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ORIGINAL ARTICLE

Alanine supplementation enhancing cordycepin production in *Cordyceps militaris* via upregulation of *Cns2* and *Cns3* genes expression levels

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Abstract

Cordycepin, a key bioactive compound produced by *Cordyceps militaris*, faces the challenge of low productivity for commercial use. In this study, alanine supplementation in *Cordyceps militaris* boosted cordycepin production, peaking at 3 mg/g with 12 g/L concentration. Transcriptome analysis revealed 1711 differentially expressed genes, Pathway analysis indicates that protein processing in the endoplasmic reticulum was the most affected pathway. In addition, the transcriptome showed that adenylosuccinate lyase is essential for the synthesis of cordycepin. The modulation of four genes (*Cns1-4*) points to a regulatory mechanism that could increase cordycepin biosynthesis, offering a strategy to overcome low productivity for commercial applications.

Keywords: Alanine, *Cordyceps militaris*, Cordycepin, Secondary metabolites, Transcriptome

1. Introduction

Cordyceps militaris (*C. militaris*) is a typical representative species of *Cordyceps* [1]. It is mainly found in South Asia, Europe and North America [2], and has served popularly as an edible mushroom of Chinese herbal medicine for a long time [3]. Moreover, it is believed to be the earliest source among some valuable components [4]. So far, considerable progress has been made in the artificial culture of *C. militaris* [5–7]. Further research indicates that *C. militaris* can produce a variety of bioactive compounds with functional properties [8], such as treating fatigue, renal dysfunction, and respiratory disease [9]. In addition, its anti-oxidation, anti-photoaging and anti-pigmentation properties has garnered a lot of attention in the medical industry. For example, bioactive compounds found in *C. militaris* are used for natural anti-cancer drug [10] and facial mask cosmetics [11–13], while byproducts of its mass production are mostly for animal feed. The development of fermentation and biological processes makes the production of these specific bioactive metabolites a prospect of functional ingredients [14].

The bioactivity diversity of *C. militaris* has demonstrated that it contains over 200 bioactive metabolites including sterol, amino acid, *Cordyceps* polysaccharides [15], cyclic peptides [16], polyketide [17], phenolics [18], terpenes [19], phenolic acids [20], and cordycepin (COR) [21]. These metabolites, especially COR, have significantly broadened the application scope of *C. militaris* as one of its most crucial secondary metabolites [22]. COR exhibits a broad spectrum of biological activities [23], including anti-inflammatory, anti-oxidation, anti-tumor [24,25], anti-proliferative [26], and anti-metastatic properties [27]. These attributes have led it to be sold as an over-the-counter drug in Western countries. The edible value of *C. militaris,* with high COR content, is far higher than that of ordinary *C. militaris* and *Cordyceps* flowers. Reports suggest that COR has the

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potential to become a cardioprotective drug [28], and can serve as escort compound [29]. Despite its powerful functions, there are still key defects such as its high cost and limited availability [30].

The biosynthetic pathway of cordycepin was elucidated with the discovery of the gene cluster responsible for its production in *C. militaris* [31]. This gene cluster consists of four genes (*Cns1*-*Cns4*) encoding proteins involved in different steps of cordycepin metabolism. *Cns1* (CCM_04436) catalyzes oxidoreduction reactions, *Cns2* (CCM_04437) acts as a phosphohydrolase, *Cns3* (CCM_04438) consists of two functional domains: an N-terminal domain exhibiting nucleoside/nucleotide kinase (NK) activity and a C-terminal domain, and *Cns4* (CCM_04439) functions as an ATP-binding cassette transporter. The synthesis of cordycepin begins with adenosine, which is converted to adenosine-3′-monophosphate (3′-AMP) by *Cns3*. Phosphohydrolase activity of *Cns2* further transforms 3′-AMP into 2′-carbonyl-3′-deoxyadenosine (2′C-3′dA). Finally, *Cns1* mediates oxidoreduction reactions to produce cordycepin. In addition, the presence of pentostatin (PTN), an inhibitor of adenosine deaminase, coupled with cordycepin by the phosphoribosyltransferase domain of *Cns3* [32]. This inhibits the deamination of cordycepin and helps maintain its stability within the cell [33]. When cordycepin reaches toxic levels, *Cns4* transports PTN out of the cell, neutralizing cordycepin to nontoxic 3′-deoxyinosine [34].

With the increasing market demand for cordycepin, it is imperative to take measures to enhance the production of COR [35]. This necessitates a thorough comprehension of the intricate biosynthetic process of COR, which encompasses the regulation of multiple enzymes and transcription factors at various molecular levels, including environmental signaling, transcriptional control, and epigenetic regulation [36]. For example, Tan et al. reengineered the metabolic pathways of *Komagataella phaffii* in 2023, enabling the microorganism to efficiently convert methanol into cordycepin. This innovative biotransformation process has been shown to trigger a cascade of global metabolic adjustments, which are orchestrated at the transcriptional level [37]. A study on transcriptional regulation has demonstrated that the production of COR could be increased by overexpressing two key Zn_2Cys_6-type transcription factors, CmTf1 and CmTf2, in the wild type of *C. militaris* [38]. Moreover, Cordycepin production was enhanced by overexpressing *Cns1, Cns2, Cns3*, and a fusion gene combining *Cns1 and Cns2* in a control strain of *C. militaris*. The transformant with the *Cns1*/*Cns2* fusion gene showed a 2.7-fold increase in cordycepin yield compared to the wild-type strain. However, no significant differences were observed in cordycepin yield between the control strain and the transformants with single genes [39]. To further enhance COR yield, other methods have been explored, such as environmental signals regulation [36]. These include the addition of selenium [40] and nucleoside analogs [41]. These substances have shown potential in increasing the production of COR [42]. Additionally, new techniques such as 'ion beam', proton beam [43], and UV irradiation [44], can be utilized to harvest *C. militaris* mutant for the enhancement of COR yield. These techniques provide alternative strategies for improving the efficiency of COR production. By continually improving the production process and exploring new approaches, the commercial production of COR can become more efficient and cost-effective. This will contribute to meeting the growing demand for cordycepin in various industries, including pharmaceuticals and nutraceuticals.

A pivotal factor in enhancing COR production is the presence of alanine in the culture medium, which serves a critical role as a precursor in the biosynthesis of COR. The addition of alanine has been demonstrated to significantly boost COR yield; specifically, supplementing the culture with 8 g/L L-alanine has resulted in a remarkable twofold increase in COR concentration [38]. The enhancement underscores the importance of alanine in the metabolic pathways leading to COR synthesis. Recent advances in understanding of *C. militaris* metabolism have shed light on the intricate relationship between amino acid supplementation and secondary metabolite production. For instance, the role of amino acids as regulators of enzyme activity and transcription factor expression has been increasingly recognized [45]. Moreover, the addition of the precursor adenosine could significantly boost cordycepin production by 51.2% in *C. militaris*. This enhancement occurred without upregulating the four key genes in the main biosynthetic pathway, suggesting that adenosine supplementation may activate a parallel route to increase cordycepin yield. These findings underscore the potential of targeted metabolic interventions to optimize the production of valuable secondary metabolites like cordycepin [46].

In this study, we aimed to investigate the impact of different alanine concentrations in the culture medium on cordycepin production in *C. militaris*. We conducted a transcriptome analysis using RNA sequencing to identify differentially expressed genes (DEGs) associated with cordycepin biosynthesis under varying alanine concentrations.

Additionally, we performed gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis to gain insights into the molecular processes and pathways involved in cordycepin synthesis. The findings of this study not only enhance the yield of COR in *C. militaris*, but also have the potential to deepen our understanding of the mechanism of COR synthesis with alanine supplementation.

2. Materials and methods

2.1. Experimental setup and culture condition

In the study, the commonly used CM01 strain of *C. militaris* was employed for the experiments. A basal medium (PDA) composed of glucose (20 g/L), peptone (5 g/L), KH_2PO_4 (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L) and Vitamin B1 (0.05 g/L) was employed [47]. A liquid medium was prepared by milling and boiling 200 g of potatoes and filtering the resulting solution. The filtrate was mixed with the basal medium, and water was added to make a final volume of 1000 mL. The pH of the liquid medium was adjusted to 6.6, which was utilized for strain activation. Adding 100 mL the prepared liquid medium to each flask along with 1.8 g of agar, then the complete medium was sterilized using an autoclave with moist heat sterilization at 121 °C for 20 mins.

For the preparation of the standard alanine solution, a total of 400 g of alanine was dissolved in 1 L of distilled water to achieve a concentration of 400 g/L. For the experimental setup mentioned, 20 g of alanine was accurately weighed and dissolved in 50 mL of distilled water to reach the desired concentration for the study. When the sterilized PDA solid medium was cooled down to 60 °C, 1 mL, 2 mL and 3 mL of standard alanine solution was added to the PDA solid medium, at which time the concentration of alanine in the medium was equivalent to 0, 4, 8 and 12 g/L, respectively. One bottle of medium was poured into four petri dishes equally, solidified and then spread on a thin layer of cellophane, and then activated strains were inoculated on individual PDA plates supplemented with alanine. The plates were incubated in the dark at 22 °C for 7 days until the medium was completely covered with mycelium. Afterward, the plates were transferred to the light and incubated at 22 °C for 10 h per day, with a light intensity of 1200 lx. This light exposure was maintained for three days. Subsequently, mycelium was collected by scraping from the plates into 50 mL centrifuge tubes, and the centrifuge tubes with mycelium were used to determine the biomass after three days in a vacuum lyophilizer. Each experiment was performed in triplicate to ensure reproducibility and accuracy.

2.2. HPLC detection of cordycepin from Cordyceps militaris

After harvesting the mycelium of *C. militaris*, after which the content of COR was determined for subsequent analysis. Specifically, the mycelia were vacuum freeze-dried to a constant weight, and ground to a fine powder. Two grams of the dry powder were then weighed from each sample for High Performance Liquid Chromatography (HPLC) detection.

To conduct HPLC detection, a Waters Alliance e2695 HPLC (Milford, MA, USA) equipped with a ZorbaxSB-C18 column (4.6 × 250 mm, 5 μm) was utilized. The UV detector was set to 260 nm, and the analytical conditions were as follows: mobile phase consisting of 88% ultrapure water/methanol (88:12, v/v) at an elution rate of 1.5 mL/min, with an injection volume of 10 μL. To generate a standard curve, the COR standard ranging from 30 to 600 μg/mL (Sigma–Aldrich, Burlington, MA, USA) was used. The COR yield was calculated based on the detected peak area using the standard curve. The COR concentration of the mycelia in our study was normalized by equal biomass.

2.3. Transcriptome sequencing

Total RNA extraction was conducted using a fungal RNA kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's instructions, including DNA digestion. The concentration and integrity of the extracted RNA were assessed using a NanoDrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA), respectively [48]. Subsequently, mRNAs were enriched using Oligo-deoxythymidine (Oligo dT) beads, and cDNA synthesis was performed using fragmented mRNAs as templates [49]. The cDNA fragments were then refined using the QiaQuick PCR extraction kit, followed by end-repair, single-nucleotide adenine addition, and ligation to Illumina sequencing adapters to construct the cDNA library [50]. To select the majority of the ligation products, agarose gel electrophoresis was employed, followed by amplification and sequencing on an Illumina HiSeqTM2500 (Biomarker Biotechnology Co., Beijing, China) platform [50]. The generated RNA-Seq data for *C. militaris* with alanine supplementation have been deposited in the NCBI/SRA database

(https://www.ncbi.nlm.nih.gov/sra) under the Bioproject accession number PRJNA902538.

2.4. Quantitative analysis of the transcriptome

The transcriptome datasets initially contained raw reads with adapters or sub-standard bases. To obtain clean reads, a filtering step was performed to remove these contaminants [51]. Additionally, Bowtie2 software (http://bowtie-bio.sourceforge.net/bowtie2) was utilized to eliminate reads that mapped to the ribosomal RNA (rRNA) database, resulting in the generation of final clean reads. These clean reads were then used for assembly and transcriptome analysis [52]. For gene expression analysis, the clean read datasets from the four different cultures were mapped to the reference genome *C. militaris* CM01 (NCBI accession number AEVU00000000) using Tophats2 (v2.0.3.12) [53]. The quantification of gene expression levels was carried out using RSEM software, and the Fragments Per Kilobase of transcript per Million mapped reads (FPKM) method was employed to normalize the gene abundances [54,55].

2.5. Differentially expressed genes analysis and annotation

Based on the RNA-Seq by Expectation-Maximization results [56], differentially expressed genes (DEGs) among samples were detected using edgeR on the R package (version 3.4.2). To identify significant DEGs among the four groups (CK vs. Ala-1, 2, and 3), a threshold of absolute fold change $\geq$2 and a false discovery rate (FDR) of up to 0.05 were set [57]. The identified DEGs were then subjected to hierarchical clustering analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed for further annotation and analysis of the DEGs. The KEGG pathway enrichment analysis was conducted by referencing the KEGG database [58].

2.6. Quantitative real-Time PCR validations of differentially expressed genes

To validate the transcriptional levels derived from RNA-Seq data analysis, we selected four genes (*Cns1-4*), which are involved in COR biosynthesis in *C. militaris*. Real-time reverse transcription PCR (qRT-PCR) was performed to confirm these gene expressions [59]. First, cDNA templates for qRT-PCR were synthesized using the Fungus RNA Kit from 0.5 μg of total RNA. The PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China) was used for reverse transcription to obtain first-strand cDNA from each RNA sample [60]. The qRT-PCR reaction system consisted of a total volume of 10 μL, including cDNA, suitable primers, and SYBR Green Real-time PCR Master Mix (Takara). Real-time RT-PCR was conducted using the Real-Time PCR System (Bio-Rad, Hercules, CA, USA).

To normalize the target gene expression levels and correct for any variations among samples, Glyceraldehyde 3-phosphate dehydrogenase was used as the internal control/reference gene. The thermal cycling conditions for the qRT-PCR were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and extension at 72 °C for 20 s. After each PCR reaction, melting curve analyses were performed to ensure amplification of specific products. The primers for the candidate genes were designed based on Illumina sequencing data using Primer Premier 5 [61]. The relative expression levels of the target genes were determined using the comparative $2^{-\Delta\Delta CT}$ method, which allows for a comparison between the different genes.

2.7. Data analysis

In this study, three separate experiments were conducted, and the data presented are expressed as the average values ± standard error (SE) of three replicates. To analyze the data collected within the same time period, a one-way ANOVA was performed, and a post-hoc analysis using the Least Significant Difference Test (LSD) was carried out for mean comparisons. Furthermore, a one-tailed Student's *t*-test was employed to compare the control group with the alanine supplementation group, in order to calculate the *p*-value. All statistical analyses were performed using SAS 9.20 software (SAS Institute Inc., Cary, NC, USA), with a significance level set at $p < 0.05$.

3. Results

3.1. Effects of alanine supplementation on cordycepin production from Cordyceps militaris

According to Fig. 1, the addition of alanine resulted in a significant change in the diameter of *C. militaris* colonies compared to the control group. The diameter of the fungus increased as the concentration of alanine was enhanced, particularly when the concentration reached up to 12 g/L. However, the difference in colony diameter between the second and third sample was not as pronounced (Fig. 1A). Subsequently, the biomass of the

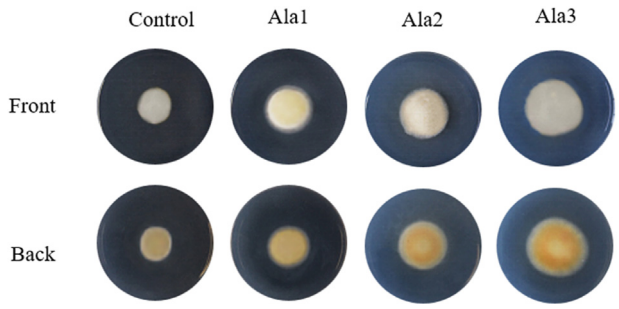

Fig. 1. ***The phenotype of Cordyceps militaris is affected by different alanine supplementation.*** *The concentration of "Ala1, Ala2 and Ala3" in the figure was equivalent to 0, 4, 8 and 12 g/L, respectively.*

harvested *C. militaris* mycelia was measured. The results showed that the biomass increased with increasing alanine concentration, similar to the trend observed in colony diameter.

To determine the production of COR in each alanine supplementation group, an HPLC detection assay was performed. The production of COR per unit mass of mycelium significantly increased with higher levels of alanine concentration. Specifically, the increase in COR content between the group supplemented with 4 g/L alanine (second sample) and the group with 8 g/L alanine (third sample) was substantial. However, the increase between the third sample with 8 g/L alanine concentration (2.51 mg/g) and the fourth sample with 12 g/L alanine (3.03 mg/g) was less pronounced. Notably, the COR content with 12 g/L alanine supplementation was approximately three times higher than that of the control group, as depicted in Fig. 2B, which illustrates the remaining COR content in mg/g dry weight.

3.2. Differentially expressed genes involved in Cordyceps militaris

To assess the impact of alanine supplementation on gene expression, the transcription levels of genes were quantified using the Reads Per Kilobase of transcript per Million mapped reads (RPKM) metric. A total of 1711 genes exhibited differential expression between the three alanine supplementation groups and the control group. Among these, the largest number of differentially expressed genes (948) was observed between the control and the group supplemented with 12 g/L of alanine. This included 597 up-regulated genes (63%) and 351 down-regulated genes (37%). At the concentrations of 4 g/L and 8 g/L of alanine supplementation, 824 and 643 DEGs were identified, respectively (Fig. 3A). These results indicate that alanine, as an additive, plays a regulatory role in the growth and metabolism of *C. militaris*, with the extent of regulation varying with the concentration of alanine.

To better understand the effects of different alanine concentrations on *C. militaris*, four groups were selected for further analysis based on their significant differences. A Venn diagram was constructed to illustrate the distribution of differentially expressed genes (Fig. 3B). The Venn diagram analysis revealed that 191 genes were commonly differentially expressed among all alanine supplementation groups when compared to the control. Among the specific differentially expressed genes, there were 548 genes uniquely differentially expressed in the Ala-3 group compared to the control, indicating the involvement of complex

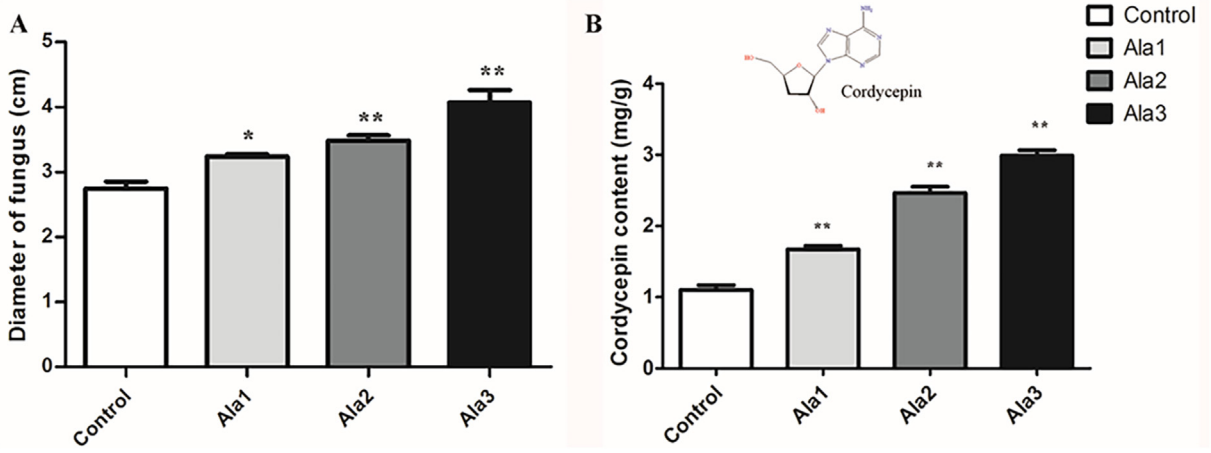

Fig. 2. ***The diameter of fungus and content of cordycepin as affected by alanine.*** *(A) The phenotype of Cordyceps militaris was affected by different alanine supplementation. Control, Ala1, Ala2, and Ala3 were equivalent to the control, slight, moderate, and severe alanine supplementation, respectively. (B) The contents of COR with mg/g dry weight 0, 4, 8, and 12 g/L were equivalent to the control, slight, moderate, and severe alanine supplementation, respectively. Asterisks indicate statistically significant differences between groups (Student's t-test): * and ** indicating significance level was accepted at $p \leq 0.05$ and $p < 0.01$, respectively, as compared to control.*

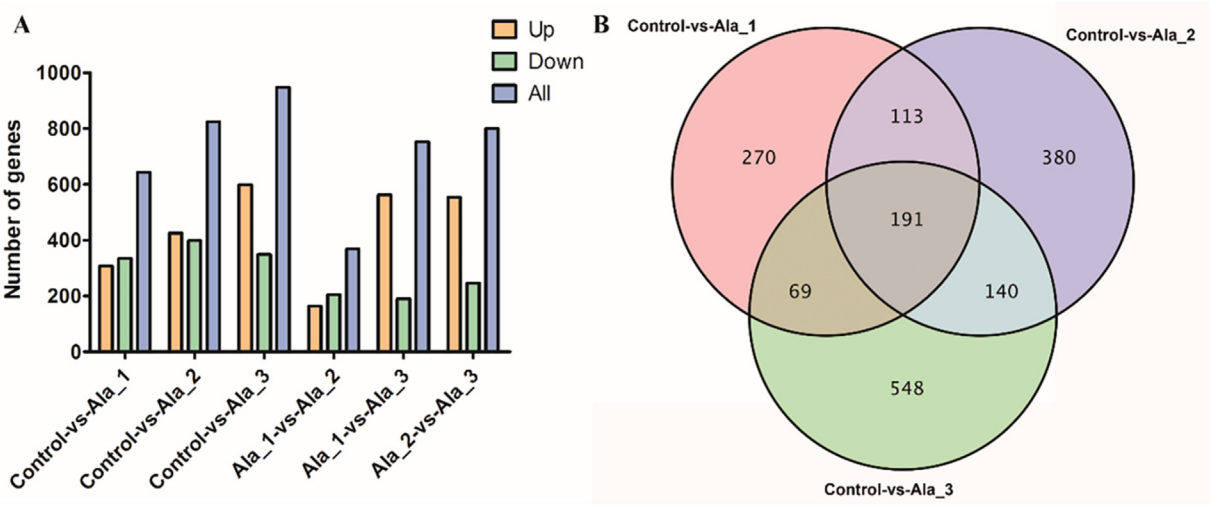

Fig. 3. ***Distribution of differentially expressed genes (DEGs) in the six samples.*** *(A) The distribution of DEGs between control and three different concentrations of alanine; (B) Venn diagram of the DEGs between samples. Ala-1, 2, and 3 represent 4, 8, and 12 g/L concentrations of alanine, respectively.*

developmental events in *C. militaris* under high concentration of alanine.

3.3. Cluster of orthologous groups of proteins classification of differentially expressed genes

The COG (Cluster of Orthologous Groups) database was constructed based on the phylogenetic relationship of bacteria, algae, and eukaryotes, and is a useful tool for gene product classification. The statistical chart of COG annotation classification of differentially expressed genes is shown in Fig. 4. The proportion of genes in different functional classes reflects the metabolic or physiological bias of *C. militaris* under specific conditions. The results reveal that genes related to posttranslational modification, protein turnover, and chaperones had the highest proportion (Fig. 4), which can be scientifically explained in combination with the distribution of research objects in each functional class. Furthermore, the majority of differentially expressed genes were related to cell synthesis and metabolic

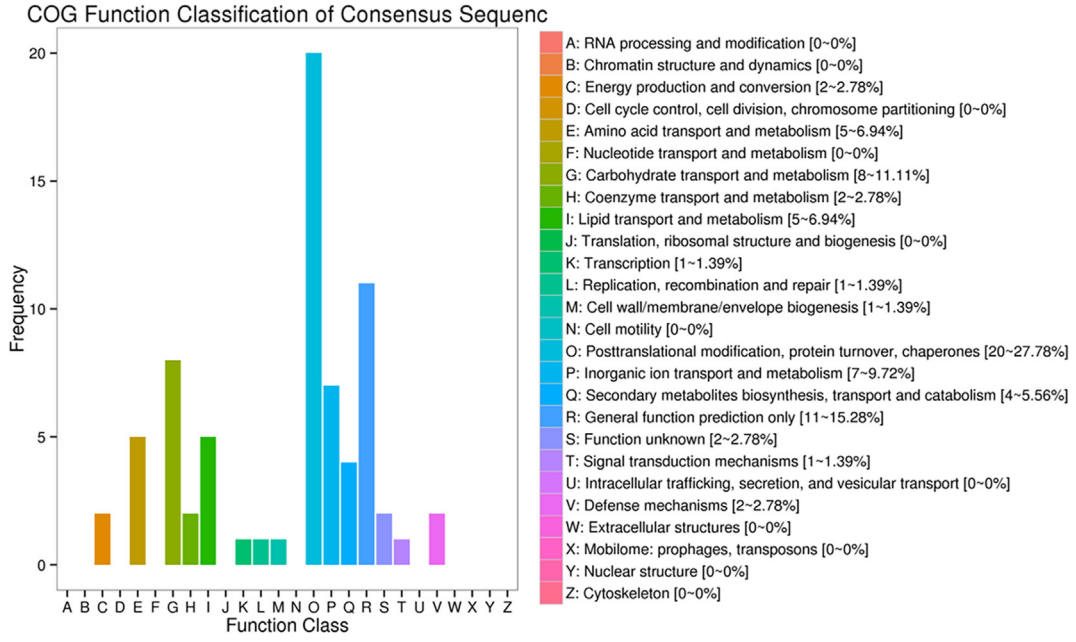

Fig. 4. COG function classification of consensus sequence.

pathways, suggesting that alanine supplementation may impact specific amino acid metabolism and energy metabolism.

3.4. KEGG pathway enrichment of differentially expressed genes

To gain further insights into the function of DEGs, KEGG pathway enrichment analysis was conducted [62]. The results of the KEGG pathway enrichment analysis, focusing on the DEGs between the alanine supplementation groups and the control group, are presented in Fig. 5. This figure displays the top 20 pathways with the lowest *q*-values, indicating significant enrichment. Interestingly, the pathway associated with protein processing in the endoplasmic reticulum exhibited the lowest *q*-value and had the highest number of DEGs. This suggests that alanine supplementation may have a profound impact on protein folding and processing within the endoplasmic reticulum. Additionally, other enriched pathways included indole alkaloid biosynthesis, staurosporine biosynthesis, and arachidonic acid metabolism, which may indicate potential alterations in secondary metabolite biosynthesis and lipid metabolism induced by alanine supplementation.

3.5. Differentially expressed genes involved in protein processing in the endoplasmic reticulum

To gain a more comprehensive understanding of the gene expression patterns in the context of the protein processing pathway in the endoplasmic reticulum, we identified twelve genes encoding proteins involved in this pathway from the transcriptome of *C. militaris*. As depicted in Fig. 6 [63], these twelve genes were analyzed for their expression profiles. The expression analysis revealed that among the identified genes, only one gene: heat shock protein (Hsp)20-1 exhibited up-regulation in response to alanine supplementation. In contrast, the expression of the remaining eleven genes (geneHsp90-1, geneHsp90-2, geneHsp70, geneHsp40, geneNEF, geneHsp20-2, geneHsp20-3, geneHsp20-4, geneHsp20-5, genep97-1, and genep97-2) was significantly down-regulated upon alanine supplementation. These findings suggest that the enhanced production of COR may be attributed to the inhibition of gene expression within the protein processing pathway. In summary, these results highlight the potential regulatory role of alanine supplementation in modulating the expression of genes involved in protein processing, thereby influencing the yield of COR in *C. militaris*.

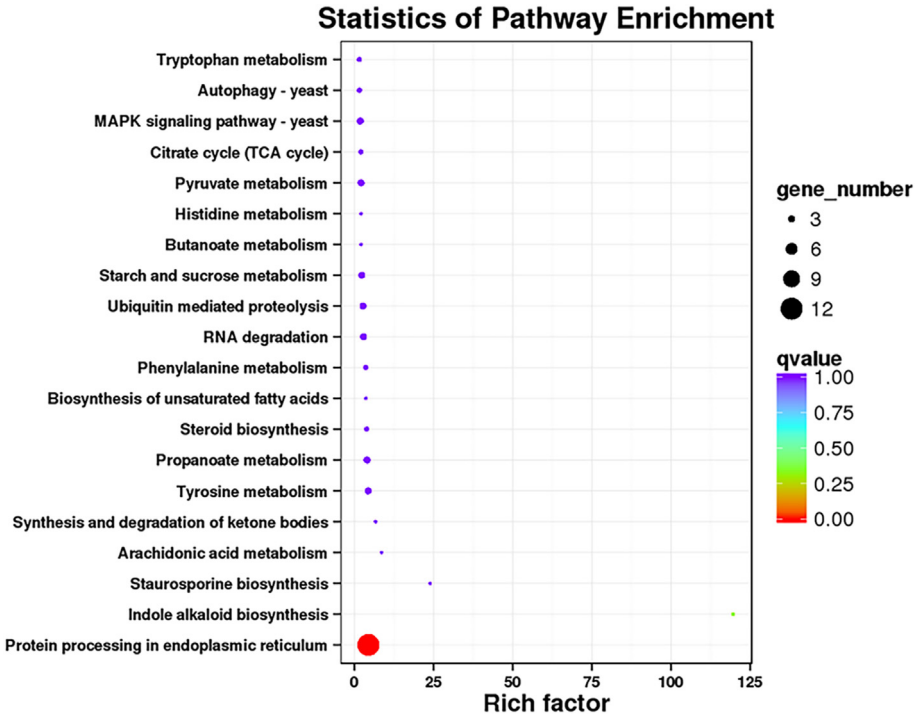

Fig. 5. ***The functional enrichment analysis of differentially expressed genes using KEGG annotation.*** *The x-axis represents the rich factor, and the y-axis represents the pathway name. DEGs, differentially expressed genes; KEGG, kyoto encyclopedia of genes and genomes.*

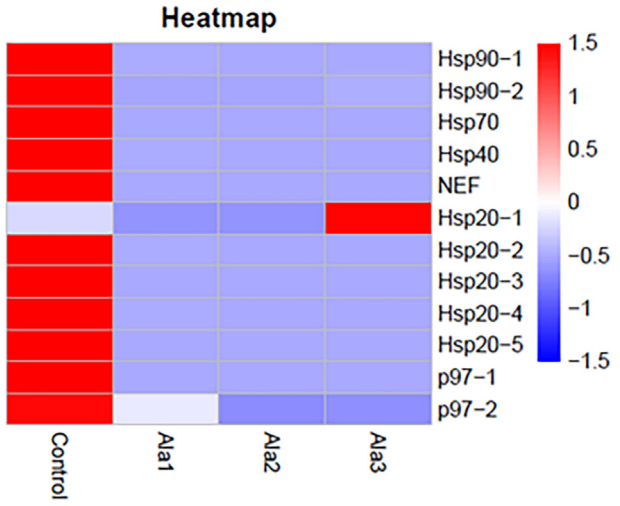

Fig. 6. ***Heatmap of the differentially expressed genes from the pathway concerning protein processing in endoplasmic reticulum (the x-axis represents each compared sample; the y-axis represents the distinct DEGs.*** *The coloring indicates the fold change: high, red; low, navy blue). Hsp90-1, Hsp90-2, heat shock 90; Hsp70, heat shock 70; Hsp40, heat shock 40; Hsp20-1, Hsp20-2, Hsp20-3, Hsp20-4, Hsp20-5, heat shock 20; NEF, hsp70-interacting protein; p97-1, p97-2, transitional endoplasmic reticulum ATPase.*

3.6. Analysis of the genes related to cordycepin synthesis pathway

In order to investigate the effect of alanine supplementation on COR production in *C. militaris*, we conducted an analysis of DEGs involved in the alanine addition to COR synthesis pathway using KEGG pathway analysis. Within this network, a total of 13 DEGs were identified. The pathway analysis revealed that the network initiates from amino acid interconversion and histidine metabolism. Through histidine metabolism, 5-aminoimidazole-4-carboxamide ribotide (AICAR) is produced, which is then converted into inosine monophosphate (IMP) by IMP cyclohydrolase (EC 2.1.2.3). Interestingly, IMP cyclohydrolase and adenylosuccinate synthase (EC 6.3.4.4) are encoded by three and five genes, respectively, while all other enzymes in this pathway are encoded by single genes (refer to Fig. 7). It was observed that the gene expression level for the pathway from IMP to adenylosuccinate (EC 6.3.4.4) was downregulated. Conversely, the expression level of adenylosuccinate lyase in the pathway from adenylosuccinate to 3′-AMP (EC 4.3.2.2) was increased. Notably, under conditions of limited adenylosuccinate, the concentration of 3′-AMP significantly increased, suggesting that the limitation of adenylosuccinate might promote transcriptional activity.

Furthermore, we validated the expression of four key genes (*Cns1-4*) involved in the COR synthesis pathway using qRT-PCR. Based on the analysis of the COR synthesis pathway and the PCR results, it was found that the expression level of *Cns1* was decreased compared to the control, while the expression levels of *Cns2-4* were upregulated with increasing concentrations of alanine compared to the control. This suggests that the activation of the pathway from adenylosuccinate to 3′-AMP could be greatly enhanced, ultimately leading to an increase in the content of COR upon alanine supplementation. In summary, our findings indicate that alanine supplementation influences the expression of genes involved in the cordycepin synthesis pathway, particularly in the regulation of adenylosuccinate-related enzymes. This knowledge contributes to a better understanding of how alanine promotes cordycepin production in *C. militaris*.

4. Discussion

Given the various applications of COR in the therapeutic and pharmaceutical fields, such as its use as an antibiotic with excellent biological and pharmacological activity [64]. A natural anticancer drug [65], and a prodrug to activate AMP-activated protein kinase (AMPK) [66], there is great interest in increasing the content of COR. Additionally, the bioactive components of COR have shown benefits in cosmeceuticals, including anti-photoaging and anti-pigmentation effects [34]. Therefore, enhancing the production of COR has become a prominent research focus. Previous studies have reported that supplementation of 2 g/L L-alanine enhanced the yield of COR in liquid static fermentation of *C. militaris* [67]. However, the optimal concentration of alanine supplementation to increase COR content and the underlying mechanism remain unclear.

In this study, we evaluated the effect of different concentrations of alanine supplementation on COR production. The results revealed that alanine served as a beneficial additive for the development of *C. militaris* strains and the formation of COR, with its effect positively correlated to the concentration of alanine. It is worth noting that alanine supplementation may also influence the biosynthetic yield of other secondary metabolites. For instance, the enrichment of alanine significantly increased the biosynthetic yield of Monaco's purpureus M3 in liquid fermentation [68]. Although current production of COR in many factories does not involve alanine supplementation, we believe that significant potential for improvement exists by exploring the mechanism we have discovered. It is crucial to determine the optimal concentration of alanine supplementation and comprehensively understand

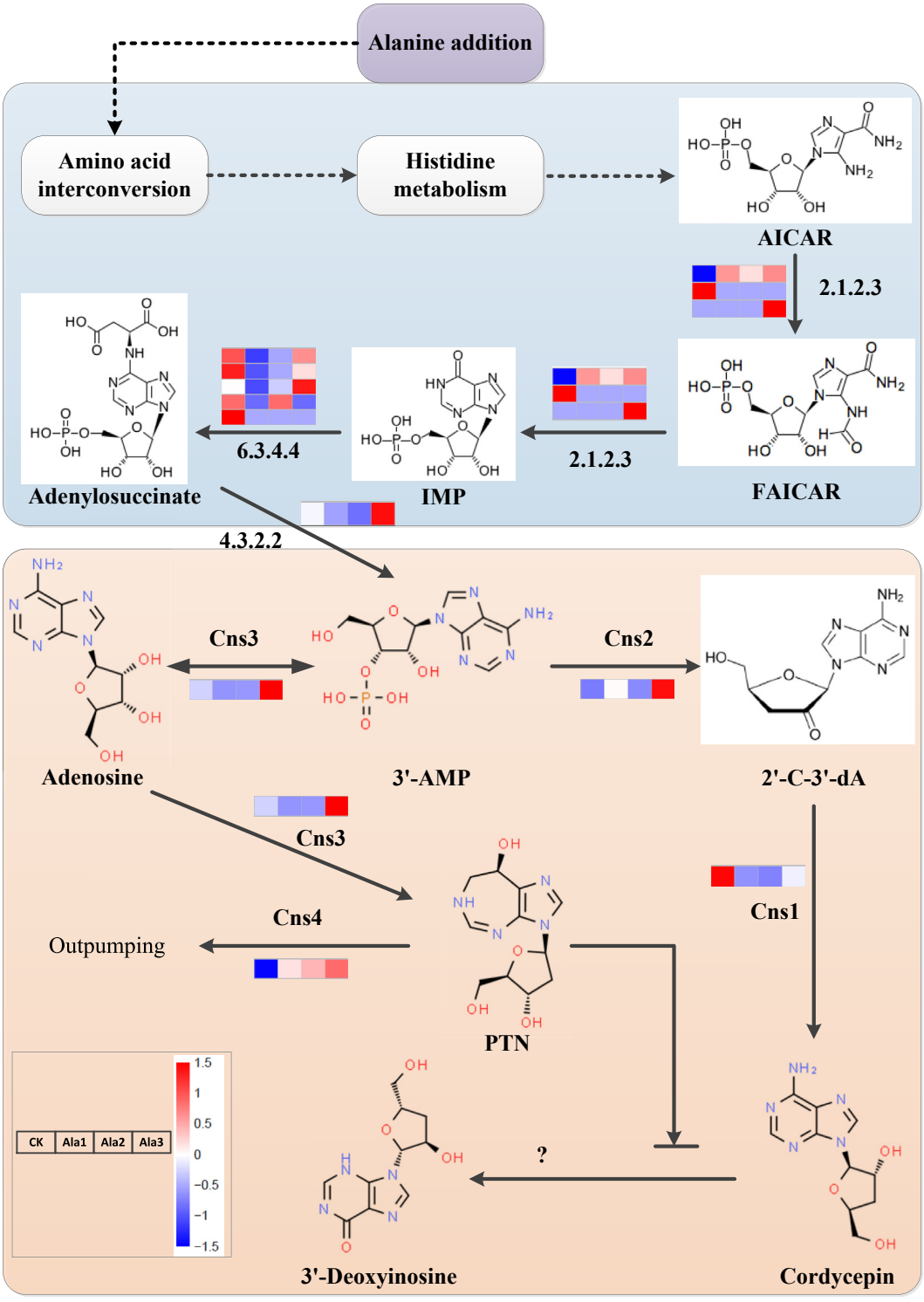

Fig. 7. ***Cordycepin metabolism and biosynthesis pathway in Cordyceps militaris and qRT-PCR results.*** *Heatmap with 4 colors represents the relative expression level of four genes in response to distinct concentrations of alanine. The heatmap represents the average (±SE) of biological repeats. Ala-1, 2, and 3 represent 4, 8, and 12 g/L alanine concentrations, respectively. The dotted lines indicate the indirect effects. The solid arrows indicate direct action. AICAR, 5-aminoimidazole-4-carboxamide ribotide; FAICAR, 5-formamidoimidazole-1-(5-phosphoribosy 1) imidazole-4-carboxamide; IMP, inosine monophosphate; 2.1.2.3, IMP cyclohydrolase; 6.3.4.4, adenylosccinate synthase; 4.3.2.2, adenylosuccinate lyase; 3′-AMP, adenosine-3′-phosphate; 2′-C-3′-dA, 2′-carbonyl-3′-deoxyadenosine; PTN, pentostatin.*

its molecular regulation mechanism in order to develop *C. militaris* strains for large-scale COR production.

In order to investigate the molecular mechanism underlying the enhancement of COR production by alanine supplementation, we employed RNA-seq analysis using the RPKM metric to analyze the DEGs in *C. militaris* under alanine conditions. Our results demonstrated that the high alanine group exhibited more significant changes in gene expression compared to the low alanine group, indicating that supplementing the growth environment of *C. militaris* with alanine leads to extensive transcriptional regulation. Recent studies have discovered several plant secondary metabolites, such as epigallocatechin gallate, celastrol, and deguelin, that exhibit inhibitory activity towards Hsp90 [69]. Hsp90 is regulated through various mechanisms that impact its transcription and undergoes post-translational modifications and regulation by co-chaperones [70]. Gene p97 acts downstream from ubiquitin signaling events and utilizes the energy from ATP hydrolysis to extract its substrate proteins from cellular structures or multiprotein complexes [71]. Transcriptome data from our study revealed that the expression of Hsp genes was inhibited, alongside the down-regulation of almost all genes related to the protein processing pathway in the endoplasmic reticulum. Furthermore, previous reports have suggested that suilloid fungi isolates from high-elevation regions thrived on alanine and glutamine [72]. Alanine is synthesized from glutamic acid and undergoes transamination with oxoglutarate to produce glutamate and pyruvate—a reversible reaction that grants alanine a dual function in carbon and nitrogen metabolism [73]. It is likely that the addition of alanine repressed amino acid synthesis or promoted amino acid transformation, ultimately leading to the utilization of excess ATP for COR synthesis. In fact, metabolomic profiling data indicated an imbalance in alanine and glutamate metabolism within the fungal hyphae, with a strong positive correlation observed between glutamate and alanine levels [74].

The purine nucleotide cycle, a metabolic network integral to cellular energy homeostasis, is orchestrated by the concerted action of three key enzymes: adenylosuccinate synthetase, adenylosuccinate lyase, and AMP deaminase [75]. This cycle is instrumental in the transformation of adenosine monophosphate (AMP) into inosine monophosphate (IMP) [76], a reaction that is more than a mere conversion—it is a critical step in the broader context of ATP regeneration [77]. Recent insights suggest that the flux through the purine nucleotide cycle is intricately linked to the adenylate kinase reaction, which is vital for maintaining cellular ATP levels and supporting energy–intensive processes [78]. The strategic upregulation of adenylosuccinate synthase, a rate-limiting enzyme in the cycle, has emerged as a metabolic maneuver to enhance microbial growth and productivity. Studies have demonstrated that this enzyme's overexpression can lead to a more efficient generation of IMP, thereby accelerating the overall purine synthesis rate and contributing to the proliferation of microorganisms [79]. The elucidation of these metabolic intricacies not only deepens our understanding of the purine nucleotide cycle's regulatory mechanisms but also unveils potential targets for metabolic engineering. By modulating the expression and activity of the enzymes involved, it is possible to fine-tune the cycle's contribution to ATP regeneration and microbial growth, offering a promising avenue for biotechnological applications.

The KEGG pathway analysis has highlighted the critical function of protein processing in the production of COR, indicating that cellular mechanisms are finely tuned to support this biosynthetic process. Our results are in concordance with recent studies that have underscored the importance of adenylosuccinate lyase in the purine nucleotide synthesis pathway [79]. Specifically, the upregulation of adenylosuccinate lyase, a key enzyme in the 3′-AMP biosynthetic pathway, has been demonstrated to markedly increase 3′-AMP levels, consistent with findings by Jiang et al. that highlight its regulatory impact on purine synthesis [80]. The enzyme's catalytic conversion of adenylosuccinate to AMP is a critical step in the de novo purine synthesis pathway, as previously described [81,82]. Our findings resonate with the established literature, illustrating a tight interplay between transcriptional regulation and metabolite dynamics in the context of secondary metabolism modulated by alanine supplementation [83]. Furthermore, we have expanded on the role of transcriptionally upregulated genes in energy metabolism and amino acid transformation, which are central to COR production. This is supported by research indicating that amino acid can modulate the expression of transport proteins involved in metabolic pathways [84]. Our results suggest that alanine supplementation may enhance the expression of such proteins, thereby influencing energy metabolism and metabolic conversions. These insights not only deepen our understanding of the intricate regulatory networks within cells but also pave the way for future research into the nuanced effects of alanine on cellular metabolism.

It has been reported that COR is synthesized from adenosine through a gene cluster consisting of three genes. Among the four genes in the COR biosynthesis gene cluster, *Cns1* and *Cns2* are indispensable for COR biosynthesis, while *Cns3* affects COR production. The *Cns1-3* genes also enable *C. militaris* to produce PTN, another adenosine analog. Additionally, the *Cns3* gene is essential for PTN production and also functions in converting adenosine to 3′-AMP [85]. When the cellular concentration of COR reaches a self-toxic level, *Cns4* may pump PTN out of the cell, potentially regulating the interaction between adenosine availability and COR production. This mechanism could link secondary metabolism and activate membrane transport to prevent excessive COR production [39]. COR is synthesized after the protein complex *Cns1-Cns2* converts 3′-AMP into the intermediate 2′-C-3′-dA [35]. It is speculated that the initial step in COR biosynthesis involves hydroxyphosphorylation at the 3′-OH position of adenosine, catalyzed by the kinase activity of *Cns3*, resulting in the production of adenosine 3′-AMP. In this study, the expression levels of COR-related genes, *Cns2-3*, were significantly upregulated, indicating that the addition of alanine may improve the metabolic process and lead to increased metabolite production. The practical application of these findings could involve genetic engineering strategies to enhance the expression of *Cns1* and *Cns2*, thereby potentially increasing COR yields in commercial settings. However, it is important to recognize that our study has limitations. Specifically, we have not yet addressed the operational and economic aspects of scaling up these findings to large-scale production. Future research should focus on these practical considerations and also explore additional genetic and environmental factors that could further optimize COR biosynthesis. For instance, investigating the role of other genes in the COR biosynthesis pathway and their interaction with *Cns1-Cns2* under different culture conditions could provide more insights into enhancing the efficiency of COR production.

5. Conclusion

To better reveal the alanine reaction mechanism of *C. militaris* and its effects on COR content, a transcriptome analysis of *C. militaris* is conducted under different alanine supplementation. The HPLC analysis indicated that high alanine supplementation to the benefit of enhancing COR production. Combined with KEGG pathway enrichment, the upregulated transcriptome level of genes responsible for the pathways of COR metabolism and biosynthesis might be a major reason for the accumulation of COR. Finally, the results showed that the transcription level of regulatory genes was related with secondary metabolites. In summary, our research presents an in-depth transcriptome analysis of the impact of alanine supplementation on *C. militaris*, unveiling the molecular underpinnings that augment COR biosynthesis. The identification of key regulatory genes linked to secondary metabolite production is a significant advancement, offering a strategic approach to engineer industrial strains designed for superior COR output. The development of these strains is poised to revolutionize the pharmaceutical industry by enabling the large-scale production of COR with enhanced efficiency and quality, thereby facilitating its integration into a range of pharmaceutical formulations. Furthermore, our insights into the role of alanine in COR synthesis establish a robust basis for future investigations, with the dual goal of refining production processes and uncovering novel medical applications for this valuable compound. Our work, therefore, not only advances the scientific understanding of the metabolic responses of *C. militaris* but also opens new avenues for innovation in medicinal development.

CRediT authorship contribution statement

Wenbin Yu: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. Zeying Zhao: Conceptualization. Bin Zeng: Project administration. Yayi Tu: Conceptualization, Supervision. Bin He: Conceptualization, Writing – review & editing, Supervision.

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.

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Appendix A. Supplementary data

RNA-Seq data of *C. militaris* with alanine supplementation were deposited in the NCBI/SRA database (https://www.ncbi.nlm.nih.gov/sra), under the Bioproject accession number PRJNA902538.

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