Research Article

Enhancing Antioxidant Activity and Bioactive Compound Production of Cordyceps Mushroom Using Quinoa as an Alternative Substrate

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Abstract
In the commercial production of *Cordyceps militaris*, cereal grains are commonly used as cultivation substrates, influencing both growth and bioactive metabolite profiles. This study evaluated the effectiveness of pseudocereals as alternative substrates by cultivating *C. militaris* on solid media containing different ratios of jasmine brown rice to quinoa (0%, 25%, 50%, 75%, and 100% w/w) and assessing the impact on bioactive metabolite production, including cordycepin, adenosine, total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activities. The methanolic extract of fruiting bodies grown on 75% quinoa medium (75-SMQ) exhibited the highest cordycepin (8.71 ± 0.80 mg/g dry weight [DW]) and adenosine (0.20 ± 0.01 mg/g DW) levels ($p \leq 0.05$). Meanwhile, the 100% quinoa medium (100-SMQ) enhanced TPC (13.90 ± 0.02 mg GAE/g DW), TFC (16.70 ± 0.17 mg RE/g DW), and antioxidant activities, with the methanolic extract showing a strong DPPH radical scavenging capacity (3.22 ± 0.02 mg AAE/g DW; IC_{50} = 0.71 ± 0.01 mg/mL; % inhibition = 77.20 ± 0.11) and a ferric-reducing ability power of 4.42 ± 0.01 mg AAE/g DW. These findings demonstrate the feasibility of using quinoa as an effective substrate for large-scale *C. militaris* cultivation, providing an efficient approach to enhance bioactive compound production and antioxidant activities for various industrial applications.

Keywords: Antioxidant activities, Cordycepin and Adenosine, *Cordyceps militaris*, Cultivation method, Medicinal mushroom, Quinoa

1 Introduction

Medicinal mushrooms have recently received more attention due to their bioactive compounds and pharmacological benefits. According to the World Health Organization (WHO), a significant portion of the global population currently uses mushrooms and herbs to treat various diseases [1]. Numerous studies have highlighted the health benefits, nutritional value, and therapeutic potential of mushrooms for drug development and healthcare products, identifying them as valuable sources of bioactive compounds with medicinal properties [2]–[4]. Among medicinal mushrooms, *Cordyceps militaris* is both edible and medicinal, widely valued for its health benefits and therapeutic properties, especially in East Asia [5]. *C. militaris* belongs to the phylum Ascomycota [6] and grows as a parasite in Lepidopteran pupae, producing yellow to orange fruiting bodies [6]. These fruiting bodies are widely utilized in the pharmaceutical and healthcare industries due to their abundance of bioactive compounds, including secondary metabolites, nucleosides, cordycepic acid, carotenoids, enzymes, sterols, ergosterol, and superoxide dismutase (SOD) [2], [7]. The mycelium and fruiting bodies contain key bioactive compounds and nutrients, including cordycepin (3'-deoxyadenosine), adenosine, proteins, polysaccharides, phenolic compounds, and other essential nutrients [8]–[12]. These compounds exhibit significant bioactivity and pharmacological effects. Recent studies have shown that *C. militaris* extracts possess antioxidant, antimicrobial, anti-aging, antibacterial, neuroprotective, anti-inflammatory, antitumor, and anticancer properties, primarily due to their rich bioactive components, such as adenosine, cordycepin, and polysaccharides [7]. Among these, cordycepin is the key active compound and has been extensively studied for its medicinal and nutraceutical potential. Additionally, *C. militaris* extracts contain potent antioxidants, including phenolic compounds, ergothioneine, glutathione, selenium, vitamins, and carotenoids, which help neutralize free radicals [7], [8]. Moreover, *C. militaris* is recognized for its ability to enhance energy levels, boost metabolism, and improve physical and sexual performance, making it a valuable functional ingredient for promoting vitality and overall well-being.

Currently, numerous manufacturers commercially produce Cordyceps products using fruiting bodies and mycelium cultivated on artificial media (both solid and liquid) through highly optimized cultivation methods [2]. Consequently, market demand is continuously increasing, prompting extensive development of artificial cultivation for large-scale production. *C. militaris* is known for its adaptability and well-documented ability to produce high-value bioactive compounds. Its cultivation is more feasible in controlled environments compared to other mushroom species, increasing the likelihood of obtaining reliable and reproducible experimental results [13], Fungal growth and metabolite production are influenced by various factors, including light, temperature, media composition, pH, and environmental conditions, with media composition being one of the most critical. Several studies have investigated the effects of different carbon and nitrogen sources on bioactive compound production. The use of food grains as substrates for mushroom cultivation is increasingly recognized for its potential to enhance yield and bioactive compound production. Using the grains as a substrate for mushroom cultivation is promising, as the resulting compost can be repurposed as a supplement, animal feed, or health-promoting product rich in bioactive compounds. This approach also supports the principles of a circular economy. Additionally, mushroom cultivation plays a key role in enhancing food security by providing a high-protein, nutrient-rich food source. By optimizing the use of food grains, we can improve yields, support sustainable practices, and contribute to a more efficient and resilient food production system, reducing waste and maximizing the value of agricultural resources.

Formulating a new recipe for *C. militaris* production is essential to enhance yield, bioactive compound content, and economic viability. Conventional cultivation techniques may not fully exploit the mushroom's biosynthetic potential, particularly with respect to the production of valuable metabolites such as cordycepin and antioxidants. By incorporating novel substrates, it can create a more nutrient-rich growing medium, leading to improved growth rates and higher bioactive compound production. Additionally, optimizing the formula can differentiate the final product in the functional food or pharmaceutical markets. Pseudocereals present an intriguing option, as they differ from traditional cereals in both their morphological characteristics and chemical compositions, particularly their higher protein content. Notably, quinoa (*Chenopodium quinoa* Willd.) has gained recognition as one of the "grains of

the twenty-first century," with its cultivation expanding globally [14]. Quinoa seeds are rich in high-quality proteins, containing all nine essential amino acids, unlike common cereals [15]. They are primarily composed of soluble proteins and have a carbohydrate content ranging from 51% to 61% on a dry weight basis [16]. Additionally, quinoa provides essential vitamins (e.g., B and E), antioxidants, and minerals such as potassium, magnesium, calcium, and manganese [17]. Despite its potential, little is known about using quinoa as a substrate for *C. militaris* cultivation. The presence of bioactive compounds, including polyphenols and saponins, may further stimulate the biosynthesis of secondary metabolites such as cordycepin and antioxidant compounds in *C. militaris* [18]. This study aims to develop an optimal solid medium formulation using brown rice and quinoa to maximize the production of bioactive compounds, such as cordycepin and adenosine, and enhance antioxidant activity. Our findings may offer valuable insights for optimizing *C. militaris* cultivation, contributing to various industries, and fostering economic development.

2 Materials and Methods

2.1 *Chemicals and materials*

A strain of *C. militaris* was obtained from Sunanta Farm in Thailand and cultured in a liquid medium. The cultivation substrates included jasmine brown rice varieties purchased from local stores in Nonthaburi province, Thailand, and white quinoa varieties bought from Raw Food brand stores in Thailand. These substrates were washed three times with tap water. Methanol (99.9% purity, analytical reagent grade) and methanol for HPLC were used in the study. Standards of cordycepin and adenosine were acquired from Sigma Chemical Corporation (Saint Louis, MO, USA).

2.2 *Preparation of seed culture of C. militaris*

The fungus was cultured on solid potato dextrose agar (PDA) in petri dishes. The mycelium was allowed to grow in the dark at 20 °C for 7 days. Small sections of mycelium (2–3 pieces) were inoculated into 100 mL fresh potato dextrose broth (PDB) and incubated at 22 °C for 7–14 days under agitation conditions. The culture was routinely performed to prepare intact mycelium for being used in further experiments.

2.3 *Cultivation of C. militaris in solid media*

The fungus was cultured on modified solid medium (MSM) using jasmine brown rice (SMJ) and modified quinoa media (SMQ) with varying substrate ratios of jasmine brown rice to quinoa, specifically in the following conditions: 100:0 (100-SMJ), 75:25 (25-SMQ), 50:50 (50-SMQ), 25:75 (75-SMQ), and 0:100% (100-SMQ) w/w. These substrate ratios were placed into 18-ounce bottles. Modified potato dextrose broth (MPDB) was prepared by combining three eggs (approximately 180–200 g), 5 g of glucose, and 1000 mL of distilled water. The mixture was blended, filtered, and added into the bottles at a volume of 30 mL per bottle. After sterilization, 5 mL of liquid mycelium culture was aseptically introduced into each MSM bottle, which were then incubated at 20 °C with 70–80% humidity in the dark for two weeks to allow the medium to become overgrown with white mycelium. Following this, the bottles were exposed to light at an intensity of 500 lux to induce stroma development [19]. Harvesting occurred after eight weeks, with the height of the *C. militaris* fruiting bodies measured to assess growth characteristics and morphology within the bottles. The average height for each treatment was estimated, and the dried weight was evaluated after dehydration at 60 °C for 6 h [20].

2.4 *Preparation of C. militaris extracts*

The dried fruiting body (2 g) was ground and placed in a clean volumetric flask. It was then extracted using 60% methanol at a 1:10 (w/v) ratio as the solvent, and the flask was tightly sealed. Ultrasonication was done at 35 kHz and 320 watts at 30° for 25 min. The mixture was clarified by centrifugation. The supernatants were harvested and filtered through a 0.22 µm nylon membrane filter. The crude extracts of *C. militaris* were kept at 4 °C for further analysis.

2.5 *The analysis of bioactive compounds quantity*

The content of cordycepin and adenosine was quantified using an Agilent 1200 series HPLC system (Agilent Technologies, USA). The chromatographic conditions were optimized as follows: a C18 column (150 mm × 4.6 mm, 5 µm, Agilent) was utilized, with the column temperature precisely controlled at 30 °C. The mobile phase comprised 20% methanol (v/v, pH 6), and the flow rate was maintained at 1 mL/min. A 20 µL volume of the sample extract was injected into the

system, with detection carried out at a wavelength of 260 nm using a UV-visible detector. Quantitative analysis was performed by constructing a calibration curve (0.2–1 mg/mL) to accurately determine the concentrations of cordycepin and adenosine, which were then expressed as mg/g dry weight (DW) based on the peak area.

2.6 *Determination of total phenolic content (TPC)*

The total phenolic content was determined following the Folin–Ciocalteu reagent method with minor modifications [21]. Briefly, the extract was mixed with 10% Folin-Ciocalteau reagent at a 1:7.5 ratio and incubated at room temperature for 15 min. At the indicated time, sodium carbonate solution (6% Na_2CO_3) was added and incubated in the dark for another 90 min. The reaction was measured at a 725 nm wavelength using a spectrophotometer (Hanon Instruments, Japan). Various concentration of gallic acid was used to generate a calibrating curve for quantification. TPC was expressed as gallic acid equivalents (GAE) in mg/g dry weight.

2.7 *Determination of total flavonoid content (TFC)*

TFC determination was performed following the aluminum colorimetric assay as previously described with minor modifications [22]. Briefly, 200 µL of *C. militaris* extract was mixed with 2.3 mL of a methanol-water solution (30:70, v/v) and 100 µL of 0.5 M $NaNO_2$ solution, followed by 100 µL of 0.3 M $AlCl_3$ solution. The mixture was vortexed and incubated for 5 min in the dark at room temperature. Absorbance was measured immediately at a 506 nm wavelength against a blank sample. Normalized data were calculated using a rutin standard calibration curve (0.1–1 mg/mL) and expressed as rutin equivalents per gram of extract (mg RE/g dry weight).

2.8 *Determination of antioxidant activities of C. militaris extracts*

2.8.1 DPPH radical scavenging activity assay

The *in vitro* scavenging capability of *C. militaris* extracts against free radicals was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, following the method described by Tinrat [21]. Briefly, 100 µL of sample extracts were mixed with 900 µL of 0.1 mM DPPH reagent solution in methanol. After thorough mixing, the solution was left undisturbed in the dark at room temperature for 30 min. The change in color of the mixture was then measured immediately using a spectrophotometer at 517 nm. The IC_{50} values were determined through non-linear regression analysis as a measure of antioxidant activity. Ascorbic acid (0.1–0.8 mg/mL) was used as a calibrating curve, and results were reported as milligrams of ascorbic acid equivalent antioxidant capacity per gram of extract (mg AAE/g dry weight). %inhibition was calculated according to the formula in Equation (1)

$$\% \text{ Inhibition} = \frac{\text{Ac - As}}{\text{Ac}} \times 100 \quad (1)$$

where Ac represents the absorbance of the blank group (DPPH solution), and As represents the absorbance of the sample extracts at 517 nm.

2.8.2 Ferric-reducing antioxidant power assay (FRAP)

The reducing power of the sample extracts was determined using the Fe(II)-TPTZ complex method, following the protocol by Tinrat [21]. A freshly prepared FRAP reagent in 0.3 M acetate buffer at pH 3.6 was warmed to 37 °C and mixed with 300 µL of *C. militaris* extracts at a concentration of 0.2 mg/mL, then incubated for 30 min in a water bath. The discoloration of the blue-colored solution was measured at 596 nm. A standard curve was prepared using ascorbic acid solutions ranging from 0.05 to 0.8 mg/mL. The results, reflecting the antioxidative capacity of the samples, were reported as mg AAE/g dry weight.

2.9 *Mycelium characteristics of C. militaris*

The MSM samples containing *C. militaris* mycelium were analyzed using a scanning electron microscope (SEM) with a Quanta 450 series (FEI, USA) at a magnification of 5000x, 9.4 nm resolution, and 10.0 kV. Prior to analysis, the MSM samples were coated with a layer of gold, attached to a carbon tape-covered stub, and mounted onto a sample holder.

2.10 *Statistical analysis*

The tests were performed in triplicate, and differences observed from cultivating *C. militaris* on different substrates were reported as mean ± standard deviation. All experimental statistical analyses were conducted using one-way ANOVA with a confidence level of

95% ($p \leq 0.05$). The study compared variations among multiple groups and assessed the significance of these differences using the Tukey-Kramer multiple comparison test at a 95% confidence interval. Data analysis was carried out with SPSS software version 28.0 (SPSS Inc., USA), and differences with *p*-values less than 0.05 were considered statistically significant.

3 Results and Discussion

3.1 *Growth and biomass of C. militaris*

The fruiting bodies of *C. militaris* were cultivated on modified solid media (MSM) prepared with varying ratios of a cereal (jasmine brown rice) and a pseudo-cereal (quinoa) across five conditions: 100:0, 75:25, 50:50, 25:75, and 0:100% (w/w).

Mycelial growth was observed to completely colonize the surface of the medium within six days under all treatment conditions. Following two weeks of cultivation under light exposure, the mycelium began to exhibit yellow pigmentation, gradually darkening from white to orange-yellow as the culture matured. Concurrently, the mycelial mat became visibly thicker.

The fruiting bodies, which generally exhibited a club-shaped morphology, were harvested after eight weeks of cultivation (Figure 1). All substrate formulations successfully supported fruiting body development, with consistent morphological features including orange-yellow coloration and clustered strip formations. These findings suggest that quinoa is a viable component in MSM formulations for *C. militaris* cultivation, without compromising key structural or visual characteristics of the fruiting bodies. The comparable appearance across treatments further supports the feasibility of using quinoa as a substrate to potentially enhance cultivation outcomes.

Table 1 shows the growth performance of *C. militaris* cultivated on various modified solid media (MSM) containing different proportions of steamed jasmine brown rice and quinoa. Among all formulations, the 50-SMQ (50% steamed jasmine brown rice and 50% quinoa) yielded the longest fruiting bodies, with an average length of 5.40 ± 0.10 cm, significantly higher than all other treatments ($p \leq 0.05$). This result suggests that a balanced mixture of jasmine brown rice and quinoa provides an optimal nutrient composition that supports the vertical growth of the fruiting bodies. In contrast, the 100-SMJ control group (100% jasmine brown rice) produced the shortest fruiting bodies at 4.17 ± 0.29 cm, indicating that quinoa supplementation enhances morphological development. Other treatments, including 25-SMQ, 75-SMQ, and 100-SMQ, exhibited intermediate lengths ranging from 4.13 to 4.87 cm.

In terms of yield, the highest dry weight of fruiting bodies was recorded in the 100-SMQ (4.87 ± 0.16 g DW) and 50-SMQ (4.80 ± 0.29 g DW) groups. These yields were significantly greater than that of the 100-SMJ control (2.72 ± 0.20 g DW), further confirming the growth-promoting effect of quinoa. Although the 100-SMQ formulation resulted in the highest yield, the fruiting body length was shorter than in the 50-SMQ group, indicating that a balanced ratio of quinoa and brown rice may provide a better overall morphology-to-yield outcome. The 75-SMQ group had a slightly lower yield (4.10 ± 0.07 g DW), suggesting that an excess proportion of quinoa may not further enhance biomass production.

The enhanced growth performance observed in quinoa-containing formulations may be attributed to the superior nutritional profile of quinoa, which includes higher levels of protein, essential amino acids, vitamins, and minerals compared to traditional rice-based media. These nutrients likely contributed to improved mycelial metabolism and more robust fruiting body development. This observation is consistent with the findings of Sánchez-García *et al.* [17], who reported that quinoa, when used as a carbon source, significantly enhanced the growth of *Pleurotus ostreatus*. These results underscore the critical role of carbon sources in promoting fruiting body biomass, as further supported by previous studies [23]. In addition to substrate composition, the height and development of fruiting bodies are influenced by other environmental factors, including air exchange, temperature, humidity, and light intensity [24], which were controlled uniformly in this study to ensure consistency across treatments.

Figure 1: Morphology of *C. militaris* cultivated for 8 weeks on various modified solid media (MSM) with different ratios of cereal and pseudo-cereal (jasmine brown rice: quinoa), observed in glass jar (a) 100-SMJ formula (100:0), (b) 25-SMQ formula (75:25), (c) 50-SMQ formula (50:50), (d) 75-SMQ formula (25:75), and (e) 100-SMQ formula (0:100).

Table 1: Effect of various modified solid media on *C. militaris* growth.

Formulas	Length of Fruiting Body (cm)	Yield of Fruiting Body (g DW)	Fruiting Body Appearance
100-SMJ	4.17 ± 0.29^{ab}	2.72 ± 0.20^{c}	range-yellow, clustered strips
25-SMQ	4.87 ± 0.71^{ab}	4.67 ± 0.22^{a}	range-yellow, strips
50-SMQ	5.40 ± 0.10^{a}	4.80 ± 0.29^{a}	range-yellow, strips
75-SMQ	4.13 ± 0.64^{ab}	4.10 ± 0.07^{b}	range-yellow, strips
100-SMQ	4.50 ± 0.53^{b}	4.87 ± 0.16^{a}	range-yellow, clustered strips

Results are presented as the mean values of three experimental replicates with standard deviation. Significant differences are indicated by letters a, b, and c at a 95% confidence level ($p \leq 0.05$).

3.2 *Bioactive compounds content in the fruiting body of C. militaris*

Cordycepin and adenosine were identified as the primary bioactive compounds in the fruiting bodies of *C. militaris* after 8 weeks of cultivation, as determined by HPLC analysis. Their concentrations were evaluated across five modified solid media (MSM) formulations containing different ratios of jasmine brown rice and quinoa: 100-SMJ (0% quinoa), 25-SMQ, 50-SMQ, 75-SMQ, and 100-SMQ. These formulations were developed without the use of insect larvae, which are commonly used in traditional *Cordyceps* cultivation. This highlights a plant-based and potentially more acceptable method for broader consumer markets, including vegetarians and those concerned about allergen risks.

As shown in Figure 2(a), all quinoa-containing MSM formulations significantly increased cordycepin content compared to the control group (100-SMJ), which yielded 5.58 ± 0.71 mg/g dry weight (DW). The highest cordycepin content, 8.71 ± 0.80 mg/g DW, was recorded in the 75-SMQ group. These values are particularly noteworthy given that previous studies reported commercial *C. militaris* cultivated on rice or wheat substrates typically contain less than 1 mg/g of cordycepin after similar cultivation durations [25]. This demonstrates the strong potential of quinoa-enriched media for improving bioactive compound yields.

Adenosine production followed a similar pattern (Figure 2(b)). The 100-SMJ control yielded the lowest concentration (0.09 ± 0.01 mg/g DW), while the 75-SMQ formulation exhibited the highest level at 0.20 ± 0.01 mg/g DW. All quinoa-based formulations significantly outperformed the control, indicating that quinoa supplementation benefits both bioactive compounds in *C. militaris*.

These findings are especially important considering the regulatory standards set by the Thai Food and Drug Administration (Thai FDA), which stipulate minimum levels of 0.3 mg/g DW for cordycepin and 1.7 mg/g DW for adenosine in Cordyceps-based products [26]. While adenosine levels in this study remained below the regulatory threshold, cordycepin concentrations in all formulations, including the control, exceeded the minimum requirement. The ability to surpass the cordycepin benchmark without relying on insect-based substrates supports the commercial and ethical viability of this cultivation approach.

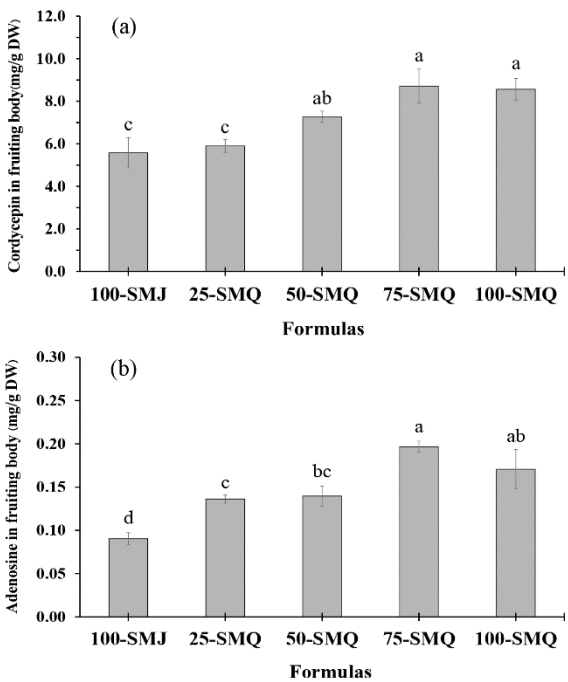

Figure 2: Comparison of *C. militaris* cultivated on different formulas (a) cordycepin production in the fruiting body, (b) adenosine production in the fruiting body. Error bars indicate the standard deviations (SD) from three independent experiments; mean significant differences are indicated by letters a, b, and c at a 95% confidence level ($p \leq 0.05$).

The enhanced production of these bioactive compounds can be attributed to quinoa's superior nutritional profile. Rich in proteins, essential amino acids, vitamins, and minerals, quinoa provides vital carbon and nitrogen sources necessary for fungal growth and metabolite synthesis [16], [27]. This aligns with previous findings showing that quinoa supplementation promotes the growth and metabolite production of fungi like *Pleurotus ostreatus* [17].

Moreover, quinoa supplementation may positively alter the physical characteristics of the substrate. Its incorporation into the solid medium can enhance the surface area for cell immobilization, improve oxygen diffusion, and stabilize the microenvironment, thereby facilitating the biosynthesis of secondary metabolites such as cordycepin and adenosine. However, it is important to note that even with the same microbial species, changes to the initial substrate, whether physical or chemical, can affect the overall cultivation process and final yields [28]–[31].

Additionally, the incorporation of quinoa into the solid medium may enhance the physical properties of the substrate by increasing the surface area available for mycelial attachment, improving oxygen diffusion, and stabilizing the internal microenvironment. These factors collectively contribute to the elevated production of cordycepin and adenosine observed in this study [32]. However, it is important to recognize that the levels of bioactive compounds can be influenced by multiple variables, including the substrate composition, cultivation techniques, fungal strain characteristics, environmental conditions, duration of cultivation, and the methods used for extraction and quantification. These factors often complicate direct comparisons across different studies [33].

3.3 *Antioxidant activity*

3.3.1 Quantification of the total phenolic and flavonoid contents of C. militaris extracts

C. militaris is widely known for its abundance of bioactive compounds such as cordycepin, ergothioneine, adenosine, γ-aminobutyric acid, sterols, phenolics, and vitamins [34], [35]. These compounds are associated with various pharmacological properties, including antioxidant, antimicrobial, and anti-inflammatory activities [21], [36]. Mushrooms and other microorganisms are increasingly recognized as valuable sources of phenolics and flavonoids, alongside traditional plant-based sources.

In this study, the total phenolic content (TPC) and total flavonoid content (TFC) of methanolic extracts from *C. militaris* cultivated on different substrate formulations were analyzed and are presented in Table 2. The results demonstrated that substrate composition had a significant effect on the levels of extracted phenolic compounds ($p \leq 0.05$). Notably, the 100-SMQ formulation (100% quinoa) yielded the highest TPC (13.90 ± 0.02 mg GAE/g DW) and TFC (16.70 ± 0.17 mg RE/g DW), followed by lower values in the 50-SMQ, 75-SMQ, and 25-SMQ formulations. The control group, 100-SMJ (100% jasmine brown rice), exhibited the lowest TFC values (4.76 ± 0.40 mg RE/g DW).

The TPC obtained in our study was significantly higher than that reported by Jędrejko *et al*. [37], who observed a TPC of only 1.49 mg GAE/g DW in the fruiting bodies of *C. militaris* grown on a brown rice-based medium supplemented with glucose, peptone, $MgSO_4 \cdot 7H_2O$, and K_2HPO_4. The elevated TPC in our 100-SMJ may be attributed to the inclusion of egg, which likely improved the nutritional composition of the substrate and supported enhanced phenolic compound biosynthesis in the fruiting bodies. Notably, the formulations containing quinoa resulted in even higher TPC levels, further highlighting quinoa's potential to stimulate fungal metabolism and promote the accumulation of phenolic compounds in *C. militaris* fruiting bodies.

The accumulation of bioactive compounds in *C. militaris* is highly dependent on the cultivation substrate and can be affected by various factors, including fungal strain, environmental conditions, light, temperature, extraction methods, and media composition [2], [38]. The marked increase in total phenolic and flavonoid contents (TPC and TFC) observed in quinoa-based formulations in this study highlights the importance of substrate optimization in enhancing the functional properties of *C. militaris* fruiting bodies. In contrast, inadequate levels of these compounds may compromise the bioactivity and therapeutic potential of the resulting extracts. Thus, selecting and tailoring suitable cultivation substrates is crucial for maximizing both the productivity and functional quality of *C. militaris*, while also ensuring product safety and minimizing environmental impact.

3.3.2 Antioxidant activities of C. militaris extracts

The antioxidant capacity of *C. militaris* extracts was evaluated using two established assays: DPPH radical scavenging and FRAP (ferric reducing antioxidant power), as summarized in Table 3. The results indicate that antioxidant activity was significantly influenced by the cultivation substrate ($p \leq 0.05$).

Table 2: The total phenolic and flavonoid contents in *C. militaris* extracts.

Formulas	Total Phenolic Content (mg GAE/g DW)	Total Flavonoid Content (mg RE/g DW)
100-SMJ	9.12 ± 0.02^{d}	4.76 ± 0.40^{d}
25-SMQ	9.68 ± 0.02^{b}	5.36 ± 0.23^{d}
50-SMQ	8.94 ± 0.02^{e}	8.60 ± 0.35^{b}
75-SMQ	9.36 ± 0.03^{c}	7.12 ± 0.29^{c}
100-SMQ	13.90 ± 0.02^{a}	16.70 ± 0.17^{a}

Results are presented as mean values from three experimental repetitions, along with standard deviations. Significant differences are indicated by letters (a, b, c, d, e) at a 95% confidence interval ($p \leq 0.05$). GAE refers to gallic acid equivalents, and RE denotes rutin equivalents.

Table 3: Antioxidant activities of *C. militaris* extracts.

Formulas	Antioxidant Capacity			
	FRAP Assay (mg AAE/g DW)	DPPH Assay (mg AAE/g DW)	%Inhibition (DPPH)	IC_{50} Sample (mg/mL)
100-SMJ	3.50 ± 0.01^{d}	0.58 ± 0.02^{d}	55.38 ± 0.20^{d}	1.04 ± 0.08^{a}
25-SMQ	3.16 ± 0.01^{e}	1.73 ± 0.02^{c}	64.88 ± 0.15^{c}	0.95 ± 0.08^{bc}
50-SMQ	3.88 ± 0.02^{c}	1.76 ± 0.01^{c}	65.13 ± 0.08^{c}	0.84 ± 0.03^{bc}
75-SMQ	4.05 ± 0.01^{b}	1.93 ± 0.02^{b}	66.54 ± 0.15^{b}	1.02 ± 0.06^{b}
100-SMQ	4.42 ± 0.01^{a}	3.22 ± 0.02^{a}	77.20 ± 0.11^{a}	0.71 ± 0.01^{c}

Results are presented as the mean values of three independent experiments with standard deviation. Significant differences are denoted by letters (a, b, c, d, e), with a confidence level of 95% ($p \leq 0.05$). AAE = Ascorbic Acid Equivalent, IC_{50} = 50% Inhibitory Concentration.

In the DPPH assay, which measures the ability of extracts to neutralize free radicals through Hydrogen donation, the 100-SMQ formulation exhibited the highest antioxidant activity. It achieved a DPPH value

of 3.22 ± 0.02 mg AAE/g DW, 77.20 ± 0.11% inhibition, and the lowest IC_{50} value of 0.71 ± 0.01 mg/mL, indicating strong radical scavenging efficiency. In contrast, the 100-SMJ control group showed the lowest activity (0.58 ± 0.02 mg AAE/g DW, 55.38 ± 0.20% inhibition, and IC_{50} = 1.04 ± 0.08 mg/mL). These results were consistent with the FRAP assay, which assesses the electron-donating ability of antioxidants through the reduction of Fe(III)-TPTZ to Fe(II)-TPTZ. Again, the 100-SMQ extract demonstrated the highest FRAP value (4.42 ± 0.01 mg AAE/g DW), with a decreasing trend observed as the proportion of quinoa decreased. The lowest FRAP value was observed in the 100-SMJ formulation (3.50 ± 0.01 mg AAE/g DW).

These findings indicate that incorporating quinoa into the cultivation medium significantly enhances the DPPH radical scavenging activity of *C. militaris* extracts. This is consistent with earlier studies demonstrating the antioxidant potential of *C. militaris*, observed in both fruiting bodies and mycelia [9], [39], [40]. The antioxidant activity of the extracts may also be influenced by various bioactive constituents present in the fruiting bodies, including phenolic compounds, ergothioneine, and carotenoids [35]. Moreover, cordycepin has been shown to promote the activity of key antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase, suggesting a strong correlation between cordycepin content and antioxidant capacity [12], [41], [42], which is further supported by our results.

3.4 *SEM Results*

The mycelia of *C. militaris* in the basal MSM (Figure 3(a)) were analyzed using scanning electron microscopy (SEM), which revealed the mycelial surface at 50 µm (Figure 3(b) and (c)). The morphology of *C. militaris* cultivated under varying quinoa ratios in the media was also assessed. The results showed that the mycelium in the basal MSM control group (100-SMJ) (Figure 3(b)) had a rough surface with a high density of the medium. In contrast, the mycelium cultivated on the 100-SMQ formula (Figure 3(c)), which exhibited the highest levels of total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activities, showed a lower medium density. This suggests that cultivating *C. militaris* with quinoa enhances the porosity of the solid medium, creating additional internal space for nutrient absorption and providing an essential carbon source for fungal growth [23]. The lower density of the medium improves gas exchange, ensuring that *C. militaris* receives adequate oxygen for growth and bioactive compound production. Additionally, the transparent structure of the medium supports mycelial expansion, allowing the mycelium to grow more effectively. As a result, the increased porosity and larger surface area of the solid media positively impacted both biomass yields and the concentrations of cordycepin and adenosine.

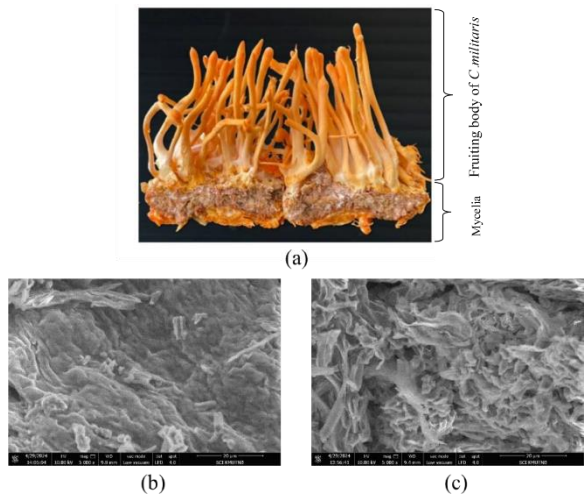

Figure 3: SEM images of mycelia obtained from different cultivated conditions (a) basal modified solid media, (b) basal solid media cultivated with 100-SMJ formula (control), and (c) basal modified solid media cultivated with 100-SMQ formula. All images are shown at a magnification of 5000x, with 9.4 nm resolution and 10.0 kV, displaying the mycelium surfaces at 50 µm.

4 Conclusions

C. militaris is highly valued for its nutraceutical properties. This study investigated various modified solid media to identify the most effective formula for cultivating *C. militaris* using different ratios of jasmine brown rice and quinoa. The results show that replacing jasmine brown rice with quinoa in the modified solid medium significantly enhances both biomass yield and the concentrations of cordycepin and adenosine. The 75-SMQ formula produced the highest levels of cordycepin and adenosine after 8 weeks of cultivation. Furthermore, cultivation on the 100-SMQ formula notably improved antioxidant activities, as demonstrated by FRAP and DPPH assays, and resulted in the highest total phenolic

content (TPC) and total flavonoid content (TFC). These findings highlight that the modified solid medium can effectively enhance the production of bioactive compounds, with potential for scaling up to larger production levels. Therefore, quinoa serves as a promising supplement that not only promotes the growth of fruiting bodies but also boosts the production of bioactive compounds, offering both health and economic benefits. However, for industrial-scale production, considerations such as production costs, nutritional composition, and other relevant factors must be carefully evaluated.

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Author Contributions

O.J.: conceptualization, investigation, methodology, experimental design, data analysis, writing-original draft; S.T.: investigation, supervision, methodology, research design, writing-reviewing and editing; V.R.: supervision, funding acquisition; P.P.: conceptualization, research design, data curation, supervision, writing-reviewing and editing, funding acquisition.

Conflicts of Interest

The authors declare no conflict of interest.

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