group and HFD + SeCM group. These groups continued to receive the HFD, while the HFD + CM group was orally administered 250 mg kg^{-1} d^{-1} of CM, and the HFD + SeCM group received SeCM, also in a dose of 250 mg kg^{-1} d^{-1}, both resuspended in 0.1% sodium carboxymethyl cellulose. Notably, CM and SeCM here referred to the powder of their fruiting bodies, and the dose was used based on the previous studies on the bioactivity evaluation of CM in mouse experiments (Liu et al., 2016; Shimizu, Mori, Ouchi, Kushida, & Tsuduki, 2018), which is approximately equivalent to a daily dose of 1.2 g of CM/SeCM for a 60 kg adult, meeting the requirements of the Document No. 10 of 2014 of National Health and Family Planning Commission (China). The body weight was recorded weekly, and food intake was measured daily. After 12 weeks of experimental treatment, all mice were sacrificed by CO_2 asphyxiation, followed by blood collection from the heart, and then liver, epididymal, perirenal and inguinal fat were precisely dissected and weighed.

2.5.2. Glucose tolerance assessment

After overnight fasting, mice were administered orally glucose (2 g kg^{-1}), and blood glucose levels were measured at 0, 30, 60, 90, and 120 min using a glucometer (ACCU-CHEK performa, Roche, Germany). The area under the curve (AUC) of the blood glucose was calculated by GraphPad Prism (v8.0, GraphPad Software, USA).

2.5.3. Serum lipid analysis

Blood samples were centrifuged (4 °C, 3000 *g*, 15 min) to separate serum. Serum triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), and high-density lipoprotein cholesterol (HDL-c) were determined by the corresponding commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.6. Network pharmacology

2.6.1. Screening of bioactive ingredients in CM and SeCM against HFD-induced obesity

The serum metabolites for each mouse group were determined and annotated as described in section 2.4. The metabolites with threshold values of VIP > 1 and p-value < 0.05 between the experimental groups and the model group and existing in CM and SeCM were considered to be the potentially bioactive ingredients in CM and SeCM to reduce the side effects induced by HFD. Meanwhile, the experimental groups removed the metabolites that were common to the model group, and the remaining metabolites were present in CM and SeCM, which were also considered to be the potentially bioactive ingredients in CM and SeCM. After combining above potentially bioactive ingredients, the effectively bioactive ingredients were determined based on the screening criteria of drug-likeness (DL) > 0.18 and percent human oral bioavailability (OB) $> 30\%$.

2.6.2. Target collection of ingredients and obesity

The bioactive ingredients were uploaded to ChEMBL database (https://www.ebi.ac.uk/chembl/), TCMIO (http://http://tcmio.xielab.net) database and YaTCM database (http://cadd.pharmacy.nankai.edu.cn/yatcm/home) to collect the ingredients-related targets. Further, associated targets of obesity were searched from TTD database (http://db.idrblab.net/ttd/) and DisGeNET database (http://www.disgenet.org). The redundancy parts in ingredients- and obesity-related targets were removed.

2.6.3. Ingredients-target network construction

The obtained ingredients-related targets and obesity-related targets were intersected using Venn analysis, and the intersected targets were considered as the candidate targets of CM and SeCM in the obesity prevention. The interaction network between obesity, candidate targets, CM/SeCM, and effectively bioactive ingredients was constructed using the Cytoscape 3.7.1 software (https://cytoscape.org/). Only the targets with connections ≥6 with active ingredients are considered key targets and displayed.

2.6.4. Protein-protein interaction (PPI) network construction

The PPI network was established by uploading the key targets the STRING 11.0 database (https://string-db.org/cgi/input.pl), of which the organism was set to Homo sapiens. Cytoscape software was employed to reestablish and analyze the PPI network.

2.6.5. GO function enrichment analysis and KEGG pathway enrichment analysis

The function annotation and signaling pathways of the key targets were performed by GO and KEGG enrichment analysis, and the first 10 items were filtered according to the fold enrichment value to manifest the signaling pathway and mechanism of CM and SeCM in the treatment of obesity-related diseases.

2.7. Statistical analysis

Data are shown as mean ± SEM. A comparison of two and more groups was performed using two-sided unpaired Student's *t*-test with Benjamini-Hochberg correction and one-way parametric analysis of variance followed by Duncan's range tests, respectively. All results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Se enrichment changes the nutrients and their composition in CM

Se enrichment significantly increased the Se content in SeCM compared to that in ordinary CM (Fig. 1A, $p < 0.001$). SeCM possessed a

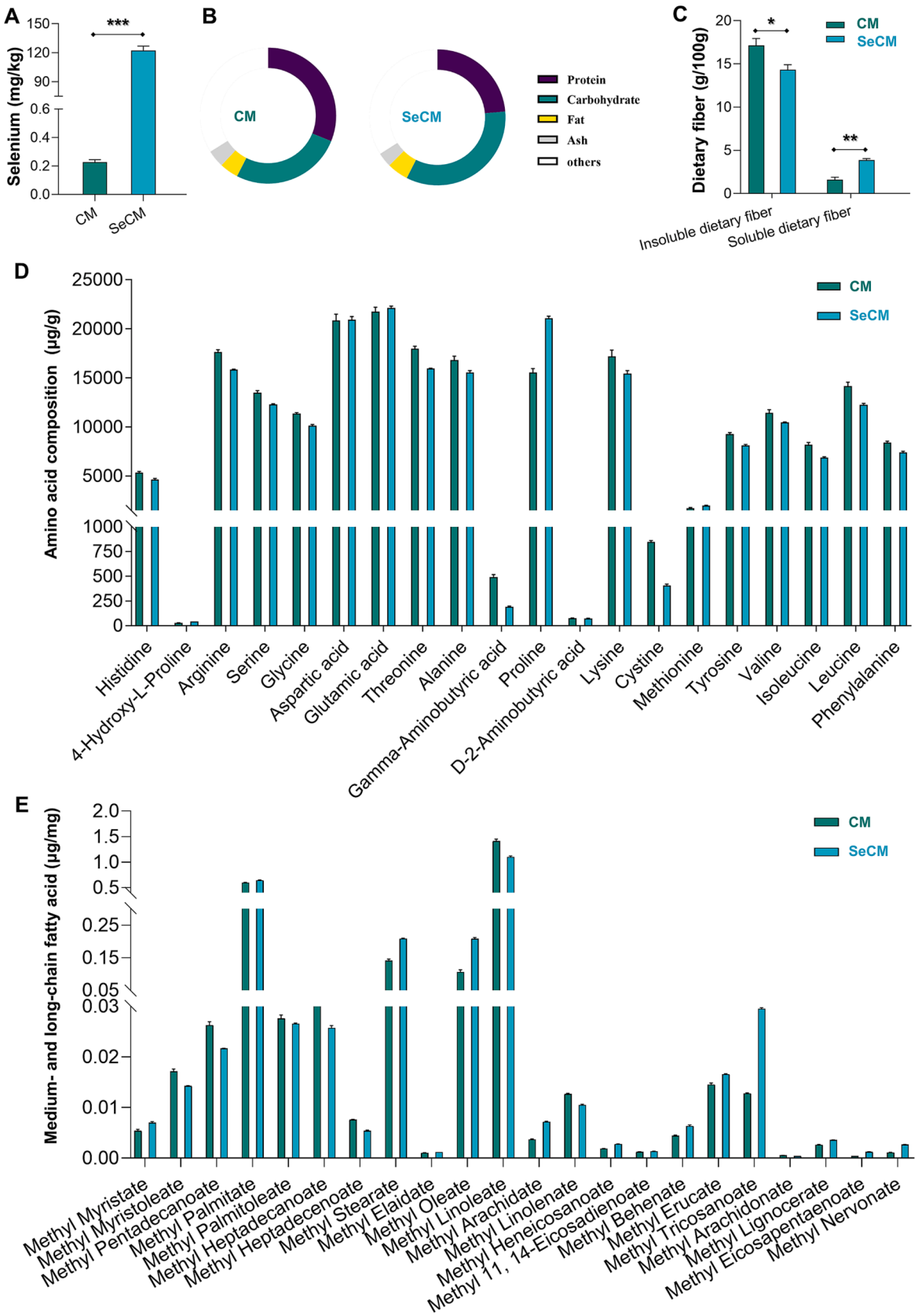

Fig. 1. Se enrichment changes the nutrients and their composition in CM. Selenium (A), major nutrients (B), dietary fiber (C), amino acid composition (D), and medium- and long-chain fatty acid composition (E). CM, *Cordyceps militaris*; SeCM, Se-enriched *Cordyceps militaris*. Data are expressed as the mean ± SEM (n = 3). Graph bars marked with different asterisks on top represent statistically significant results (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

higher content of total carbohydrate, and a lower content of protein than CM (Fig. 1B). The level of soluble dietary fiber was markedly increased in SeCM by Se-enriched, while the level of insoluble dietary fiber was decreased (Fig. 1C, $p < 0.05$). Although there are no differences in the compositions of amino acid between CM and SeCM, the contents of most amino acids are obviously different, especially 4-hydroxy-L-proline, arginine, serine, glycine, threonine, aminobutyric acid, proline, tyrosine, isoleucine, and phenylalanine (Fig. 1D). Similarly, Se enrichment did not change the compositions of MLFA in CM, but significantly changed their contents, except for methyl palmitoleate (Fig. 1E).

3.2. Se enrichment changes the secondary metabolites in CM

Secondary metabolites are mainly responsible for the medicinal properties of the organism (Abdalla & Mühling, 2023), therefore, we investigated the impact of Se enrichment on the secondary metabolites of CM by a UHPLC system coupled to Q Exactive HF-X mass spectrometer. A total of 479 secondary metabolites in CM and SeCM were identified in positive and negative ions after peak alignment (Table S2). The PCA and OPLS-DA analysis showed that the metabolic spectra of CM and SeCM were clearly disjointed (Fig. 2A and B), indicating that the secondary metabolites in CM were significantly changed by Se enrichment. Among these secondary metabolites, 264 differential secondary metabolites between two groups were initially obtained with threshold values of VIP > 1 and p-value < 0.05. Further, 49 notably differential secondary metabolites were screened through volcano analysis based on the threshold values of $|\log_2 FC| > 2$ and $-\log_{10} p\text{-value} > 2$ (Table S3 and Fig. 2C). These differential secondary metabolites were mainly belonged to the terpenoids (19) and alkaloids (7), most of which were higher in SeCM than in CM (Fig. 2D). Theses secondary metabolites such as atractylenolide III and methionine have been shown to be associated with preventing obesity (Song, Jung, Kang, & Park, 2017; Wang et al., 2020), suggesting that they play an important role in preventing obesity. In addition, partial differential secondary metabolites were identified in metabolic pathways-CM (cmt01100) and biosynthesis of secondary metabolites-CM (cmt01110) by the KEGG pathway analysis (Table S4).

3.3. CM and SeCM prevent HFD-induced obesity in mice

We found that Se-enriched treatment significantly increased the contents of total carbohydrate, soluble dietary fiber, and medium and long chain fatty acids of CM, among which some bioactive ingredients have been proven to possess a preventive effect on obesity (Basu et al., 2021; Panth, Abbott, Dias, Wynne, & Garg, 2018; Yu et al., 2021). Thus, it was further investigated whether Se enrichment has an impact on the obesity prevention of whole CM. Compared to the NMD group, mice fed with HFD had significantly increased bodyweight (Fig. 3A and B, $p < 0.001$). However, both CM and SeCM supplementations in HFD-fed mice significantly inhibited the body weight increase and final weight gain. The total energy intake and energy efficiency of HFD-fed mice were markedly higher than those of the HFD-fed mice after CM and SeCM supplementations (Fig. 2C and D, $p < 0.05$), suggesting that the effects of CM and SeCM on body weight might result from the reduction of food intake. We next examined whether CM and SeCM achieved the effects by alleviating hypertrophy of liver and fat. As shown in Fig. 3E–H, the weights of liver and epididymal, perirenal, and inguinal fat were markedly decreased after CM and SeCM supplementations ($p < 0.05$). Additionally, the alleviating effect on obesity in the HFD + CM group

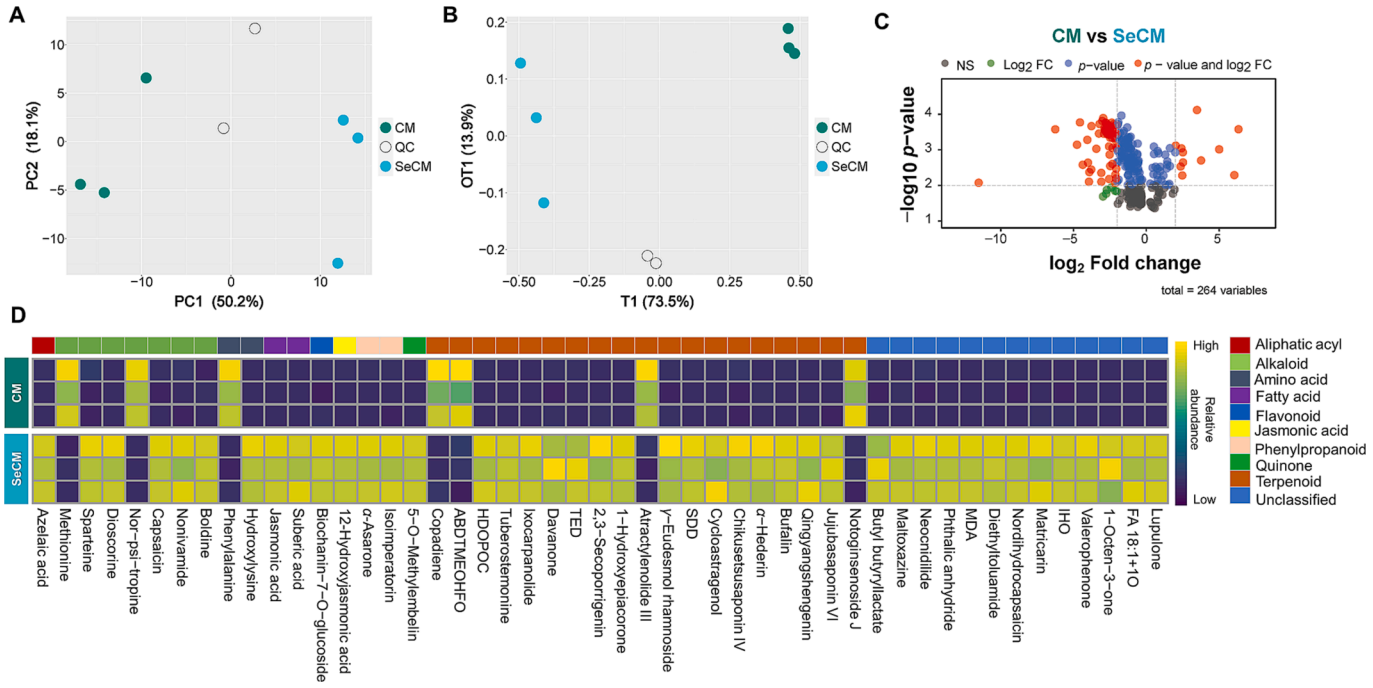

Fig. 2. Se enrichment changes the secondary metabolites in CM. PCA score plot (A), OPLS-DA score plot (B), volcano plot of altered metabolites with $|\log_2$Fold change$| > 2$, and p-value < 0.01 between CM and SeCM (C) and heatmap of the secondary metabolites in CM significantly altered by Se enrichment and the colors range from purple (low) to yellow (high) (D). CM, *Cordyceps militaris*; SeCM, Se-enriched *Cordyceps militaris;;* ABDTMEOHFO, [6−acetyloxy−4−butanoyloxy−3,3a−dihydroxy−3,6,9−trimethyl−8−[(E)−2−methylbut−2−enoyl]oxy−2−oxo−4,5,6a,7,8,9b− hexahydroazuleno[4,5−b]furan−7−yl]octanoate; HDOPOC, 7−hydroxy−1,4a−dimethyl−9−oxo−7−propan−2−yl−2,3,4,4b, 5,6,10,10a−octahydrophenanthrene−1−carboxylic acid; TED, 11,17,21−Trihydroxypregn−4−ene−3,20−dione; SDD, (22E,24R)−Stigmasta−4,22−diene−3,6−dione; MDA, (S)−p−Mentha−1,8−dien−10−yl acetate; IHO, 5−Isopropylbicyclo [3.1.0]hexan−2−oneABDTMEOHFO, [6−acetyloxy−4−butanoyloxy−3,3a−dihydroxy−3,6,9−trimethyl−8−[(E)−2−methylbut−2−enoyl] oxy−2−oxo−4,5,6a,7,8,9b− hexahydroazuleno[4,5−b]furan−7−yl]octanoate; HDOPOC, 7−hydroxy−1,4a−dimethyl−9−oxo−7−propan−2−yl−2,3,4,4b, 5,6,10,10a−octahydrophenanthrene−1−carboxylic acid; TED, 11,17,21−Trihydroxypregn−4−ene−3,20−dione; SDD, (22E,24R)−Stigmasta−4,22−diene−3,6−dione; MDA, (S)−p−Mentha−1,8−dien−10−yl acetate; IHO, 5−Isopropylbicyclo [3.1.0]hexan−2−one. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

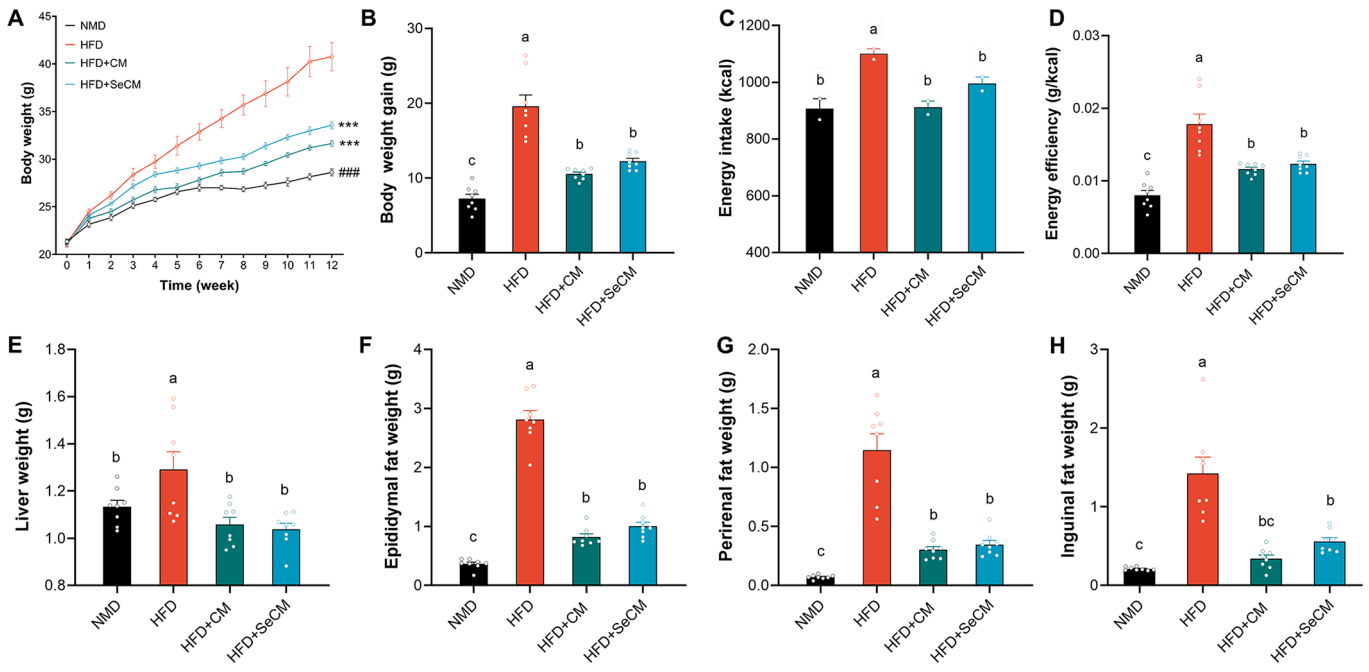

Fig. 3. Dietary CM and SeCM prevent obesity in HFD-fed mice. Body weight (A), body weight gain (B), total energy intake (C), energy efficiency (D), liver weight (E), epididymal fat (F), perirenal fat (G), and inguinal fat (H). NMD, normal diet; HFD, high-fat diet; CM, *Cordyceps militaris*; SeCM, Se-enriched *Cordyceps militaris*. Data are expressed as the mean ± SEM (n = 8). Body weight differences were analyzed using unpaired two-tailed Student's *t*-test with Bonferroni correction (*** $p <$ 0.001or ### $p <$ 0.001 versus the HFD group). Graph bars marked with different letters on top represent statistically significant results ($p < 0.05$).

was similar to that in the HFD + SeCM group.

3.4. CM and SeCM attenuate obesity-associated metabolic diseases in HFD-fed mice

Previous studies indicated that diet-induced obese mice would induce multiple metabolic diseases such as glucose tolerance and lipid disorder (Su et al., 2020; Zeng et al., 2020). Here, we found that CM and SeCM treatments significantly reduced fasting glucose (Fig. 4A, $p <$ 0.05), and the OGTT test exhibited a lower glucose level in CM- and SeCM-treated HFD-fed mice than in that fed HFD alone (Fig. 4B). In addition, the serum levels of TG, TC, LDL-c, and HDL-c in the HFD + CM

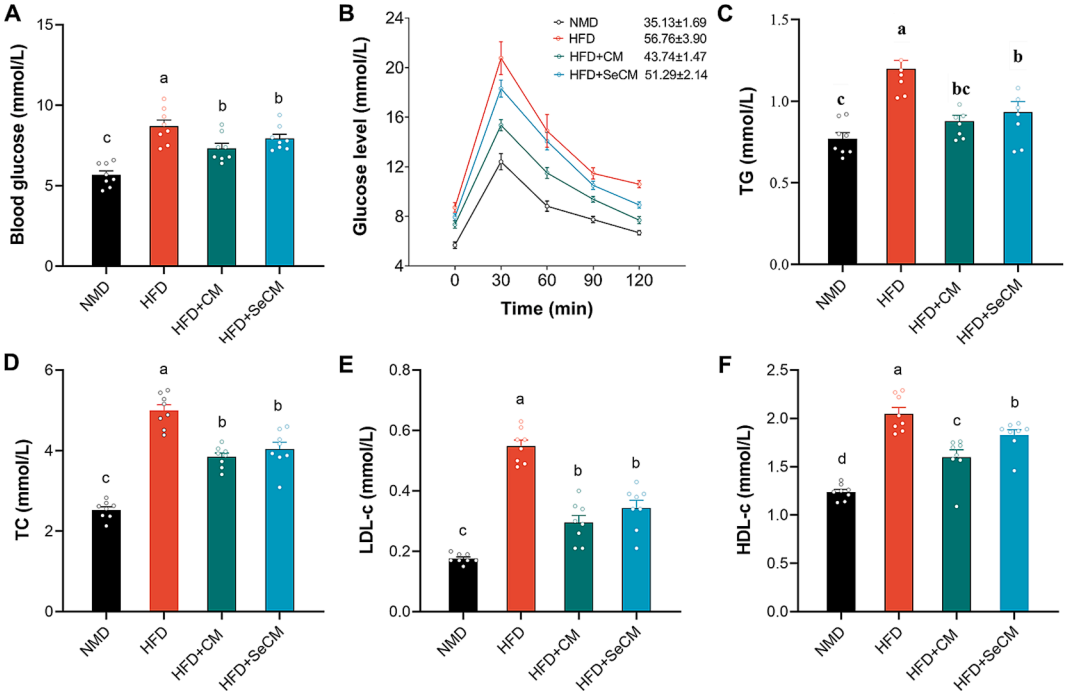

Fig. 4. Dietary CM and SeCM improve obesity-associated metabolic diseases in HFD-fed mice. Fasting blood glucose (A), oral glucose tolerance test (B), serum triglyceride (C), total cholesterol (D), LDL-c (E), and HDL-c contents (C-F). NMD, normal diet; HFD, high-fat diet; CM, *Cordyceps militaris*; SeCM, Se-enriched *Cordyceps militaris*; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol. Data are expressed as the mean ± SEM (n = 8). Graph bars marked with different letters on top represent statistically significant results ($p < 0.05$).

and HFD + SeCM groups were markedly reversed compared to those in the HFD group (Fig. 4C–F, $p < 0.05$). We also noted that CM and SeCM had similar effects on improving obesity-associated metabolic diseases.

3.5. Network pharmacological analysis of CM and SeCM on obesity

To explain the pharmacologic mechanisms of CM and SeCM on obesity, the effectively bioactive ingredients of CM and SeCM and molecular targets based on network pharmacology were performed in the present study. Based on the screening criteria procedure with OB $\geq$ 30% and DL $\geq$ 0.18, 55 and 53 effectively bioactive ingredients were identified in CM and SeCM, respectively (Table S5). After removing the duplication, a total of 805 targets related to CM and 806 targets related to SeCM were collected. Meanwhile, 2550 corresponding obesity targets were shortlisted after eliminating the duplicate values (Table S6). Finally, both CM and SeCM had 324 targets overlapping with the obesity targets after performing the Venn analysis (Fig. S1), suggesting that these molecular targets might mediate the preventive effects of CM and SeCM on obesity.

The potential mechanisms of the preventive effects of CM and SeCM on obesity were next investigated by a CM/SeCM-ingredients-obesity-targets network using Cytoscape. As shown in Fig. 5A, a total of 141 nodes and 831 edges were presented in this network, where red node represents obesity, 87 blue nodes represent the molecular targets between obesity and CM, green node represents CM, and 52 yellow nodes represent the active ingredients of CM. The nodes with the highest degree of connection to ingredients or targets were regarded as hub in the entire network, that is, the key active ingredients or targets. For instance, the ingredients genistein manifested the highest degree (84), followed by melatonine (80) and capsaicin (77), indicating that the obesity prevention effects of these key active ingredients in CM could be achieved via multiple targets. In addition, mitogen-activated protein kinase 1 (MAPK1), tumor protein P53 (TP53), and thyroid stimulating hormone receptor (TSHR) were separately linked to 41, 39, and 37 ingredients, respectively, suggesting that different ingredients could act on the same target in a synergistic manner. Notably, the above results of

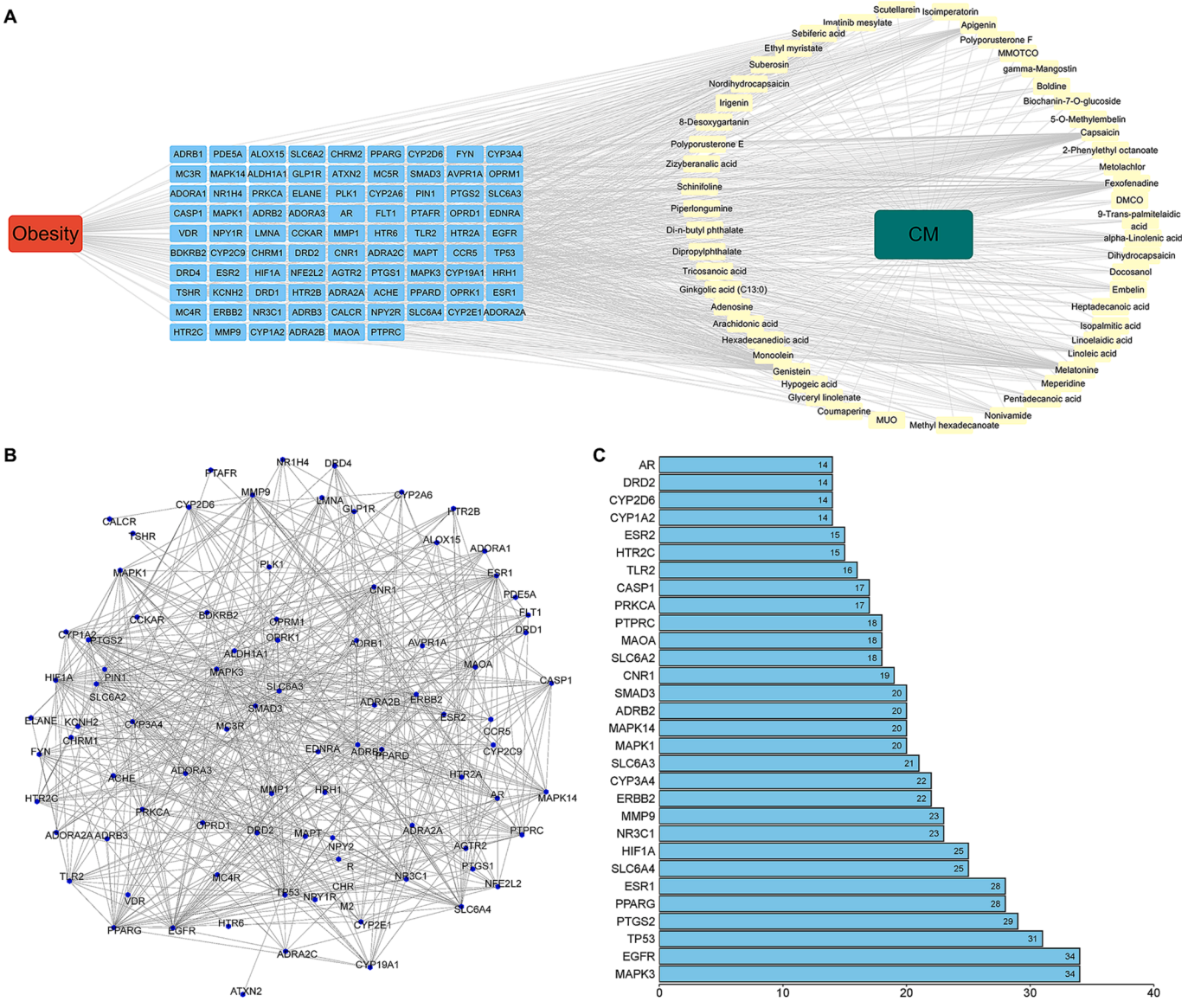

Fig. 5. Key bioactive ingredients and targets analysis of CM in preventing obesity. Bioactive ingredient-target-obesity network (A), protein–protein interaction (PPI) network plotting (B), and number of connectivity degree for the hub genes (C). Different color symbols in A represent: obesity (red), targets (blue), ingredients (yellow), and CM (green). The lines represent each side, indicating the interaction between them. CM, *Cordyceps militaris*; MMOTCO, 7-methyl-3-methylidene- 6-(3-oxobutyl)-4,7,8,8a-tetrahydro-3aH-cyclohepta[*b*]furan-2-one; DMCO, 4-(2,3-Dihydroxy-3-methyl butoxy)furo(3,2-g)chromen-7-one; MUO, 1-Methyl-2-undecylquinolin-4-one. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CM are similar to those of SeCM (Fig. S2A).

Based on STRING database, the PPI network was constructed by the molecular targets of CM and SeCM. A total of 87 nodes, 1002 edges were exhibited in the PPI network of CM (Fig. 5B), where nodes represent target protein and edges represent the protein–protein interaction. To further analyze the targets of CM, the number of proteins involved were ranked as shown in Fig. 5C. The results showed that MAPK3, epidermal growth factor receptor (EGFR), and TP53 were the top 3 targets of CM, which might be the key molecular targets of CM against HFD-induced obesity. Additionally, SeCM showed similar results (Fig. S2B and C).

To verify the biological characteristics of the molecular targets of CM, the relevant biologic processes were clarified by GO enrichment analysis. In this study, the top 10 GO terms of each category, namely biological process (BP), cellular component (CC), molecular function (MF), were illustrated in Fig. 6A. The results showed that the positive regulation of MAPK cascade was the most critical function of CM in preventing obesity. We further analyzed the potential pathways of CM involvement in obesity prevention using KEGG pathway enrichment analysis. As shown in Fig. 6B, 10 main signaling pathways were determined, of which the obesity-related calcium signaling pathway is the most significant. Similarly, these results were also found in the GO enrichment analysis and KEGG pathway enrichment analysis of SeCM (Fig. S3). Overall, these findings establish that CM and SeCM exert obesity prevention via multiple ingredients acting on multiple targets.

4. Discussion

Here, our results showed that Se enrichment significantly enhanced the content of Se in CM, consistent with prior studies (Dong et al., 2012; Wang et al., 2018). Various mushroom species, including CM, have exhibited a significant augmentation at nutritional level, such as total carbohydrates and proteins, following the process of Se enrichment (Dong et al., 2012; Dong et al., 2021; Wang et al., 2018). In this study, the enrichment of Se did indeed elevate the overall carbohydrate content in CM. However, we observed a decrease in the protein content, which could be attributed to the unique cultivation condition or the employed detection method (Wiesner-Reinhold et al., 2017). From the perspective of amino acid composition, it could be explained that Se enrichment significantly inhibited the content of most amino acids like 4-hydroxy-L-proline, arginine, serine, glycine, threonine, aminobutyric acid, proline, tyrosine, isoleucine, and phenylalanine in addition to proline. Recently, it has been reported that Se enrichment does not affect the content of total dietary fiber (Dong et al., 2021). We found, however, that Se enrichment changes the content of soluble and insoluble dietary fiber in CM, which is a novel finding. Furthermore, our results revealed that the contents of MLFA in CM and SeCM were significantly altered by Se enrichment, while the total fat content remained the same. These findings highlight that we cannot simply evaluate the impact of Se enrichment on the nutritive value of CM from its major nutrients, and need to further analyze its impact on their compositions.

Furthermore, for the first time, the impact of Se enrichment on secondary metabolites in CM was comprehensively explored in the present study. These secondary metabolites, such as phenols, flavonoids, terpenoids and alkaloids, possess favorable effects on human nutrition and health when consumed as part of our diet (Abdalla & Mühling, 2023; Kabera, Semana, Mussa, & He, 2014). Previous studies primarily focused on the impact of Se enrichment on secondary metabolites in plants (Tavakoli, Enteshari, & Yousefifard, 2020; Xiang et al., 2022; Zhu, Zhang, Liu, Chen, & Zhang, 2018). For instance, Se enrichment promoted the production of secondary metabolites such as phenols, flavonoids and theanine in tea (Xiang et al., 2022), flavonoids in tomato fruit (Zhu, Zhang, Liu, Chen, & Zhang, 2018), and terpenoids in *Melissa officinalis* (Tavakoli, Enteshari, & Yousefifard, 2020). In the current investigation, the application of Se enrichment resulted in a significant

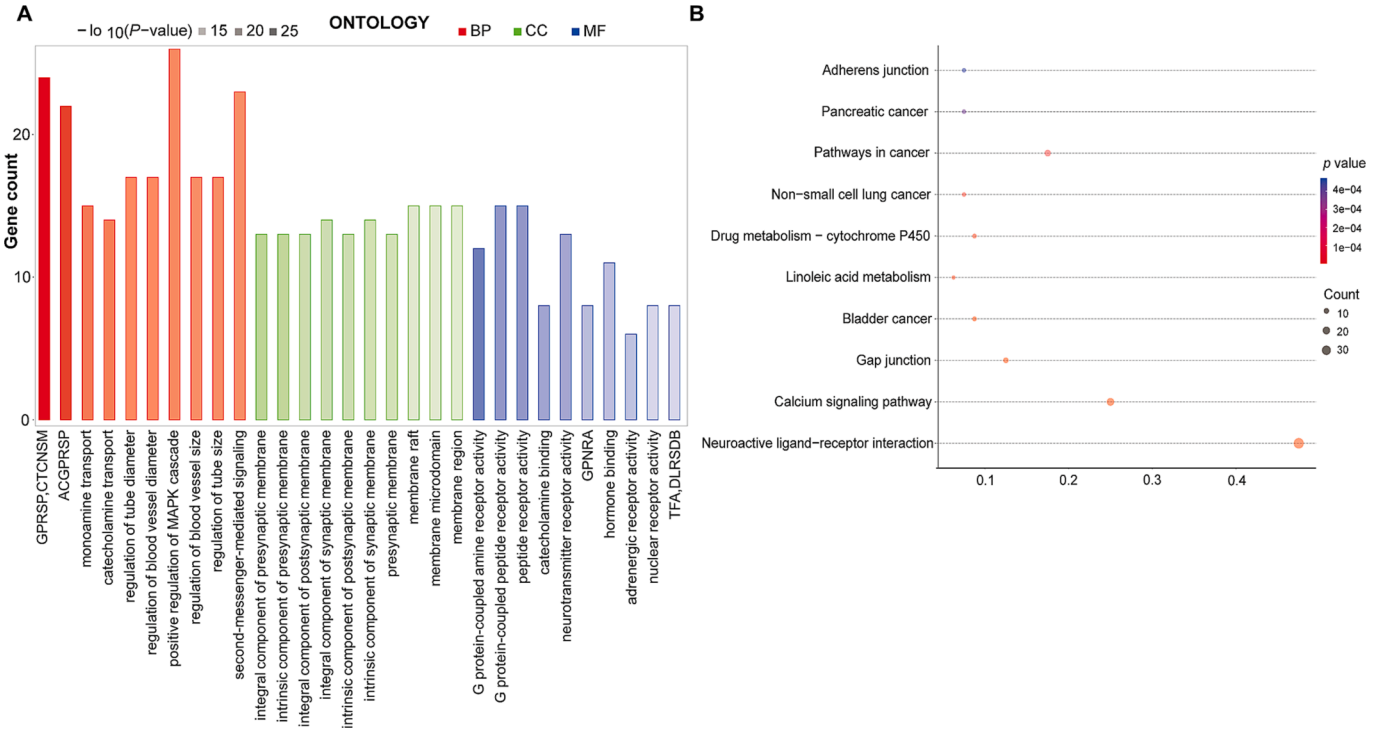

Fig. 6. Enrichment analyses of key targets of CM. GO enrichment analysis of target proteins (A), and bubble diagram of KEGG pathway annotation (B). GO: The number of GO entries in the functional categories of biological process (BP), cellular component (CC) and molecular function (MF). KEGG: Y-axis label represents signaling pathway and X-axis label represents gene ratio. Gradual changes in color represent probability changes. CM, *Cordyceps militaris*; GPRSP-CTCNSM, G protein-coupled receptor signaling pathway-coupled to cyclic nucleotide second messenger; ACGPRSP, adenylate cyclase-modulating G protein-coupled receptor signaling pathway; GPNRA, G protein-coupled neurotransmitter receptor activity; TFA-DLRSDB, transcription factor activity-direct ligand regulated sequence specific DNA binding.

elevation of secondary metabolites, specifically terpenoids and alkaloids. Jia, Liu, Shi, & Chu (2020) have demonstrated that Se enrichment could remarkably promote the synthesis of some secondary metabolites in plants, as revealed by the KEGG pathway analysis in this study. That is, the changed secondary metabolites were attributed to the metabolic pathways and biosynthesis of secondary metabolites. The present results point out the interplay between Se regulation and secondary metabolite biosynthesis in CM and help further the understanding of the molecular mechanism of how Se affects the nutritional value of CM.

Apart from the above-mentioned nutritional value being influenced by Se enrichment at both macro and micro levels, it has also been found to possess an impact on various biological activities. For example, the antioxidant activity of mulberry wine was reported to be significantly improved by Se enrichment (Ekumah et al., 2021). Likewise, Se-enriched tea mixed peptides had a superior ACE-inhibiting activity in comparison to ordinary tea (Zhu et al., 2020). These studies provide support for the notion that Se enrichment enhances the biological activities of original substances, which could be explained in part by their different nutritional ingredients (Wu et al., 2022). Nevertheless, evidence also indicates that Se-enriched and ordinary black teas have similar effects in regulating glucose and lipid metabolism (Shang, Li, Zhu, Sun, & Wang, 2022). Consistent with this finding, daily supplementation of CM and SeCM produced comparable obesity prevention effects, despite having considerable differences in nutritional ingredients between them. In the network pharmacological analysis, our study found that there was no significant difference between CM and SeCM in the bioactive ingredients that play a role in preventing obesity in the host. Therefore, we speculated that the impact of Se enrichment on their biological activities might not be affected by changes in their nutritional ingredients, but by the ingredients that could truly exert their effects in host.

Dietary supplementations of CM and SeCM markedly reduce fat accumulation, impaired glucose tolerance, and lipid levels in HFD-fed mice, which are the three main pathological phenotypes of obesity (Zhu et al., 2022). Thus, this study confirmed the beneficial effects of CM and SeCM on diet-induced obesity. Furthermore, the material basis, targets, potential molecular mechanisms of CM and SeCM in preventing obesity were also elucidated by the network pharmacological analysis. In detail, a total of 52 ingredients and 87 genes were considered to be the material basis and molecular targets of CM (taking CM as an example since the results of SeCM are basically consistent with it) in preventing obesity, respectively. Among these ingredients, genistein, melatonine, and capsaicin interact most with obesity-related targets, which have been reported to show biological activities against obesity and associated metabolic diseases (Karamitri & Jockers, 2019; Li et al., 2020; Li, Zhou, Zhang, Yu, & Xiao, 2022). In addition, MAPK1, TP53, and TSHR were found to be the most relevant molecular targets for these active ingredients. Moreover, the PPI network, used to evaluate the key biological processes in living cells (Li et al., 2021), highlighted the targets proteins of MAPK3, EGFR, and TP53. Previous studies confirmed that MAPK1, MAPK3, and TP53 are vital in regulating obesity (Suriagandhi & Nachiappan, 2022; Tian et al., 2022), suggesting that they could represent the critical molecular targets that mediate CM to prevent obesity. Indeed, GO analysis indicated that the positive regulation of MAPK cascade was the most critical function in the preventive effect of CM on obesity, which had been observed to have regulatory effects on molecular targets such as MAPK1, MAPK3, and TP53 (Wu et al., 2022). Moreover, KEGG analysis showed that the obesity-related calcium signaling pathway was significantly enriched by CM, and its role in regulating obesity is mainly through the p38-MAPK signaling pathway (Song, Wang, Zhang, Yao, & Sun, 2019). These findings also showed that the MAPK signing pathway play an important role on the obesity prevention effect of CM. Taken together, the main active ingredients in CM and SeCM, genistein, melatonine and capsaicin, exert obesity prevention by acting on the core targets of MAPK1, MAPK3, and TP53, mainly regulating the MAPK signaling pathway. However, further validation in 3T3-L1 adipocytes and in the high-fat diet-induced obesity mouse model is necessary.

5. Conclusions

Our observations suggest that Se enrichment significantly changes the nutritional ingredients of CM at both macro and micro levels, thereby improving its nutritive value to a certain extent. Nevertheless, it does not affect the preventive effect of CM on obesity, which may be ascribed to the similar bioactive ingredients shared by CM and SeCM in the host. Furthermore, network pharmacology offers a research foundation to support the therapeutic potential of CM and SeCM in preventing obesity, but these findings should be verified by *in vitro* and *in vivo* experiments in further research.

CRediT authorship contribution statement

Zhenjun Zhu: Data curation, Funding acquisition, Conceptualization, Writing - original draft. **Aohuan Huang:** Investigation, Data curation, Validation. **Mengfei Chen:** Formal analysis. **Juan Wang:** Writing – review & editing. **Zeyang Li:** Investigation, Software. **Zhongxu Sun:** Investigation. **Yiheng Ye:** Investigation. **Jingwei Nan:** Formal analysis. **Shubo Yu:** Investigation, Formal analysis. **Moutong Chen:** Methodology, Investigation. **Yizhen Xie:** Resources. **Huiping Hu:** Resources. **Jumei Zhang:** Supervision. **Qingping Wu:** Project administration, Funding acquisition. **Yu Ding:** Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was funded by the Strategic Research and Consulting Project of Chinese Academy of Engineering (2022-GD-5), State Key Laboratory of Applied Microbiology Southern China (SKLAM011-2021), Guangzhou Basic and Applied Basic Research Project (202201010197), National Natural Science Foundation of China (32202014), Guangdong Basic and Applied Basic Research Foundation (2023A1515010744), and Open Project Program of State Key Laboratory of Food Science and Technology, Nanchang University (SKLF-KF-202222).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100788.

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