PREBIOTIC PROPERTIES OF POLYSACCHARIDES ISOLATED FROM *CORDYCEPS MILITARIS* MYCELIA

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ABSTRACT:

Polysaccharides (PS) is the major bioactive ingredients in *Cordyceps militaris (C.militaris)*. This study evaluated the prebiotics activity of *C.militaris* in liquid culture. Findings indicated that PS extracted from this mycelium could promote the growth of beneficial microorganisms in the intestine, *Lactiplantibacillus plantarum (L.plantarum).* The extract from this bacterial culture medium is able to inhibit the growth of *Escherichia coli (E.coli)* and *Staphylococcus aureus (S.aureus)* with inhibition zones of 19.33 ± 0.577 mm, and 21.67 ± 1.443 mm, respectively. The solution culture of *L.plantarum* supplemented with *C.militaris's* mycelium reduces the pH of the medium and produces short-chain fatty acids (SCFAs) with acetic, butyric, and propionic acid contents of 11626 mg/L, 8047.5 mg/L and 334.78 mg/L, respectively. The results show the potential to use *C.militaris's* mycelium as a raw material for prebiotics production to replace other commercial prebiotics on the market.

Keywords: short-chain fatty acids, *L. plantarum, C. militaris,* polysaccharide, prebiotics.

1. Introduction

Cordyceps militaris is known as a medicinal mushroom with many valuable biologically active substances such as cordycepin, adenosine, ergosterol, and minerals with hypoglycemic effects, anti-inflammatory effects, inhibiting the growth of cancer cells, immune system regulation, and antioxidant properties [1]. In addition, the high content of polysaccharides (PS) from mycelium has the effect of reducing weight and blood glucose levels, enhancing tolerability of glucose, protecting immune organs, and repairing lipid disorders in the blood to control diabetes in mice [2].

Indigestible polysaccharides derived from mushrooms are potential sources of prebiotics because they may prevent viral or bacterial infections by enhancing the growth of probiotics in the intestine [3]. In the presence of intestinal microbiota fermentation, short-chain acids (SCFAs) derived from polysaccharides have positive effects on gut health, including increasing epithelial cell proliferation and lowering colonic pH. The metabolites of SCFAs have a positive effect on health as crucial and essential factors in maintaining intestinal integrity, inhibiting bacterial pathogenicity, and protecting the entire properties of intestinal epithelial cells from

mechanical, chemical, and microbial damage. Besides, SCFAs might also enhance the absorption of minerals, stimulate the host's immune system, enhance anti-inflammatory ability, and prevent cancer [4].

In parallel with the research and development of advanced technology for cultivation of edible and medicinal mushrooms, the liquid fermentation to collect mycelium biomass is opening a new potential and prospect in shortening the cultivation cycle and allowing easy control of the culture conditions on an industrial scale. A large amount of organic biomass is obtained to fulfill the increasing demand for food and pharmaceuticals, especially the utilization of polysaccharide-rich mycelium biomass for prebiotics production, which is a matter of current concern.

In this study, we investigated the activity of polysaccharides obtained from submerged cultures of *C.militaris's* mycelium, which is one of the potential prebiotics, by testing their antioxidant and prebiotic activities. Findings indicated that the polysaccharide of *C. militaris* is a potential prebiotic to inhibit the growth of harmful bacteria and stimulate the growth of beneficial bacteria through the production of metabolites.

2. Materials and Methodology

2.1. Materials

Grade 1 of *C.militaris* were supplied by the Laboratory of Mushroom Biotechnology, Faculty of Biology and Environmental Science, University of Education - University of Danang.

Commercial prebiotics, fructo-oligosaccharides (FOS) and inulin were provided by Novaco Pharmaceuticals JSC. Probiotics varieties - *L. plantarum* is provided by Vietnam Biotech Technology Joint Stock Company. The bacteria, Escherichia coli, Bacillus cereus were provided by the Laboratory of Microbiology, Faculty of Biology and Environmental Science, University of Education - University of Danang.

2.2. Methodology

2.2.1. Cultivation of C.militaris's mycelium biomass

The grade 1 of *C. militaris* was propagated in glass tubes (F1,8 cm, 180 mm) with a medium composition including potato (200 g/L), glucose (20 g/L) and agar (15 g/L), incubated at 25^{o}C for 10 days. Then, the mycelium was cultured by adding 150 mL of PD+ medium (including potato: 200 g; glucose: 20 g; yeast extract: 2 g; peptone: 2g; H_2O: 1 L) into the 500 mL-triangle flasks. Autoclaved with hot steam at 121^{o}C for 30 minutes, cooled to room temperature, and then inoculated equal quantities of mycelium from the grade 1 into each 500 mL-triangle flasks. Shaking the culture (150 rpm) at a temperature of 18^{o}C - 22^{o}C and collecting the mycelium after 7 days.

2.2.2. Collection of C.militaris's mycelium biomass

Dry the filter papers (11mm, F150 mm) to a constant weight at 50^{o}C, then weigh them to record their initial mass. Pour the entire culture solution in the conical flask through the filter funnel, then dry the biomass and filter papers at 50^{o}C to a constant weight, cool, and weigh to gain the dry mass [5].

2.2.3. Extraction and determination of total polysaccharide content

Drying C.militaris's mycelium at 50^{o}C to a constant weight before extracting the crude polysaccharide with hot water and precipitating with ethanol according to Sun's (2005) guidance with modifications.

The mycelium, after drying, was milled and soaked in 70^{o}C-water at a ratio of 1:25 for 3 hours. Then, collect the filtrate and precipitate it with 96% alcohol (1:5) for 12 hours at 4^{o}C [6]. The precipitate was washed twice with 96% alcohol and dried at 60^{o}C for 2 hours to obtain crude polysaccharide.

Protein removal by the Sevag's method. The total polysaccharide content was determined by the phenol-sulfuric acid method [7]. Based on the hydrolysis reaction of polysaccharides to monosaccharides, coloring with phenol forms a

solution with maximum absorption at λ = 490 nm. The D-glucose standard curve line was used for the determination of polysaccharides.

2.2.4. Evaluation of growth stimulation for L. plantarum.

The *L. plantarum* was propagated on MRS medium (glucose: 20 g, peptone: 10 g, yeast extract: 5 g, K_2HPO_4: 2 g, CH_3COONa: 5 g, $MgSO_4.7H_2O$: 0.58 g, H_2O: 1 L), pH=7 at 37°C for 24 h under anaerobic conditions (controlling experiments), and MRS medium supplemented with 10 mg/mL extract of *C. militaris' s* mycelium.

The cell concentration of *L. plantarum* was determined through optical density at 620 nm (OD 620) by the UV-VIS spectrophotometer [8]. MRS medium supplemented with 10 mg/mL FOS (fructo-oligosaccharide) and inulin was used as a positive control experiment.

After determining the cell concentration above, plate culture and count the clumps to determine the cell density according to the following formula (2).

$$M_i(CFU/mL) = \frac{A_i \times D_i}{V} \qquad (2)$$

Which A_i is the average number of clumps per plate; D_i is the dilution; V is the volume of cell suspension added to each plate (mL). The experiment was arranged on four formulas as followed:

Formulas	Compositions
CT1	MRS medium
CT2	MRS medium added 10mg/mL FOS
CT3	MRS medium added 10mg/mL inulin
CT4	MRS medium added 10mg/mL PS's extract from C. militaris

2.2.5. Evaluation of growth inhibition against E. coli and S. aureus

L. plantarum in the above 4 formulas were cultured at 37°C for 48 hours. The transparent solution after centrifugation (5000 rpm for 20 min at 4°C) was used to determine the growth inhibition against *E. coli* and *S. aureus.* Inoculate a 50 µL solution of *E. coli* and *S. aureus* on plates containing NA medium (yeast extract: 3 g/L, peptone: 5 g/L, and agar: 15 g/L) by plate culture technique.

Place the transparent solution obtained from the above centrifugation into the agar holes (9 mm) on plates containing those bacteria and incubate at 37°C for 24h. Inhibitory capacity was determined by measuring and comparing the sterile ring diameters on plates with PS supplementation and controls [9].

2.2.6. Quantification of SCFAs

In four experiments, cultures of *L. plantarum* were filtered through a 0.44 µm membrane. Then, centrifuge these solutions (5000 rpm for 10 min at 4°C), collect the supernatant for analysis of lactic, acetic, propionic, and butyric acids by HPLC (Agilent 1200) with a 0.20 µm acetate cellulose membrane filter. The RI detector and Aminex HPX87H column (BioRad, Hercules, CA, USA) were used to analyze SCFAs, with the mobile phase being 3 mM H_2SO_4 at 0.6 mL.min^{-1}, 50°C [10].

3. Results and Discussion

3.1. Total polysaccharide content of C.militaris's mycelium

The total polysaccharide content obtained from the *C.militaris's* mycelium when extracted with alcohol 96% was 3204.65 ± 214.48 µg/mL. This result is lower than Liu's study, 5660 µg/mL when extracting polysaccharide by ethanol 96°C [11]; higher than Wang's study, 390 µg/mL in the study of polysaccharide extraction from Yunzhi mycelium [12]. The difference in those findings may be due to differences in the varieties used, the growing conditions, and the extraction methods and techniques applied. The high content of soluble polysaccharide in *C.militaris's* mycelium pointed out a great potential for obtaining bioactive compounds cultivated from mycelium.

3.2. Effect of polysaccharides on the growth of beneficial microorganisms

Gut microbiotas have numerous functions in metabolism and the immune system to protect the host body. The symbiotic relationship between the

gut microbiota and the host is regulated and maintained by cross-communication. This exchange is mediated through metabolites synthesized by the microbiome as well as the host organism, such as neuro-immune-regulating signaling molecules, inflammatory responses that connect intestinal communication with other organs [13].

L. plantarum is one of the beneficial microorganisms in the intestinal system. Polysaccharide extracted from the *C.militaris's* mycelium has the ability to stimulate the growth of *L. plantarum.* Table 1 shows the growth of *L. plantarum* in MRS medium with and without adding polysaccharides extracted from *C. militaris* compared with MRS medium supplemented with FOS and Inulin.

The cell density was highest in the MRS medium supplemented with PS extracted from *C. militaris* mycelium (CT4), 2.59 × 10^{10} CFU/mL, higher than CT3, 2.16 × 10^{10} CFU/mL, CT2, 1.23 × 10^{10} CFU/mL and CT1, 1.06 × 10^{10} CFU/mL.

When investigating the growth-stimulating activity of beneficial microorganisms from the extracts of seven fungi, Sawangwan found that the cell density on all media supplemented with mushroom extract was higher than that on the medium without adding this extract [14]. Even, in our experiment, the cell density in formulas supplemented with PS extracted from *C.militaris's* mycelium was higher than that in formulas supplemented with commercial prebiotics-FOS.

These could be explained by the fact that polysaccharides extracted from mycelium have a stronger ability to stimulate the growth of beneficial microorganisms than commercial prebiotics such as FOS and inulin. The findings are also consistent with the study of Sawangwan [14].

Table 1. The cell density of *L. plantarum* in formulas cultured at 37°C for 24h

Formulas	Optical density	Cell density (CFU/mL)
CT1 (n=30)	3.1768 ± 0.0028[a]	1.06× 1010
CT2 (n=30)	3.1866 ± 0.0112[a]	1.23× 1010
CT3 (n=30)	3.2648 ± 0.0140[b]	2.16× 1010
CT4 (n=30)	3.2292 ± 0.0013[c]	2.59× 1010

Note: letters a, b, and c represent the significant difference between the formulas ($p < 0.05$)

Figure 1. The number of L.plantarum clumps cultured on the MRS medium

(Note: A, B, C, and D are L.plantarum cultured in CT1, CT2, CT3, and CT4)

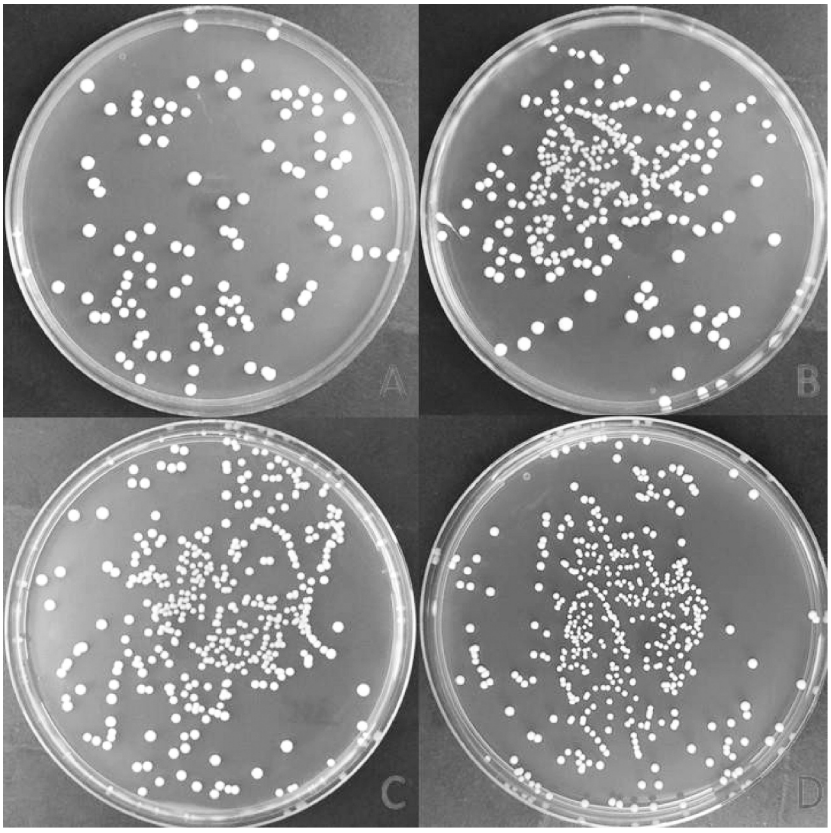


Figure 2. The inhibitory zone diameters of E. coli, S. aureus from the L. plantarum culture in experimental formulas

(Note: CT1 was the MRS medium; CT2, CT3, and CT4 were the MRS medium supplemented with FOS, Inulin, and PS extract from C.militaris's mycelium, respectively)

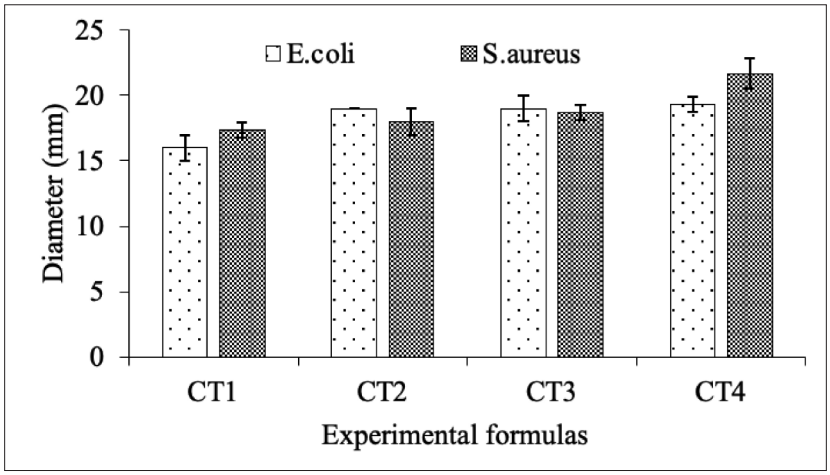
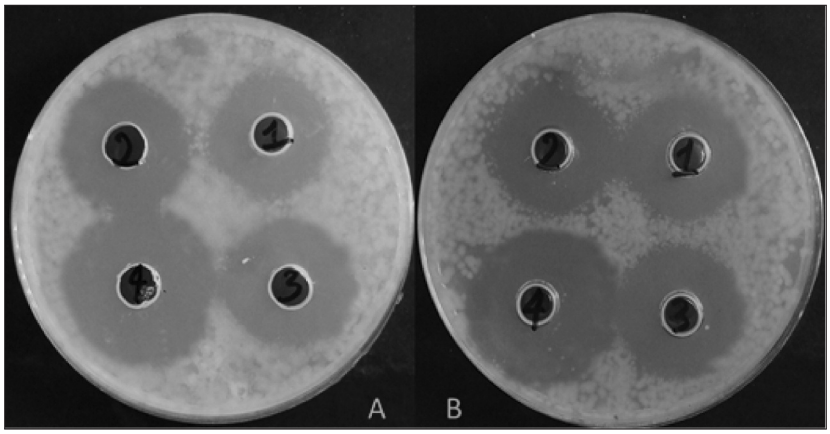


Figure 3. Sterile rings of E. coli (A), S. aureus (B) cultured on plates containing NA medium (Note: 1=CT1, 2=CT2, 3=CT3, 4=CT4)



The number of *L. plantarum* clumps were highest when cultured on the medium supplemented with PS extract from *C.militaris's* mycelium (Figure 1-D, CT4) was 2,59× 10^{10} (CFU/mL), corresponding to the OD was 3.2292 ± 0.0013, greater than the other formulas. This indicated that PS extracted from *C.militaris's* mycelium not only stimulates the growth of beneficial bacteria but also prolongs their growth time. PS extracted from *C.militaris's* mycelium can stimulate the growth of *L. plantarum* better than available commercial prebiotics sources.

3.3. The ability to inhibit the growth of pathogenic bacteria

E. coli and *S.aureus* are bacteria that cause intestinal diseases. They often cause poisoning, abdominal pain, vomiting, and diarrhea. The growth of *L. plantarum* both creates a quantitative balance of the intestinal microflora and produces substances that inhibit the growth of these pathogenic microorganisms.

Cultures of *L. plantarum* on formulars containing PS extracts from *C.militaris's* mycelium, FOS and inulin were able to inhibit the growth of *E. coli* and *S. aureus*. In CT4, where *L. plantarum* cultured on MRS medium supplemented with PS extract, the inhibitory zones (the sterile ring diameter) against *E. coli* and *S. aureus* were 19.33 mm and 21.67 mm respectively, higher than in CT1. This may be because PS stimulated the production of more compounds capable of inhibiting these bacteria than the medium would have without this supplementation.

The diameter of the sterile rings for *E. coli* at CT4 was almost equal to those of CT2 and CT3. This indicated that the addition of PS extracted from the *C.militaris's* mycelium had facilitated *L. plantarum* in producing compounds that have the ability to inhibit pathogenic bacteria, equivalent to commercial prebiotics, FOS and inulin. Meanwhile, the inhibitory effect of *L. plantarum* extract on *S. aureus* in CT4 was higher than that of CT2 and CT3, indicating that the addition of PS extracts from *C.militaris's* mycelium to

Table 2. Contents of SCFAs in the *L. plantarum* culture medium (after 48 hours)

Formulas	Acid acetic (mg/L)	Acid propionic (mg/L)	Acid butyric (mg/L)
CT1 (n=30)	2095.3 53.9^{c}	153.90 21,80^{c}	4427.1 25.60^{d}
CT2 (n=30)	10753 284^{b}	267.58 11,78^{b}	5916.3 79.10^{c}
CT3 (n=30)	11611 348^{a}	327.65 17,20^{a}	7406.3 71.70^{b}
CT4 (n=30)	11626 235^{a}	334.78 15.65^{a}	8047.5 92.60^{a}

Note: letters a, b, c, and d represent the significant difference between formulas ($p < 0.05$)

L. plantarum culture medium increased the production of beneficial substances that inhibit the growth of this harmful bacteria.

Besides, the sterile ring diameters for *E. coli* in formulas supplemented with FOS and inulin were also different. This proves that each type of prebiotic has a different structure that will facilitate beneficial microorganisms to metabolize and secrete inhibitory compounds into the culture medium against different strains of pathogenic bacteria. These findings were consistent with Sawangwan's previous study [14].

Alves reported that several bioactive compounds such as peptides (plectasin), polysaccharides (beta-glucan), organic acids (benzoic acid), and phenolic compounds (catechins) from mushroom extracts have broad-spectrum antimicrobial potential, inhibiting growth not only against *E. coli,* but also against other intestinal bacteria such as *Klebsiella pneumonia, Pseudomonas aeruginosa* and *Salmonella typhimurium* [15].

3.4. The content of SCFAs during the culture of L. plantarum

SCFAs have anti-inflammatory and antibacterial effects that can reduce the severity of many pathogens, such as gastrointestinal diseases, cancer, and cardiovascular disease [16]. Polysaccharide extracted from the *C. militaris's* mycelium promotes the production of acetate, propionate, and butyrate. Therefore, it can be used as one of the potential types of prebiotics beneficial to human health.

Cultivation of *L. plantarum* with supplementation of *C. militaris's* mycelium produced SCFAs, including acetic, propionic, and butyric acids. which butyric acid content was highest in CT4 (8047.5 mg/L). In CT1, without the addition of prebiotics, the SCFAs content was lower than that of the remaining formulas, CT2, CT3, and CT4. The study by Wang indicated that the content of SCFAs produced by resistant starch after 48 hours of culture were 758 mg/L (acetic acid), 615 mg/L (propionic acid), and 360 mg/L (butyric acid) [17].

4. Conclusions

The total polysaccharide content extracted from the *C.militaris's* mycelium was 3204.65±214.48 μg/mL. This polysaccharide proved to have a higher growth stimulant capacity for *L. plantarum* than commercial prebiotics (FOS and Inulin). Cultivation of *L. plantarum* in MRS medium supplemented with this polysaccharide produced a culture solution that effectively inhibits the growth of *E. coli* and *S. aureus*. Notably, the culture of *L. plantarum* in the medium supplemented with this polysaccharide promoted the production of SCFAs, especially butyric acid. Therefore, *C. militaris* has the potential to be a new source of prebiotics in the future, contributing to the diversification of sources of prebiotics on the market and enhancing the value of agricultural products ■

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KHẢO SÁT HOẠT TÍNH PREBIOTIC CỦA POLYSACCHARIDE CHIẾC XUẤT TỪ SỢI NẤM ĐÔNG TRÙNG HẠ THẢO (*CORDYCEPS MILITARIS*)

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TÓM TẮT:

Polysaccharide (PS) là thành phần chính có hoạt tính sinh học cao trong nấm Đông trùng hạ thảo (*C. militaris*). Nghiên cứu này đánh giá hoạt tính prebiotics của sinh khối hệ sợi nấm *C. militaris* trong môi trường nuôi cấy dịch thể. Kết quả nghiên cứu chỉ ra rằng, PS chiết xuất từ hệ sợi nấm *C. militaris* có khả năng thúc đẩy sự sinh trưởng của chủng vi sinh vật đường ruột *L. plantarum*. Dịch chiết từ môi trường nuôi cấy *L. plantarum* có khả năng ức chế sự sinh trưởng của *Escherichia coli* và *Staphylococcus aureus* với vùng ức chế lần lượt là 19,33±0,577 mm, và 21,67±1,443 mm. Quá trình lên men *L. plantarum* có bổ sung hệ sợi nấm *C. militaris* làm giảm pH môi trường và sản xuất ra các axít béo mạch ngắn (SCFAs) với hàm lượng axít axetit thu được là cao nhất với 11626 mg/L, axit butyric và axit propionic lần lượt là 8047,5 mg/L và 334,78 mg/L. Kết quả cho thấy tiềm năng sử dụng sợi nấm *C. militaris* làm nguồn nguyên liệu sản xuất prebiotics bên cạnh các nguồn prebiotics thương mại khác trên thị trường.

Từ khóa: Axít béo mạch ngắn, *Lactobacillus plantarum, C. militaris,* Polysaccharide, Prebiotics.