

Review

# Research Progress in Understanding the Molecular Biology of *Cordyceps militaris*

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**Abstract:** *Cordyceps militaris* (*C. militaris*) is a valued medicinal fungus that can be traced back thousands of years in traditional Chinese medicine (TCM). Both TCM and modern scientific research have confirmed the positive effects of *C. militaris* on human health. In recent years, *C. militaris* has gained wide popularity; unfortunately, strains often degrade during cultivation, resulting in a decline in fruiting bodies and active components that negatively impacts the development of *C. militaris* in the health food and medicine industries. This review summarizes the current progresses in research on the genomic, transcriptomic, proteomic, and genetic manipulation of *C. militaris* and discusses its primary metabolites and strain degradation mechanisms. The current challenges and future prospects of *C. militaris* research are also discussed.

**Keywords:** *Cordyceps militaris*; microbiome; genetic manipulation; microbial metabolism; degradation



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## 1. Introduction

*Cordyceps militaris* (*C. militaris*), belonging to Cordycipitaceae (Hypocreales, Ascomycota), is a well-known entomopathogenic fungus with rich nutritional value [1–3]. In Asia, especially in China, *C. militaris* is a famous tonic food and traditional Chinese medicine (TCM); it contains essential amino acids, with the most abundant being tyrosine and leucine [4]. *C. militaris* also contains a higher content of polysaccharides, vitamins, carotenoids, and other compounds compared to common plant foods. Considering its medicinal value, it has been used in alternative medicines to treat kidney, liver, neurological, and cardiovascular diseases, as well as to treat tumors and improve immunity [5,6]. Although a variety of compounds have been extracted from *C. militaris*, it is not the most popular member of the *Cordyceps* family. Similar to *C. militaris*, *Cordyceps sinensis* (*C. sinensis*) has been used to improve renal, hepatic, and respiratory functions; delay fatigue; and reduce type 2 diabetes symptoms. It has also been used as an aphrodisiac [7]. In China, *C. sinensis* has been listed as an endangered species due to a sharp decline in production and over-harvesting caused by high market demand. For these reasons, there is an active search for alternative sources that are already being produced under large-scale conditions, and *C. militaris* is used as an artificial cultured alternative due to its ease of production. As a model *Cordyceps* species, *C. militaris* is relatively more widely studied compared to *C. sinensis* [8]. There has been increasing attention paid to its unique pharmacological properties. More research has been conducted on the active metabolites of *C. militaris* [9], such as cordycepin (COR), cordycepic polysaccharide, and cordycepic acid [10–17], which are not

significantly different from those in *C. sinensis*. Among these compounds, COR is regarded as an important active secondary metabolite with therapeutic potential. Pharmacological studies have reported that COR has antibacterial [18,19], tumor-relieving [20,21], antioxidant [22,23], anti-inflammatory [24,25], immune-regulatory [22], and lipid-lowering [26] properties. It also shows antiviral effects towards several viruses including influenza virus [27], HIV [28], dengue virus [29], Epstein–Barr virus (EBV) [30,31], and hepatitis C virus (HCV) [32]. Recent studies indicate that COR may also have effective treatment potential against coronavirus disease 2019 (COVID-19) [33,34]. Notably, the COR content in *C. militaris* is higher than that in wild *C. sinensis* while its price is much lower. In recent years, novel carotenoids with good water solubility have been found in *C. militaris* fruiting bodies, which have different characteristics from fat-soluble carotenoids. These carotenoids have expanded the application of *C. militaris* in healthcare, where it is considered to be a safe natural drug without side effects. Its fruiting body can be easily cultivated and contains more metabolites than the vegetative mycelium. *C. militaris* fruiting body powder and capsules have been found to improve micturition and prostate size in patients with benign prostatic hyperplasia [35].

The popularity and demand for *C. militaris* have led to its scarcity, also leading it to be listed as a protected endangered species. To meet the demand, significant research has been conducted on artificial cultivation and fermentation. Several challenges encountered during cultivation, especially strain degradation, affect its large-scale production. Strain degradation in the subculture process usually manifests as the absence or reduction of fruiting body production [36], which not only leads to a decrease in the yield of *C. militaris* metabolites but also affects the strain quality and causes economic losses [37]. To eliminate these issues, it is necessary to study the degradation mechanism and related genes that affect the biosynthesis of important metabolites. Many studies have focused on the molecular analysis of *C. militaris*, including manipulation of its genome, transcriptome, proteome, and genetics. Genetic engineering is a positive choice to increase production performance. However, the understanding of its molecular biology has been limited due to the lack of genome sequence information. It was not until 2011 that Zheng et al. [38] analyzed the complete molecular mechanism of COR biosynthesis through bioinformatics and gene function analysis of 18 *Cordyceps* species, including *C. militaris*, *C. sinensis*, and *Cordyceps cicadae* (*C. cicadae*) [39,40]. There has been great progress in transcriptomic and proteomic studies on *C. militaris*. Advances in multi-omics technology have enabled the analysis of the secondary metabolite biosynthesis pathways and their regulatory mechanisms, and the published genome has facilitated further molecular biological study [38].

This review primarily focuses on recent findings from genomic, transcriptomic, proteomic, and genetic manipulation studies of *C. militaris*. Additionally, the biosynthesis mechanisms of COR and carotenoids are discussed along with recent advances in understanding strain degradation. This study highlights the recent advancements in *C. militaris* research and its application prospects, providing a reference for further exploration of the mechanisms and interactions of its active components.

## 2. *C. militaris* Genome Analysis

In 2011, Zheng et al. [38] performed second-generation high-throughput whole-genome sequencing of the *C. militaris* strain Cm01. This strain has seven chromosomes ranging between 2.0 and 32.2 Mb that encode 9684 protein-producing genes. The publication of the genome of this strain has greatly promoted *C. militaris* research using other omics and molecular biology techniques. Cm01 has a similar genome structure to other *Cordyceps* strains (Table 1). In 2017, Kramer and Nodwell [41] used third-generation single-molecule sequencing to conduct chromosome-level assembly of the genome of the *C. militaris* strain ATCC 34164, which contained seven chromosomes ranging between 1.9 and 33.6 Mb with 9371 genes. In 2019, Chen et al. [42] conducted whole-genome sequencing of the *C. militaris* strain HN, assembling a total of 14 contigs ranging between 0.35 and 4.58 Mb with an N50 of 2.86 Mb and a genome size of 32.6 Mb. This strain was predicted to contain

approximately 10,095 genes, which is higher than that found in Cm01 and ATCC 34164. Through comparison with Cm01 and ATCC 34164, it was found that there might be inter-chromosomal translocation among different strains of *Cordyceps*. Identification of genomic structural variants of *Cordyceps* showed that there are 406 structural variations between HN and Cm01, and 1114 structural variations between HN and ATCC 34164. The translocation and structural variants often lead to changes in the number and order of related genes, resulting in changes in the function of these genes that might be associated with secondary metabolism [43]. These findings contribute to our understanding of *Cordyceps* biology and the biosynthesis pathways of bioactive components.

**Table 1.** Information on *C. militaris* genome sequencing.

Strain	Size/Mb	Coverage/Fold	Assembly Level	GenBank Assembly Accession	Ref.
Cm01	32.2	147×	33 scaffolds	GCA_000225605	[38]
ATCC34164	33.6	180×	7 chromosomes	GCA_008080495	[41]
HN	32.6	300×	14 contigs	GCA_003332165	[42]

*Cordyceps* and *Metarhizium* species are well known entomopathogenic fungi that have independently evolved insecticidal properties [44]. Until recently, the genes responsible for insect pathogenicity and bioactive component biosynthesis have been identified through comparative genome analysis; this provides a basis for studying the biological characteristics of *C. militaris* and will promote the development of the medicinal compounds it produces. Through the analysis and comparison of the genomes of several entomopathogenic fungi, it was found that the number of protein-coding genes in *C. militaris* is lower than that in *Metarhizium anisopliae* (*M. anisopliae*, 10817) [45], *Metarhizium robertsii* (*M. robertsii*, 10582) [46], and *Metarhizium acridum* (*M. acridum*, 9849) (Table 2) [47]. However, *C. militaris* has more species-specific genes (1328, 13.7%) compared to *M. acridum* (343, 3.5%) and *M. anisopliae* (509, 4.8%) [38]. Significant differences were observed in gene contents between these two genera that might be related to different survival strategies. In 2014, Staats et al. [45] compared the genome sizes of these entomopathogenic fungi and found that the *C. militaris* genome (32.2 Mb) is smaller than that of *M. robertsii* (39.04 Mb), *M. anisopliae* (38.5 Mb), and *M. acridum* (38.05 Mb); it also has the highest number of species-specific genes (2245) compared to *M. acridum* (875), *M. anisopliae* (690), and *M. robertsii* (603). This is consistent with the findings of Zheng et al. [38]. Gene family expansions of proteases and chitinases in *C. militaris* were identified with comparison to *Metarhizium* species. These two types of enzymes can degrade insect body cell walls, indicating that the pathogenic strategy of *C. militaris* is to invade insects by breaking down their epidermis. The pathogenic strategies of *Cordyceps* and *Metarhizium* species may differ due to differences in gene content. In addition, Xia et al. [39] conducted a comparative analysis of *C. militaris* with *Aspergillus nidulans* (*A. nidulans*, another COR-producing fungus) to identify four physically linked genes (CCM\_04436–CCM\_04439 vs. AN3333–AN3329), respectively, which were associated with COR biosynthesis (discussed in detail in Section 6).

**Table 2.** Comparison of *C. militaris* genome with other fungi.

Strain	Size/Mb	Coverage/Fold	%G + C Content	Protein-Coding Genes	NCBI Accession	Ref.
<i>C. militaris</i> Cm01	32.2	147×	51.4	9684	AEVU000000000	[38]
<i>M. robertsii</i> ARSEF23	39.0	100×	51.5	10582	ADNJ000000000	[46]
<i>M. acridum</i> CQMa102	38.1	107×	50.0	9849	ADNI000000000	[45,47]
<i>M. anisopliae</i> E6	38.5	19×	51.0	10817	AZNF000000000	[45]

### 3. *C. militaris* Transcriptome Analysis

*C. militaris* shows typical sexual and asexual stages, which manifest as fruiting bodies and mycelia, respectively. The transcriptome profiles of *C. militaris* at specific developmental stages are distinct (Table 3) [48]. Differentially expressed gene (DEG) analysis was performed, and a transcription-level comparison between mycelia and fruiting bodies showed significant differences: *C. militaris* TBRC6039 had 1877 DEGs, among which 935 and 942 genes were up-regulated in the mycelia and fruiting bodies, respectively [49]. In addition, genes involved in lipid metabolism were up-regulated in fruiting bodies compared to mycelia. It has been reported that the neutral lipids in lipid droplets were required during COR accumulation in *C. militaris* [50]. This might explain why the phenomenon of high COR content was associated with lipid accumulation in the fruiting body stage. These studies suggest that these stage-specific genes may have important functions in fungal development; however, they did not give insights into many other biological processes, such as strain degradation.

Strain degradation is a common phenomenon during *C. militaris* subculture. Several complex physiological and biochemical changes occur during strain degradation, along with changes in the expression of related genes. Overcoming strain degradation has become a primary target for the factory production of *C. militaris*. Yin et al. [36] subcultured the *C. militaris* strain YCC for six successive generations and compared the growth of its fruiting bodies. They found that *C. militaris* begins to retrogress from the third generation, with its fruiting bodies being underdeveloped in the fourth generation and absent in the fifth generation. Transcriptome profiles of these six generations revealed 1892, 2498, 2006, 2273, and 2188 DEGs in the second, third, fourth, fifth, and sixth generations, respectively, compared with the first generation. The number of DEGs in the third generation was the highest, indicating that an increase in passage numbers (and, consequently, an increase in DEGs) exacerbates strain degradation. The morphological changes and mechanisms of *C. militaris* strain degradation will be discussed in detail in Section 8.

Mycelium growth and bioactive metabolite biosynthesis are also affected by medium and culture conditions. In our previous study, *C. militaris* growth was inhibited by salt treatments, but COR production increased significantly with an increase in salt concentration. Transcriptome analysis of the salt-treated and control *C. militaris* groups revealed 3885 DEGs, among which DEGs associated with amino sugar and nucleotide sugar pathways were significantly enriched in the salt-treated group [51]. Salt treatment might increase energy molecule production and further promote COR accumulation. There exists sufficient evidence that COR biosynthesis is related to different carbon sources, but its regulatory mechanisms have rarely been studied. To explore these, Raethong et al. [52] cultured the *C. militaris* strain TBRC6039 using sucrose, glucose, and xylose as different carbon sources. It was found that the growth rate and biomass of xylose were much lower than those of sucrose and glucose. Thus, the cultivation time in xylose was much longer than that in sucrose and glucose for COR measurement, and the results showed the extracellular COR titer (mg/L) in xylose culture was the highest. However, the extracellular COR productivity (mg/L/day) was still lower than that of sucrose and glucose due to the prolonged cultivation in xylose culture [52]. Consistent with the results, Wongsas et al. [53] also found xylose inhibited mycelial growth and biomass. However, the xylose culture showed the highest yield of extracellular COR on dry biomass, although it was a poor carbon source for mycelial growth [53]. Transcriptome analysis showed that there were much fewer DEGs between glucose and sucrose cultures (858) than in cultures grown with xylose versus sucrose (1799) and xylose versus glucose (2098). This finding might be related to the hydrolysis of sucrose to glucose and fructose [54]. Overall, the effects of xylose metabolism on COR production might be a complex process, and further studies are needed to gain a better understanding.

**Table 3.** Transcriptome of *C. militaris*.

Strain	Sample Information	Main Results	Year	Ref.
<i>C. militaris</i> TBRC6039	Mycelia and fruiting bodies	Compared the metabolic functions and regulation of <i>C. militaris</i> relevant to its developmental stages	2023	[49]
<i>C. militaris</i> YCC	Mycelia from 6 generations of subculture	Compared the changes in <i>C. militaris</i> for six generations during fruiting body growth	2017	[36]
<i>C. militaris</i> CM10	Mycelia (cultured under light and dark conditions)	Discovered the genes related to the biosynthesis of <i>C. militaris</i> carotenoid	2021	[55]
<i>C. militaris</i> Cm01	Mycelia with different salt treatment	Provided a global transcriptome characterization of the salt treatment adaptation process in <i>C. militaris</i>	2021	[51]
<i>C. militaris</i> TBRC6039	Mycelia (cultured under three carbon sources)	Uncovering sucrose- and glucose-mediated changes in the transcriptional regulation of central carbon metabolism in <i>C. militaris</i>	2018	[52]
<i>C. militaris</i> TBRC7358	Mycelia (cultured under three carbon sources)	Proposed putative alternative routes in formation of precursors for COR production on xylose	2020	[53]

#### 4. *C. militaris* Proteome Analysis

Presently, proteomic studies on *C. militaris* are limited. One proteomic study found 359 and 214 proteins in the *C. militaris* mycelium and fruiting body, respectively [56], among which 98 were expressed at both stages. There were also significant differences in protein expression between the two stages; this is consistent with the results of the transcriptomic analysis by Zheng et al. [38]. The *C. militaris* strain Cm01 contains 73 glycosylated phospholipid acyl-anchored proteins and is predicted to have 301 secretion-related proteins, accounting for 3.1% of the total number of proteins (9651) [45]. Similarly, *Cordyceps kyushensis* Kob (*C. kyushuensis* Kob) has 445 differentially expressed proteins [57], among which 224 are up-regulated and 221 are down-regulated between the two stages. These findings provide insights into the molecular mechanisms underlying the different life stages of *C. militaris*.

#### 5. Genetic Manipulation of *C. militaris*

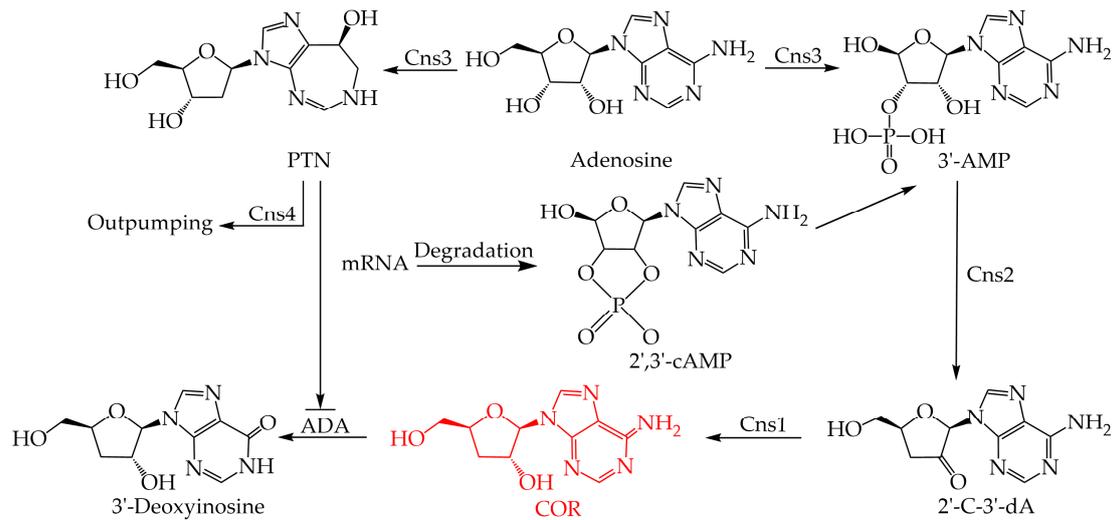
Commonly used methods for fungal transformation include *Agrobacterium tumefaciens*-mediated transformation (ATMT), polyethylene glycol (PEG)-mediated protoplast transformation (PMPT), CRISPR-Cas9, electroporation transformation, and gene gun-mediated transformation. At present, ATMT is the most developed technology and has been widely used in *C. militaris*. As early as 2011, Zheng et al. [58] constructed a *C. militaris* mutant library and identified the functions of these genes. He et al. [59] obtained a defective mutant using ATMT with a colony phenotype that was white after light exposure; the results showed that the *Cmcrf1* gene was involved in fruiting body development and carotenoid biosynthesis. In 2019, Lou Haiwei et al. [55] transformed *Cmtns* into a deleted mutant ( $\Delta$ Cmtns) using ATMT, discovering that *Cmtns* significantly affects carotenoid biosynthesis; this provides an important basis for the future study and promotion of carotenoid production. It is necessary to further study these genes involved in carotenoid biosynthesis and their interaction with other genes in order to better understand the growth and development of *C. militaris*. ATMT is not only an effective tool to study gene functions, but it is also an important method to increase the production of secondary metabolites. Chen et al. [60] transferred ergothioneine (EGT) synthesis genes into the *C. militaris* genome using ATMT,

increasing the production of EGT and COR. They also found that the overexpression of EGT synthesis genes in degraded strains of *C. militaris* could restore their production capacity, verifying its protective effect in degraded strains. PMPT methods using protoplasts as receptor cells have also been reported. Choi et al. [61] obtained green fluorescent protein (GFP) mutants using PMPT and established the genetic transformation system in *C. militaris*. Most transformation methods still face problems with low editing efficiency due to the difficulty of foreign gene transformation. Recently, CRISPR-Cas9 has been known as a site-specific genome editing method and is more accurate and efficient than ATMT and PMPT. In 2021, an optimized RNP-based method increased transformation efficiency in *C. militaris*, with editing efficiency reaching 100% and demonstrating the feasibility of gene editing in this species [62]. In summary, ATMT has more applications and higher transformation efficiency, followed by PMPT, and great challenges are faced in the application of CRISPR-Cas9 technology to fungal genome editing. However, it is not necessary to extract protoplasts for electroporation and gene gun-mediated transformation; their transformation efficiencies are low and they are rarely applied to fungal genetic transformation. The above studies provide important information for the establishment and development of fungal genetic transformation systems, contributing to gene isolation and functional identification in order to engineer *C. militaris* strains.

## 6. COR Biosynthesis in *C. militaris*

COR was isolated and identified from cultures of *C. militaris* for the first time in 1950 [63]. Over the past half-century, there has been great interest in detailing the COR biosynthesis pathway. However, few complete biosynthesis pathways have been uncovered due to the lack of *C. militaris* genomic data; it was not until 2011 that the whole genome of *C. militaris* was sequenced [38]. Subsequent studies identified gene clusters responsible for COR biosynthesis through comparative genomic analysis with *A. nidulans* and proposed a novel COR biosynthesis pathway (Figure 1) [39]. The COR biosynthesis gene cluster includes *Cns1*, *Cns2*, *Cns3*, and *Cns4*. COR is synthesized from adenosine and phosphorylated by *Cns3* to produce the intermediate product adenosine-3'-monophosphate (3'-AMP). Subsequently, 3'-AMP is dephosphorylated by *Cns2* and eventually reduced by *Cns1* to the final product COR. In addition, *C. militaris* also has a self-detoxification mechanism that is used to regulate the excessive production of COR. The gene cluster of *C. militaris* can also synthesize penstatin (PTN, deoxycoformycin), which can inhibit the degradation of COR into 3'-deoxyinosine (3'-dI). When the intracellular COR content is too high and causes cytotoxicity, the intracellular PTN content decreases, and COR is catalyzed to produce non-toxic 3'-dI through adenosine deaminase (ADA) intracellular deamination. Similar biosynthesis pathways for COR have been found in other COR-producing fungi such as *C. kyushensis Kob* [57] and *C. cicadae* [40]. Four related genes, *ck1-ck4*, were identified in *C. kyushensis Kob* as being involved in the synthesis of COR, similar to *Cns1-Cns4* in *C. militaris*, suggesting that there may be other routes involved in the COR biosynthesis pathway in *C. militaris*.

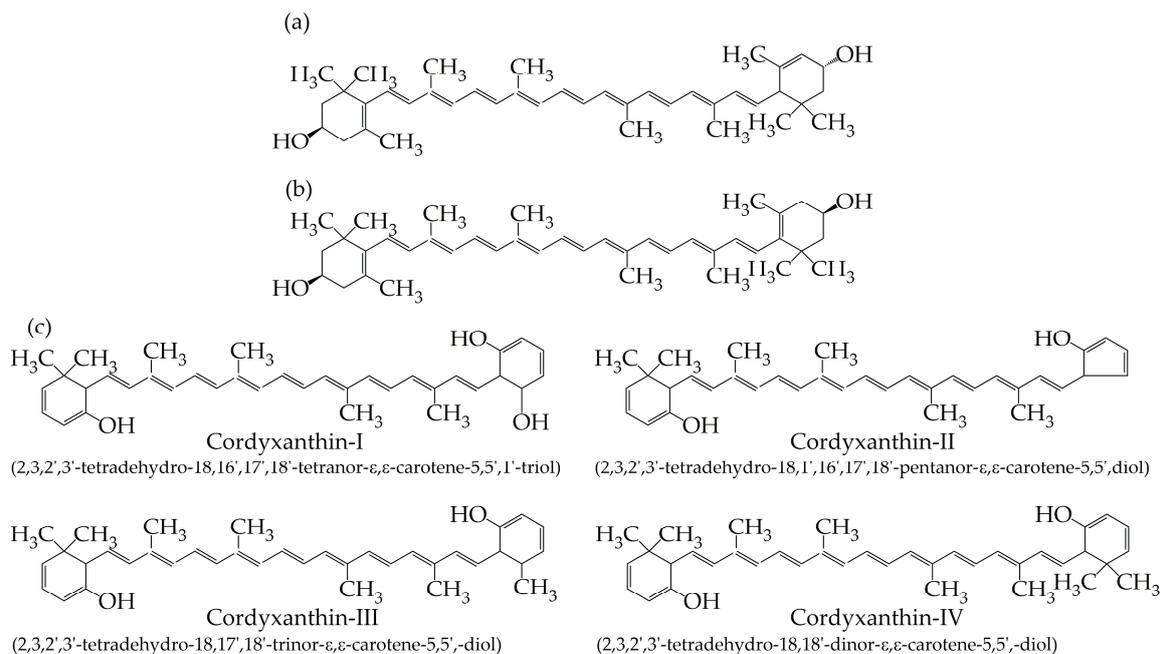
Differences in mycelial growth and COR biosynthesis are seen with different carbon sources. As described above, Raethong [51] and Wongsu et al. [53] found xylose inhibited the growth and biomass of *C. militaris* but increased COR biosynthesis on dry biomass. Consistent with this, transcriptome sequencing found that xylose significantly up-regulated genes involved in cell growth and COR biosynthesis, suggesting an important role for xylose in the formation of the precursor 3'-AMP. In addition, alternative routes involved in COR biosynthesis were proposed via transcriptome analysis of this two strains of *C. militaris*; this is consistent with the results of Xia et al. [39], who found that the COR biosynthesis pathway is highly correlated with the formation of the precursor 3'-AMP, which can be synthesized by 2',3'-cyclic monophosphate (2',3'-cAMP), a byproduct of mRNA degradation. However, the biochemical function and catalytic mechanism of the enzymes required for this pathway remain unknown. Further research is needed to prove whether this pathway is also present in other fungi.



**Figure 1.** The biosynthesis pathway of cordycepin. Abbreviations: PTN: pentostatin; 3'-AMP: adenosine-3'-monophosphate; 2',3'-cAMP: 2',3'-cyclic monophosphate; 2'-C-3'-dA: 2'-carbonyl-3'-deoxyadenosine; COR: cordycepin; ADA: adenosine deaminase.

### 7. Carotenoid Biosynthesis in *C. militaris*

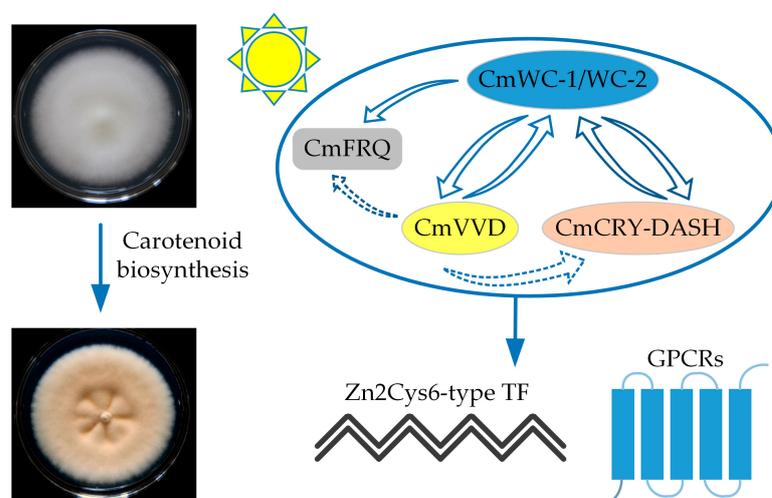
*C. militaris* contains six types of pigments, including lutein, zeaxanthin, and four cordyxanthins (Figure 2) [64–66]. Lutein and zeaxanthin are insoluble in water, while cordyxanthin I, II, III, and IV are water-soluble carotenoids; they contain 36, 35, 37, and 38 carbon atoms, respectively, compared to the 40 carbon atoms usually found in most carotenoids [66]. As a class of rare water-soluble carotenoids, they potentially have higher physiological activity, which will expand the application potential of the *C. militaris* pigments used in food, cosmetics, and other fields. Until now, the content of carotenoids in *C. militaris* has remained low, and still cannot meet the market demand. It is necessary to study the biosynthesis pathways of *C. militaris* carotenoids and the related genes to improve the content of carotenoids through genetic engineering.



**Figure 2.** Lutein (a) and zeaxanthin (b) in *C. militaris*; (c) chemical structures of four cordyxanthins in *C. militaris*.

*C. militaris* pigments are considered to be carotenoids. However, there are few reports on carotenoid biosynthesis genes in *C. militaris*; only two genes at the end of the carotenoid biosynthesis pathway have been identified that have little effect on the entire pathway [67,68]. The pathway of carotenoid biosynthesis in *Neurospora crassa* (*N. crassa*) has been well studied. In *N. crassa*, carotenoid biosynthesis involves at least three enzymes, among which geranylgeranyl diphosphate synthase genes (*CCM\_03059*, *CCM\_03697*, and *CCM\_06355*) are present in *C. militaris* [69]; the other two key enzymes, phytoene synthetase and phytoene dehydrogenase, are not found in *C. militaris*, indicating that its carotenoid biosynthesis pathway is different [55]. Based on the above, the absence of genes involved in the biosynthesis of carotenoids in *C. militaris* suggests that its biosynthesis pathway might be different from that of other species. Because of this, it is challenging to characterize the carotenoid biosynthesis pathway in *C. militaris* and explore the underlying mechanisms to solve the problem of low carotenoid yield.

Light has a significant influence on *C. militaris* pigment formation [70]. The circadian clock protein FREQUENCY (FRQ) and blue light receptors White Colar (WC)-1 and WC-2 are important photoreceptors in fungi. Shrestha et al. [71] found that wild-type *C. militaris* colonies are orange or orange-yellow. The colony color is associated with the light intensity during culturing, which influences carotenoid production of *C. militaris*, resulting in orange pigmentation [67,72,73]. A subsequent study found an albino strain (505CGMCC) of *C. militaris* with a mutation in the photoreceptor gene *Cmwc-1* [68]; the colony of the albino strain remained white under light while that of the wild-type strain (498CGMCC) changed from white to yellow. The carotenoid content was significantly lower in this mutant compared with the wild-type strain, but the COR content was high. This finding suggested that the formation of carotenoids in *C. militaris* was dependent on the photoreceptor CmWC-1. Yang et al. also found the colony color of the albino strain and a degraded one did not change or changed slower after exposure to light, and the light-induced transcription of *Cmwc-1* is significantly increased in *C. militaris* [74]. This is consistent with the findings of Wang et al. [68]. Additionally, *Cmwc-1* knockout strains have albinism, reduced fruiting body production, and significantly reduced carotenoid and COR production [67]. These results suggested that the *Cmwc-1* gene is involved in the regulation of fruiting body development and carotenoid biosynthesis in *C. militaris*. In 2020, it was found that the interaction of CmWC-1 and CmFRQ promoters regulates fruiting body development and carotenoid and COR production [75]. These studies indicate that light greatly influences the production of *C. militaris* pigments and the development of its fruiting bodies (Figure 3) [76].



**Figure 3.** Putative model for light-induced carotenoid biosynthesis in *C. militaris*. White Colar (WC)-1/2, *Drosophila-Arabidopsis-Synechocystis*-human type cryptochromes (CRY-DASH), VIVID (VVD),

FREQUENCY (FRQ). Photoreceptors CmWC-1/2, CmFRQ, and CRY-DASH perceive and transmit the light signal. The CmWC-1/2 complex activated the transcription of photoreceptor VVD gene cascades. Additionally, Zn2Cys6-type TFs and GPCRs functioned downstream of the CmWC-1/2 complex to regulate carotenoid biosynthesis.

### 8. *C. militaris* Strain Degradation Mechanisms

Filamentous fungi usually undergo degradation during the subculture process, leading to a sharp decline in fungal production that causes severe economic losses and drastically affects quality control. The colony morphology and physiological characteristics of degraded strains differ from those of wild-type strains [37]. This affects external and internal mycelial morphology. *C. militaris* degradation usually leads to the absence or reduction of fruiting body production [36,77] and decreased sporulation [78]. Studies have found significant decreases in the dehydrogenase activity and decolorization ability of degraded strains [79]; while wild-type strains gradually turn orange after light exposure, degraded strains only slightly change color or do not change color at all. In addition, strain degradation also leads to slower mycelial growth and metabolic capacity, and product yield decline, affecting production efficiency [37].

Strain degradation is a complex process involving many factors, including mating type, external environmental factors, and alternative splicing (AS) events [37]. Fungi can be divided into homokaryotic or heterothallic based on mating type; homokaryotic strains contain only one mating-type locus, whereas heterokaryotic strains contain both loci [80]. Wang et al. [81] found that wild-type strains have both mating-type loci, while degraded strains only have one mating-type locus and cannot produce fruiting bodies; this is consistent with the results of Yin et al. [14], who also found that degraded strains are homokaryotic. These results indicate that variations in the mating type may induce *C. militaris* degradation. It was unexpectedly observed in one study that the single mating strain *C. militaris* Cm01 could grow fruiting bodies without mating [38]. Additionally, only one mating-type locus has been identified in *C. militaris*, containing either MAT- $\alpha$  (*MAT 1-1* gene) or MAT-hmg (*MAT 1-2* gene) [82]. Overall, these results indicate that strain degradation is caused by many factors that may be associated with the change of mating type, though this needs to be further verified.

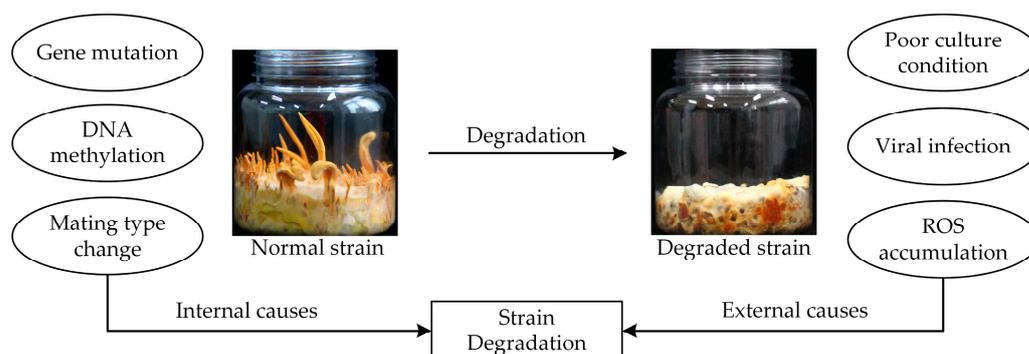
The external environment also influences *C. militaris* development and fruiting body production. Poor environmental conditions may lead to strain death or gene mutations that trigger strain degradation. Reactive oxygen species (ROS) are closely related to spontaneous degradation in *C. militaris* [37]. Exogenous melatonin (MT) is reported to scavenge oxygen-free radicals in fungal cells, and Zu et al. [83] found that the degradation characteristics of a degraded strain of *C. militaris* under MT treatment were significantly lessened. RNA-seq revealed that compared with the control, *glutathione s-transferase* genes were up-regulated under MT treatment. These studies indicate that ROS damage is one of the key factors affecting *C. militaris* strain degradation.

As mentioned above, over 5000 AS events were detected after each subculture of *C. militaris*; 6230, 6170, and 6320 AS events were detected in the second, fourth, and sixth generations, respectively. These results indicate an increased number of passages leads to enhanced strain degradation, suggesting that degradation may be directly associated with AS events [36]. In a previous study, the DNA methylation levels of a wild-type and degraded strain were found to be 0.48% and 0.56%, respectively, with 188 differential methylation regions (DMRs) [84]. DNA methylation has important effects on cell differentiation and development; the methylation level of wild-type strains was stable while that of the degraded strain was increased. This indicates that DNA methylation and recombination can induce *C. militaris* strain degradation.

Appropriate preservation methods can prevent fungal strain degradation. Since the number of generations is directly associated with enhanced strain degradation, maintaining the number of passages to  $\leq 3$  can be an appropriate strain preservation strategy. Additionally, repeated subculturing is prone to contamination, and genetic and physiological

changes often occur during long-term preservation. Cryopreservation seems to be a very efficient way to maintain the purity and vitality of strains for a long time [85]; for example, the activity of *C. militaris* strains has been shown to be maintained for  $\geq 1$  year in sterile water or liquid nitrogen at  $-196\text{ }^{\circ}\text{C}$  [86]. However, some studies have suggested that ultra-low-temperature freezing at  $-80\text{ }^{\circ}\text{C}$  is not suitable for the preservation of *C. militaris* strains [87]. As results are contradictory, there is an urgent need to determine an appropriate culture and preservation method. Although freezing can delay degradation, it cannot restore the performance of degraded strains; breeding optimal strains of *C. militaris* is key to overcome strain degradation. In a previous study, cross-mating of a single ascospore from a degraded strain restored its ability to produce fruiting bodies, COR, and adenosine [77], indicating that cross-mating is an effective method to rejuvenate degraded strains.

In summary, gene mutation is the primary cause of strain degradation, followed by poor culture and strain preservation conditions, which can induce genotypic or phenotypic changes, affecting production performance (Figure 4) [36,88–90]. Although factory production in the *C. militaris* industry has been successfully established, the key technology to overcome strain degradation has not yet been discovered, and the mechanisms associated with degradation are still unknown; the above studies contribute to understanding the causes of strain degradation and the countermeasures that can be used to counteract and prevent it.



**Figure 4.** Manifestations and causes of *C. militaris* strain degradation. Strain degradation was manifested as loss of the ability to produce fruiting bodies. Several factors might lead to strain degradation of *C. militaris* including external and internal causes.

## 9. Conclusions

At present, artificial culture and large-scale production of *C. militaris* have somewhat alleviated issues of low availability and high prices in wild *Cordyceps*; however, low metabolite yield, strain degradation, and complex biosynthesis pathways continue to be challenges facing the *Cordyceps* industry. Although several attempts have been made to enhance *C. militaris* yield, most studies were conducted with different strains; the physiological characteristics of strains vary greatly, and it is not applicable to generalize the results from one strain to others, even within the same species. Additional studies examining multiple aspects of *C. militaris* should be carried out. There have been many studies on the cultivation of *C. militaris*; considering the strain degradation, it is necessary to systematically optimize factory production and further promote the healthy and stable development of the *C. militaris* industry. *C. militaris* has great application value in various industries, including medicine, beauty, and healthcare fields, making it a key organism of interest for further study. It contains multiple secondary metabolite gene clusters, indicating its potential for secondary metabolism studies. The development of molecular biology and genetic technology will allow researchers to explore new synthetic routes and obtain new compounds. In this study, recent findings in genomic, transcriptomic, proteomic, and genetic manipulation studies on *C. militaris* were discussed, along with its primary metabolites and strain degradation mechanisms. This review will help increase the understanding of the research developments and applications of *C. militaris* cultivation.

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