EFFECT OF *CORDYCEPS MILITARIS* HYDROLYSATE ON PROBIOTIC GROWTH

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Abstract

The glucogalactomannan is the main type of polysaccharide found in *Cordyceps militaris* that exhibits prebiotic properties. A prebiotic is a nondigestible dietary fiber that selectively stimulates probiotics' growth and activity in the colon. Mannanase is an enzyme that hydrolyzes mannans and heteromannans to produce mannooligosaccharides which are prebiotics. This study investigated the impact of *Cordyceps militaris* hydrolysate, derived from mannanase hydrolysis, on the growth of probiotic bacteria. Four strains of probiotic bacteria, namely *Lactobacillus plantarum* TISTR 543, *Lactobacillus casei* TISTR 390, *Lactobacillus acidophilus* TISTR 1338, and *Lactobacillus bulgaricus* TISTR 451, were utilized in the study. The *Cordyceps militaris* hydrolysate was obtained by subjecting dried *Cordyceps militaris* powder to hydrolysis using mannanase-producing bacteria, *Bacillus methylotrophicus* KS1. The resulting culture media were filtered to obtain the crude mannooligosaccharide (MOS). Various concentrations of the crude MOS were added to the MRS medium used for probiotic cultures to assess their effects on probiotic growth. The findings revealed that the highest growth was presented in *Lactobacillus acidophilus* TISTR 1338 when a 6.67% concentration of crude MOS was added. In conclusion, this study provides evidence that *Cordyceps militaris* hydrolysate, particularly the crude MOS obtained from mannanase hydrolysis, can selectively stimulate the growth of specific probiotic bacteria, such as *Lactobacillus acidophilus* TISTR 1338. This novel finding suggests that *Cordyceps militaris* may possess prebiotic properties, which could contribute to a healthier gut microbiota when incorporated into the diet.

Keywords: ***Cordyceps militaris* hydrolysate; Prebiotic; Mannooligosaccharide; Probiotic bacteria; *Lactobacillus acidophilus***

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Introduction

Cordyceps militaris is a parasitic fungus, primarily known for its entomopathogenic tendencies, as it predominantly infects insects and other arthropods. Taxonomically, *Cordyceps militaris* belongs to the Ascomycota phylum, Sordariomycetes class, Hypocreales order, and Ophiocordycipitaceae family (Shrestha *et al*., 2017). Its natural life cycle involves infecting insect larvae, such as caterpillars, ultimately leading to the demise of its host (Wongsorn *et al*., 2021). The fungus then grows out of the host's body and produces a fruiting body, which resembles a small, elongated club-shaped mushroom. These fruiting bodies are often harvested and used in traditional Chinese and Tibetan medicine due to their purported health benefits. *C. militaris* has a distinctive bright orange to red fruiting body that emerges from the host insect or its pupal stage. The fruiting body consists of a slender stalk with a club-shaped head where spores are produced.

C. militaris is believed to have various potential health benefits, such as boosting energy, improving lung function, antimicrobial, anticancer, and enhancing athletic performance. However, scientific research on its efficacy is ongoing (Zheng *et al*., 2011). It is also used in some culinary dishes, particularly in Chinese cuisine, where it is considered a delicacy and is used in soups, stews, and other dishes. The cultivation of *C. militaris* has become a growing industry, as it allows for the controlled production of these fungi for medicinal and culinary purposes. It is cultivated on substrates like grains and rice, and the resulting fruiting bodies are harvested for consumption or extraction of bioactive compounds such as cordycepin, mannitol, peptides, adenosine, cordycepic acid, macrolides, ergosterol, polysaccharides (Zheng *et al*., 2011; Lo *et al*., 2013).

Glucogalactomannan is a type of polysaccharide found in *C. militaris*, among other fungi. This compound is a key component of the fungal cell wall and may have various biological activities including immunomodu- latory activity, antioxidant activity, antitumor activity and anti-inflammatory activity (Liu *et al*., 2019; Zhang *et al*., 2019; Miao *et al*., 2022). Mannanase is an enzyme that specifically hydrolyzes and breaks down mannan, a type of complex carbohydrate or polysaccharide composed of mannose sugar units linked together (Rattanasuk *et al*., 2014; Rattanasuk *et al*., 2015; Singh *et al*., 2019). The result of glucogalactomannan hydrolysis using mannanase was mannooligosaccharide which act as a prebiotic (Phothichitto *et al*., 2006).

Prebiotics are a type of non-digestible dietary fiber that not broken down by human digestive enzymes in the small intestine, but instead pass undigested into the colon. Prebiotics selectively nourish and support the growth of probiotic bacteria, while discouraging the growth of potentially harmful microorganisms (Chen *et al*., 2023). The beneficial gut bacteria ferment prebiotics and produces various metabolites, including short-chain fatty acids, which have been associated with health benefits including improved gut health and balance, enhanced digestion and nutrient absorption, potential contributions to the immune system and support for overall well-being (Ballini *et al*., 2023). Research aimed to determine the effect of *C. militaris* hydrolysate, derived from mannanase hydrolysis, on the growth of probiotic bacteria.

Materials and Methods

Cordyceps militaris liquid inoculum preparation

The liquid culture medium for *C. militaris* comprised boiled potato water (200 g potato), 25 g glucose, 2 g yeast extract, and 2 g peptone. Water was added to 1 liter and was steriled by autoclave at 121℃ for 20 min before used. After the liquid culture was cooled, 3 pieces of 1×1 cm *C. militaris* cultured on agar were transferred into a liquid culture medium. The bottle was cultured in the dark condition, shaking at 22℃ for 7 days.

Cordyceps militaris rice culture medium preparation and cultivation

The culture medium was prepared followed to Wen's (Wen *et al*., 2014). Twenty milliliters of culture medium were added to the bottle containing 20 g of jasmine rice. The bottles were steriled at 121℃ for 30 min. After the rice culture medium was cooled, 3 ml of *C. militaris* inoculum was added using a sterile technique. The bottles were incubated in the dark condition at 22℃ for 14 days, 18℃ for 14 days, and in light conditions at 22℃ for 45 days. The C. militaris fruiting body was collected.

Cordyceps militaris powder preparation

For *C. militaris* powder preparation, the *C. militaris* fruiting body was dried at 45℃ for 72 hr. The dried *C. militaris* was ground to a fine powder and stored for later use. For boiled *C. militaris* powder preparation, the dried *C. militaris* was boiled 5 times at 100℃ and dried at 45℃ for 72 hr before being powdered.

Cordyceps militaris hydrolysis using mannanase enzyme

Bacillus methylotrophicus KS1 is a mannanase-producing bacteria isolated from soil. *B. methylotrophicus* KS1 was cultured using sterilized Luria Bertani (LB) and incubated at 37℃, 150 rpm for 24 hr. One milliliter of *B. methylotrophicus* KS1 was added to each bottle containing 100 ml LB +1 g *C. militaris* powder, 100 ml LB +1 g boiled *C. militaris*, and 100 ml LB +1 g copra meal (control). The bottles were incubated at 37℃ for 7 days. One milliliter of cultured broth was collected every 24 hr for reducing sugar determination using the DNS method (Phothichitto *et al*., 2006; Rattanasuk *et al*., 2020).

Effect of *Cordyceps militaris* hydrolysate on probiotic growth

Five probiotic bacteria were used including *Lactobacillus plantarum* TISTR 543 *Lactobacillus casei* TISTR 390, *Lactobacillus acidophilus* TISTR 1338, *Lactobacillus bulgaricus* TISTR 451, *Bacillus subtilis* TISTR 28960. The culture broth obtained from *C. militaris* hydrolysis was steriled by a 0.22 µm sterile syringe filter and used as crude mannooligosaccharide (MOS). All probiotic bacteria were cultured with an appropriate culture broth medium overnight. The probiotic bacterial concentration was adjusted by measuring OD_{600} to 0.1. The crude MOS from *C. militaris* and boiled *C. militaris* hydrolysates were added to 96 well culture plates to the final concentration of 3.33 - 66.67% (v/v). The crude MOS from copra meal hydrolysate was used as a positive control. All experiments were triplicate. The 96 well culture plates were measured with the optical density at 600 nm before and after being incubated at 37℃ for 2 days.

Statistical Analysis

The data for optical density at 600 nm was expressed as mean ± standard error.

Results and Discussion

Reducing sugar determination

Cordyceps militaris, a medicinal mushroom, contains a glucogalactomannan. The monosaccharide composition of this glucogalactomannan is mannose (56.7%), galactose (34.5%), and glucose (8.8%) (Smiderle *et al*., 2013). Glucogalactomannan was hydrolyzed using mannanase obtained from *B. methylotrophicus* KS. The hydrolysates were mannooligosaccharides that function as prebiotics (Rattanasuk *et al*., 2015; Prayoonthien *et al*., 2019). The result of reducing sugar determination indicated that the highest amount of the reducing sugar at 0.94 mg/ml was obtained from *C. militaris* hydrolysis on day 6, followed by boiled *C. militaris* (0.23 mg/ml) and Copra meal (0.14 mg/ml) on day 7 (Table 1).

Probiotic bacterial growth

The hydrolysates were obtained from copra meal, boiled *C. militaris*, and *C. militaris* hydrolysis using mannanase-producing bacteria, *B. methylotrophicus* KS1 The hydrolysate concentrations at 3.33-33.33% were added to determine the probiotic bacterial growth (*L. plantarum* TISTR 543, *L. casei* TISTR 390, *L. acidophilus* TISTR 1338, *L. bulgaricus* TISTR 451, *B. subtilis* TISTR 28960). The results indicated that the hydrolysate obtained from *C. militaris* hydrolysis is suitable to promote probiotic growth. The highest probiotic growth was presented *in L. acidophilus* TISTR 1338 when *C. militaris* hydrolysate at 6.67%. Followed by *L. casei* TISTR 390 (20%). *L. plantarum* TISTR 543 (20%), *L. bulgaricus* TISTR 451 (26.67%), respectively. Only *B. subtilis* TISTR 28960 which showed high growth when boiled *C. militaris* was added at 33.33% (Table 2-6).

Table 1. Reducing sugar concentration of copra meal, boiled *C. militaris*, and *C. militaris* after hydrolysis using mannanase enzyme from *B. methylotrophicus* KS1

Day	Copra meal (mg/ml)	Boiled *C. militaris* (mg/ml)	*C. militaris* powder (mg/ml)
1	0.06	0.05	0.20
2	0.02	0.08	0.34
3	0.06	0.13	0.31
4	0.07	0.19	0.44
5	0.08	0.16	0.72
6	0.11	0.18	0.94
7	0.14	0.23	0.72

Table 2. The average values of OD600 of *Lactobacillus plantarum* TISTR 543

Hydrolysate concentration (% v/v)	Copra meal hydrolysate (Control)		Boiled *C. militaris* hydrolysate		*C. militaris* hydrolysate	
	Before	**After ($\bar{x}$±SD)**	**Before**	**After ($\bar{x}$±SD)**	**Before**	**After ($\bar{x}$±SD)**
3.33	0.10	0.72+0.02	0.10	0.59+0.01	0.10	0.67+0.01
6.67	0.10	0.65+0.01	0.10	0.58+0.02	0.10	0.68+0.01
10.00	0.10	0.52+0.03	0.10	0.58+0.01	0.10	0.72+0.02
13.33	0.10	0.42+0.02	0.10	0.58+0.03	0.10	0.75+0.03
16.67	0.10	0.31+0.03	0.10	0.55+0.03	0.10	0.80+0.01
20.00	0.10	0.29+0.01	0.10	0.48+0.01	0.10	0.90+0.02
23.33	0.10	0.28+0.02	0.10	0.48+0.01	0.10	0.75+0.04
26.67	0.10	0.26+0.02	0.10	0.48+0.04	0.10	0.75+0.01
30.00	0.10	0.25+0.03	0.10	0.47+0.03	0.10	0.73+0.01
33.33	0.10	0.22+0.02	0.10	0.47+0.01	0.10	0.68+0.02

Table 3. The average values of OD600 of *Lactobacillus casei* TISTR 390

Hydrolysate concentration (% v/v)	Copra meal hydrolysate (control)		Boiled *C. militaris* hydrolysate		*C. militaris* hydrolysate	
	Before	**After ($\bar{x}$±SD)**	**Before**	**After ($\bar{x}$±SD)**	**Before**	**After ($\bar{x}$±SD)**
3.33	0.10	0.73±0.02	0.10	1.20±0.03	0.10	1.27±0.4
6.67	0.10	0.65±0.03	0.10	1.34±0.02	0.10	1.34±0.01
10.00	0.10	0.48±0.01	0.10	1.37±0.04	0.10	1.37±0.02
13.33	0.10	0.42±0.02	0.10	1.41±0.02	0.10	1.39±0.03
16.67	0.10	0.21±0.04	0.10	1.42±0.04	0.10	1.51±0.02
20.00	0.10	0.29±0.03	0.10	1.44±0.03	0.10	1.56±0.03
23.33	0.10	0.29±0.02	0.10	1.46±0.02	0.10	1.43±0.02
26.67	0.10	0.29±0.03	0.10	1.46±0.01	0.10	1.41±0.01
30.00	0.10	0.36±0.01	0.10	1.47±0.02	0.10	1.43±0.03
33.33	0.10	0.36±0.02	0.10	1.44±0.01	0.10	1.41±0.03

Table 4. The average values of OD600 of *Lactobacillus acidophilus* TISTR 1338

Hydrolysate concentration (% v/v)	Copra meal hydrolysate (control)		Boiled *C. militaris* hydrolysate		*C. militaris* hydrolysate	
	Before	**After ($\bar{x}$±SD)**	**Before**	**After ($\bar{x}$±SD)**	**Before**	**After ($\bar{x}$±SD)**
3.33	0.10	0.14±0.02	0.10	1.01±0.03	0.10	1.24±0.02
6.67	0.10	0.17±0.01	0.10	1.13±0.04	0.10	1.97±0.03
10.00	0.10	0.18±0.01	0.10	1.26±0.01	0.10	1.71±0.04
13.33	0.10	0.18±0.02	0.10	1.32±0.02	0.10	1.64±0.04
16.67	0.10	0.20±0.04	0.10	1.34±0.01	0.10	1.50±0.01
20.00	0.10	0.23±0.03	0.10	1.34±0.02	0.10	1.58±0.05
23.33	0.10	0.24±0.05	0.10	1.38±0.03	0.10	1.47±0.02
26.67	0.10	0.26±0.02	0.10	1.39±0.02	0.10	1.44±0.01
30.00	0.10	0.30±0.03	0.10	1.40±0.02	0.10	1.46±0.02
33.33	0.10	0.31±0.02	0.10	1.41±0.04	0.10	1.47±0.03

Table 5. The average values of OD600 of *Lactobacillus bulgaricus* TISTR 451

Hydrolysate concentration (% v/v)	Copra meal hydrolysate (control)		Boiled *C. militaris* hydrolysate		*C. militaris* hydrolysate	
	Before	**After ($\bar{x}$±SD)**	**Before**	**After ($\bar{x}$±SD)**	**Before**	**After ($\bar{x}$±SD)**
3.33	0.10	0.10±0.02	0.10	0.05±0.01	0.10	0.14±0.02
6.67	0.10	0.07±0.01	0.10	0.11±0.03	0.10	0.19±0.01
10.00	0.10	0.03±0.03	0.10	0.12±0.03	0.10	0.26±0.01
13.33	0.10	0.01±0.03	0.10	0.21±0.05	0.10	0.31±0.04
16.67	0.10	0.25±0.02	0.10	0.17±0.03	0.10	0.34±0.04
20.00	0.10	0.07±0.02	0.10	0.27±0.03	0.10	0.43±0.03
23.33	0.10	0.09±0.04	0.10	0.37±0.03	0.10	0.45±0.03
26.67	0.10	0.12±0.03	0.10	0.42±0.02	0.10	0.61±0.02
30.00	0.10	0.10±0.02	0.10	0.48±0.01	0.10	0.56±0.04
33.33	0.10	0.09±0.02	0.10	0.53±0.02	0.10	0.56±0.03

Table 6. The average values of OD600 of *Bacillus subtilis* TISTR 28960

Hydrolysate concentration (% v/v)	Copra meal hydrolysate (control)		Boiled *C. militaris* hydrolysate		*C. militaris* hydrolysate	
	Before	After ($\bar{x}$+SD)	Before	After ($\bar{x}$+SD)	Before	After ($\bar{x}$+SD)
3.33	0.10	0.36+0.01	0.10	0.85+0.01	0.10	0.85+0.03
6.67	0.10	0.38+0.03	0.10	1.05+0.01	0.10	0.97+0.01
10.00	0.10	0.41+0.02	0.10	1.11+0.01	0.10	1.02+0.02
13.33	0.10	0.55+0.03	0.10	1.15+0.02	0.10	1.06+0.02
16.67	0.10	0.57+0.03	0.10	1.21+0.02	0.10	1.09+0.03
20.00	0.10	0.72+0.03	0.10	1.24+0.03	0.10	1.12+0.03
23.33	0.10	0.80+0.02	0.10	1.31+0.03	0.10	1.12+0.04
26.67	0.10	0.83+0.02	0.10	1.34+0.03	0.10	1.14+0.02
30.00	0.10	0.85+0.04	0.10	1.38+0.02	0.10	1.16+0.01
33.33	0.10	0.95+0.02	0.10	1.47+0.02	0.10	1.28+0.02

Mannooligosaccharides (MOS) are a specific type of prebiotic that consists of short chains of mannose. Human enzymes do not digest them in the small intestine and instead reach the colon intact, where they serve as a source of nutrition for beneficial gut bacteria, including certain strains of probiotics (Rana, *et al*., 2023; Rana *et al*., 2023). The results from this research were similar to Plangklang *et al*. that MOS can be promoted for the growth of *L. plantarum* TISTR 1265, *L. rhamnosus* TISTR 2443, *Bifidobacterium bifidum* TISTR 2129 (Plangklang *et al*., 2023). Pongsapipatana *et al*. was reported MOS that obtained from hydrolysis using mannanase stimulated the growth of the probiotic, *Limosilactobacillus reuteri* KUB-AC5 (Pongsapipatana *et al*., 2023). Villéger *et al*. reported that isomaltooligosaccharides can improve the fitness of Bacillus probiotics, *Bacillus subtilis* CU1 and improve the germination when present of bile salts in vitro (Villéger *et al*., 2022).

C. militaris is known to contain glucogalactomannan, a specific type of polysaccharide. This glucogalactomannan is a key component of the fungal cell wall in C. militaris. Glucogalactomannans are one of the bioactive compounds found in *C. militaris*. This glucogalactomannan from *C. militaris* is beneficial for the growth of probiotics (Miao *et al*., 2022).

This makes *C. militaris* a potentially valuable resource in the development of probiotics and related health products. However, more research is needed to fully understand the implications and potential applications of these findings.

Conclusions

This research indicated that the hydrolysate obtained from boiled *C. militaris* and *C. militaris* hydrolysis using mannanase can stimulate the growth of probiotics, *Lactobacillus plantarum* TISTR 543 *Lactobacillus* casei TISTR 390, *Lactobacillus acidophilus* TISTR 1338, *Lactobacillus bulgaricus* TISTR 451, *Bacillus subtilis* TISTR 28960.

Glucogalactomannan, a type of polysaccharide found in *C. militaris*, is beneficial for the growth of probiotics. However, more research is needed to fully understand the implications and potential applications of these findings. This highlights the importance of continued exploration in the field of probiotics and functional foods.

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