Effects of Cordyceps militaris Mycelia on Fibrolytic Enzyme Activities and Microbial Populations In vitro

Joon Mo Yeoa, Shin Ja Lee1,*, Sung Hwan Shin1, Sung Hoon Lee2, Jong Kyu Ha3, Wan Young Kim and Sung Sill Lee1, *
Department of Animal Science, Korea National Agricultural College, RDA, Suwon 445-893, Korea

ABSTRACT : An experiment was conducted to examine the effects of Cordyceps militaris mycelia on microbial populations and fibrolytic enzyme activities in vitro. C. militaris mycelia was added to buffered rumen fluid with final concentrations of 0.00, 0.10, 0.15, 0.20, 0.25 and 0.30 g/L and incubation times were for 3, 6, 9, 12, 24, 36, 48 and 72 h. At all incubation times, the supplementation of C. militaris mycelia linearly increased the number of total viable and cellulolytic bacteria; maximum responses were seen with 0.25 g/L supplementation of C. militaris mycelia. The addition of C. militaris mycelia above the level of 0.20 g/L significantly (p<0.01) increased the number of total and cellulolytic bacteria compared with the control. On the other hand, the response of fungal counts to the supplementation of C. militaris mycelia showed a linear decrease; the lowest response was seen with 0.30 g/L supplementation of C. militaris mycelia. It would seem that C. militaris mycelia possess a strong negative effect on rumen fungi since the lowest level of C. militaris mycelia supplementation markedly decreased fungal counts. Carboxymethyl cellulase activities were linearly increased by the addition of C. militaris mycelia except at 3 and 9 h incubation times. At all incubation times, the supplementation of C. militaris mycelia linearly increased the activities of xylanase and avicelase. In conclusion, the supplementation of C. militaris mycelia to the culture of mixed rumen microorganisms showed a positive effect on cellulolytic bacteria and cellulolytic enzyme activities but a negative effect on fungi. (Key Words : Cordyceps militaris, Rumen Microorganisms, Enzyme)

* Corresponding Author : Sung Sill Lee. Tel: +82-55-751-5411, Fax: +82-55-751-5410, E-mail: lss@gnu.ac.kr
1 Division of Applied Life Science (BK21 Program), Graduate School of Gyeongsang National University, Jinju 660-701, Korea.
2 Gyeongsangnam-Do Livestock Veterinary Research Institute, Sancheong 666-962, Korea.
3 Department of Agricultural Biotechnology, Seoul National University, Seoul 151-741, Korea.
* These authors contributed equally to this work.
Received February 2, 2009; Accepted December 7, 2010

INTRODUCTION

Cordyceps species are medical fungi well known for their pharmacological actions such as immunomodulatory (Koh et al., 2002; Yu et al., 2003), anti-inflammatory (Yu et al., 2004a, b), antitumor (Nakamura et al., 1999), antifungal (Kneifel et al., 1977) and antibacterial (Ahn et al., 2000) activities, and contain biologically active components such as nucleosides (cordycepin; 3’-deoxyadenosine, and adenosine), polysaccharides and ergosterol (Li et al., 2006). Although the pharmacological actions of Cordyceps may also affect livestock beneficially, the application of Cordyceps in livestock has received little attention. Recently, however, it has been reported that an oral dose of a hot-water extract of mycelia from C. sinensis, in an attempt to substitute for antibiotic growth promoter, improved bodyweight gain and the immune system in broiler chicks (Koh et al., 2003).

For ruminants, we investigated the effects of Cordyceps militaris mycelia on rumen microbial fermentation by measuring in vitro gas production, cellulose digestion and VFA concentrations (Yeo et al., 2009). The results of a previous study showed that C. militaris mycelia altered the mixed rumen microbial fermentation with increased production of gas and VFA, and cellulose digestion. In a continuing series of studies, the effects of C. militaris mycelia on in vitro rumen microbial growth and hydrolytic enzyme activities are reported here.

MATERIALS AND METHODS

Sample preparation

Dried C. militaris mycelia cultured on a medium composed of corn gluten, soybean protein, beer yeast and
corn steep liquor (culturing method and medium composition are patent pending in Korea) were obtained from EuGene Bio Farm (Hwaseong City, Gyeonggi Province, Korea). The mycelia were composed of 761.6 g CP, 122.4 g crude fat, 9.6 g ether extract, 32.1 g crude ash, 74.3 g nitrogen-free extract, and 1.64 g cordycepin per kg of dry matter. The manufacturer reported that *C. militaris* mycelia used in the present study contained about 2.3 times more cordycepin (1.6 mg/g DM) than *C. militaris* traditionally cultured on faunal pupae (0.7 mg/g DM).

**In vitro batch fermentation**

The anaerobic culture techniques of Hungate (1966) were used for all incubations with rumen fluid from a 515 kg Korean native steer (Hanwoo) fed a basal diet consisting of rice straw and concentrates mixed in a ratio of 4:6 (fresh weight). The steer was housed in an individual metabolic stall and given the diet at 1.75% of body weight. Rumen fluid was collected via a rumen cannula before the morning feeding into a vacuum flask that was flushed with O₂-free CO₂, and squeezed through four layers of cheese cloth into an Erlenmeyer flask in an anaerobic glove box. The fluid was then mixed with buffer (pH 6.9; containing 292 mg of K₂HPO₄, 240 mg of KH₂PO₄, 480 mg of (NH₄)₂SO₄, 480 mg of NaCl, 100 mg of MgSO₄·7H₂O, 64 mg of CaCl₂·2H₂O, 4,000 mg of Na₂CO₃, and 600 mg of cysteine hydrochloride in 1,000 ml of O₂-free distilled water) at a ratio of 1:2. After mixing, 30 ml aliquots of diluted rumen fluid were transferred to 60 ml serum bottles containing 750 mg of Whatman No. 1 cellulose filter paper (FP) as a sole carbon source. Weighed amounts of dried *C. militaris* mycelia were added to achieve final concentrations of 0.00 (control), 0.10, 0.15, 0.20, 0.25 and 0.30 g/L. The bottles (three replicates per treatment) were sealed with butyl rubber stoppers and aluminum caps, and placed in an incubator at 38°C for 3, 6, 9, 12, 24, 36, 48 and 72 h without shaking.

**Sampling and analysis**

At each sampling time, enzyme activities were determined. Supernatant from each culture was separated from sedimentable materials by centrifugation at 3,000 rpm for 20 min. Avicelase activity was determined by incubating 0.96 ml of culture supernatant with 1.0 ml of acetate buffer (0.2 M, pH 5.0) and 10 mg of Avicel-PH101 microcrystalline cellulose in 5 ml serum vials. After incubating for 16 h at 39°C with 200 rpm shaking, 1.5 ml samples were centrifuged at 12,000×g for 5 min and reducing sugars in the supernatant were determined colorimetrically using the dinitrosalicylic acid method of Miller et al. (1960). For carboxymethyl cellulase (CMCase) analysis, 0.5 ml of the supernatant was mixed with 0.5 ml of 1.0% carboxymethyl cellulose (CMC) solution in 0.05 M citrate buffer (pH 5.5). The reaction proceeded for 1 h at 55°C without shaking and was stopped by boiling for 5 min. Samples were centrifuged at 7,000 rpm for 5 min and the reducing sugars were measured as described above. Xylanase activity was assayed with 0.5 ml of 2.0% oat spelts xylan in 0.5 M potassium phosphate buffer (pH 6.5). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μg of glucose equivalent of reducing sugar per minute under the above conditions.

Micropopulations were enumerated using a roll tube and microscopy. Total bacteria and fungal zoospores were enumerated microscopically with a glass slide using a modification of the procedures of Holdman et al. (1977). Viable cells were counted by the cell- or thallus forming unit method for bacteria or fungi, respectively. Cellulolytic bacteria were enumerated by most probable number (MPN; using 10⁻⁶ to 10⁻⁹ serial dilution) procedures. MPN broth was similar to the cellulosomal medium of Dehority et al. (1989) containing carbohydrates sources, glucose, cellobiose and xylose, added to 0.1% final concentration, and a strip (70-100 mm) of filter paper cellulose. After incubation for 12 days, cellulolytic numbers were estimated by visual loss from a cellulose strip.

**Statistical analysis**

Data obtained from the experiment were analyzed using the SAS (1996) software package and differences were tested by Duncan's multiple range test, and p<0.05 was considered significant. Linear, quadratic and cubic responses to *C. militaris* mycelia level were tested by orthogonal contrast.

**RESULTS AND DISCUSSION**

The numbers of total viable and cellulolytic bacteria and anaerobic fungi in the culture of mixed rumen microorganisms at 24 h after incubation are shown in Table 1. At all incubation times, the supplementation of *C. militaris* mycelia linearly increased the number of total viable and cellulolytic bacteria; maximum responses were seen with 0.25 g/L supplementation of *C. militaris* mycelia. Especially, the increase of cellulolytic bacteria number for *C. militaris* supplementation was almost three times higher than for the control. The addition of *C. militaris* mycelia above the level of 0.20 g/L significantly (p<0.01) increased the number of total and cellulolytic bacteria compared with the control. On the other hand, the response of fungal counts to the supplementation of *C. militaris* mycelia showed a linear decrease; the lowest response was seen with 0.30 g/L supplementation of *C. militaris* mycelia. The
difference of fungal counts between the control and 0.3 g/L supplementation was very large, being 26 times lower for the 0.3 g/L supplementation than for the control. It would seem that C. militaris mycelia possess a strong negative effect on rumen fungi since the lowest level of C. militaris mycelia supplementation markedly decreased fungal counts. Rumen bacteria play a particularly important role in fiber degradation because of their much larger biomass and higher activity (Oripin and Joblin, 1997). The increased number of total and cellulolytic bacteria by the addition of C. militaris mycelia supports the results of the previous study (Yeo et al., 2009) where the addition of C. militaris mycelia to rumen fluid inoculum caused a marked increase in cellulose degradation, and production of gas and VFA. Furthermore, the reduced fungal counts observed here suggest that increased cellulose degradation in the previous study was largely attributable to an increased population of cellulolytic bacteria. The reasons for the increased bacteria and decreased fungi in response to C. militaris mycelia are not known, since there is no report on the effect of Cordyceps on rumen microbes. In studies searching for antibiotic alternatives, Cordyceps were shown to have not only antifungal (Kneifel et al., 1977) but also antibacterial activity (Ahn et al., 2000; Koh et al., 2003; Yeon et al., 2007) or even with an increase in lactic acid-producing bacteria (Ahn et al., 2000; Yeon et al., 2007) or even with an increase in Lactobacillus sp. (Koh et al., 2003). For the response of antifungal activity, an indirect effect via increased cellulolytic bacteria cannot be excluded since cellulolytic bacteria and anaerobic fungi are antagonistic (Williams et al., 1990; Bernalier et al., 1993). Rumen fungi possess superior ability to penetrate the plant cell wall and solubilize lignin (Oripin and Joblin, 1997), and therefore, may play an important synergistic role in the ruminal digestion of fiber by physically disrupting the lignified stem tissue. In the present study, since the carbohydrate source was only filter paper (pure cellulose), it should be noted that the response of cellulose digestion to C. militaris mycelia supplementation might not be the same if other carbohydrate sources, such as forage, were used.

The effects of the supplementation of C. militaris mycelia on enzyme activities are shown in Table 2, 3, and 4. The CMCase activities were linearly increased by the supplementation of C. militaris mycelia except at 3 and 9 h incubation times. At 24 h incubation, the response of CMCase activities to the supplementation of C. militaris mycelia was similar to that of total and cellulolytic bacterial

| Incubation times (h) | Supplement levels (%) of C. militaris | SEM | Trend (p) |
|----------------------|--------------------------------------|-----|-----------|
|                      | 0.00 | 0.10 | 0.15 | 0.20 | 0.25 | 0.30 | Linear | Quadratic | Cubic |
| 3                    | 5.07 | 5.42 | 5.03 | 5.68 | 4.61 | 4.98 | 0.75 | 0.490 | 0.652 | 0.523 |
| 6                    | 5.84^b | 6.04^b | 6.26^b | 6.50^a | 4.84^b | 5.55^b | 0.38 | 0.011 | 0.125 | 0.004 |
| 9                    | 6.78^b | 7.85^b | 7.67^b | 9.52^c | 6.67^b | 7.98^b | 0.76 | 0.443 | 0.049 | 0.029 |
| 12                   | 7.26^c | 8.59^c | 8.56^c | 10.06^c | 9.73^b | 9.70^c | 0.76 | 0.001 | 0.026 | 0.701 |
| 24                   | 8.80^d | 9.69^c | 9.31^c | 11.62^c | 11.18^b | 10.57^b | 0.86 | 0.004 | 0.025 | 0.326 |
| 36                   | 9.11^b | 9.83^b | 10.44^c | 12.81^c | 12.29^b | 10.43^b | 0.88 | 0.005 | <0.001 | 0.184 |
| 48                   | 9.08^c | 9.86^c | 10.89^c | 14.39^c | 12.68^b | 10.57^b | 1.34 | 0.023 | 0.002 | 0.402 |
| 72                   | 8.77^d | 9.78^d | 10.29^a | 13.43^a | 12.00^b | 10.50^b | 0.75 | <0.001 | <0.001 | 0.285 |

SEM = Standard error of the mean. *a,b,c,d,e* Means within a row with different superscripts differ significantly (p<0.05).
Table 3. Effects of supplement levels (g/L) of *Cordyceps militaris* on xylanase activities (U/ml h⁻¹) in the supernatant of the medium *in vitro*

| Incubation times (h) | Supplement levels (g/L) of *C. militaris* | SEM | Trend (P) |
|----------------------|------------------------------------------|-----|-----------|
|                      | 0.00 | 0.10  | 0.15  | 0.20  | 0.25  | 0.30  | Linear | Quadratic | Cubic |
| 3                    | 4.38ᵇ | 5.10ᵇ | 5.44ᵇ | 6.02ᵇ | 7.84ᵇ | 5.68ᵇ | 0.88   | 0.005     | 0.020 | 0.068 |
| 6                    | 4.63ᵃ | 5.19ᵇ | 5.97ᵇ | 6.85ᵇ | 7.88ᵇ | 6.29ᵇ | 0.92   | 0.003     | 0.014 | 0.243 |
| 9                    | 8.04ᵇ | 10.54ᵇ| 13.49ᵇ| 22.93ᵇ| 24.23ᵇ| 29.15ᵃ| 4.20   | <0.001    | 0.332 | 0.980 |
| 12                   | 8.89ᵇ | 10.94ᵇ| 13.89ᵇ| 29.58ᵇ| 28.86ᵇ| 30.19ᵇ| 3.58   | <0.001    | 0.009 | 0.209 |
| 24                   | 9.93ᵇ | 12.04ᵇ| 13.39ᵇ| 29.28ᵇ| 31.95ᵇ| 31.95ᵇ| 1.99   | <0.001    | <0.001| 0.002 |
| 36                   | 12.57ᵇ| 14.75ᵇ| 14.50ᵇ| 30.57ᵇ| 36.64ᵇ| 35.30ᵇ| 2.44   | <0.001    | 0.009 | <0.001|
| 48                   | 10.73ᵇ| 12.84ᵇ| 14.19ᵇ| 30.08ᵇ| 32.75ᵇ| 32.75ᵇ| 1.99   | <0.001    | <0.001| 0.002 |
| 72                   | 12.64ᵇ| 18.50ᵇ| 23.86ᵇ| 32.04ᵇ| 34.09ᵇ| 33.24ᵇ| 2.88   | <0.001    | <0.001| 0.849 |

SEM = Standard error of the mean.ᵃᵇᶜ Means within a row with different superscripts differ significantly (p<0.05).

Table 4. Effects of supplement levels (g/L) of *Cordyceps militaris* on avicelase activities (U/ml h⁻¹) in the supernatant of the medium *in vitro*

| Incubation times (h) | Supplement levels (g/L) of *C. militaris* | SEM | Trend (P) |
|----------------------|------------------------------------------|-----|-----------|
|                      | 0.00 | 0.10  | 0.15  | 0.20  | 0.25  | 0.30  | Linear | Quadratic | Cubic |
| 3                    | 2.30ᵇ | 2.44ᵈ | 2.66ᵈ | 3.01ᵇ | 3.21ᵈ | 3.39ᵇ | 0.35   | <0.001    | 0.488 | 0.988 |
| 6                    | 2.82ᵇ | 2.82ᵇ | 3.07ᵇ | 3.29ᵇ | 4.26ᵇ | 3.55ᵇ | 0.39   | <0.001    | 0.179 | 0.039 |
| 9                    | 3.24ᵇ | 3.37ᵇ | 3.79ᵇ | 3.99ᵇ | 4.86ᵇ | 3.96ᵇ | 0.55   | 0.011     | 0.077 | 0.184 |
| 12                   | 3.53ᵇ | 3.66ᵇ | 4.11ᵇ | 4.26ᵇ | 5.46ᵇ | 4.58ᵇ | 0.57   | 0.002     | 0.150 | 0.145 |
| 24                   | 3.70ᵇ | 3.90ᵇ | 4.41ᵇ | 4.61ᵇ | 5.30ᵇ | 4.59ᵇ | 0.43   | 0.002     | 0.025 | 0.282 |
| 36                   | 4.10ᵇ | 4.19ᵇ | 4.50ᵇ | 4.79ᵇ | 5.51ᵇ | 4.64ᵇ | 0.38   | 0.003     | 0.022 | 0.054 |
| 48                   | 4.29ᵇ | 4.51ᵇ | 4.73ᵇ | 4.98ᵇ | 5.92ᵇ | 5.36ᵇ | 0.46   | <0.001    | 0.224 | 0.172 |
| 72                   | 4.39ᵇ | 4.58ᶜ | 4.78ᶜ | 5.02ᶜ | 5.79ᶜ | 5.15ᶜ | 0.35   | <0.001    | 0.065 | 0.084 |

SEM = Standard error of the mean.ᵃᵇᶜ Means within a row with different superscripts differ significantly (p<0.05).

numbers, above 0.20 g/L of supplement being significantly higher than the control. This was true also for the activities of xylanase and avicelase; at all incubation times, the supplementation of *C. militaris* mycelia linearly increased the activities of xylanase and avicelase. The increased cellulolytic bacteria coupled with increased cellulolytic enzyme activities could facilitate the enhanced cellulose digestion and gas production observed in the previous study (Yeo et al., 2009). The relationships between gas production (Yeo et al., 2009) and enzyme activities in the present study showed a positive effect on cellulolytic bacteria and cellulolytic activity could facilitate the enhanced cellulose digestion and cellulolytic bacteria. However, further research is required to determine underlying mechanisms of the effects of *C. militaris* mycelia on changes of rumen fermentation and the effects of *C. militaris* mycelia on animal performance.

**ACKNOWLEDGMENTS**

This experiment was supported by a grant from the Special Grants Research Program of the Ministry of Agriculture and Forestry. S. J. Lee and S. H. Shin are supported by scholarships from the BK21 Program, Ministry of Education & Human Resources Development, Korea.

**REFERENCES**

Ahn, Y. J., S. J. Park, S. G. Lee, S. C. Shin and D. H. Choi. 2000. Cordycepin: selective growth inhibitor derived from liquid culture of *Cordyceps militaris* against *Clostridium spp*. J. Agric. Food Chem. 48:2744-2748.

Bernailier, A., G. Fonty, F. Bonnemoy and P. Gouet. 1993. Inhibition of the cellulolytic activity of *Neocallimastix frontalis* by *Ruminococcus flavefaciens*. J. Gen. Microbiol. 139:873-880.

Dehority, B. A., P. A. Tirabasso and A. P. Grifo Jr. 1989. Most probable number procedures for enumerating ruminal bacteria, supported by scholarships from the BK21 Program, Ministry of Education & Human Resources Development, Korea.
numbers in one medium. Appl. Environ. Microbiol. 55:2789-2792.

Holdman, L. V., E. P. Gato and W. E. C. Moore. 1977. Anaerobic laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Hungate, R. E. 1966. The rumen bacteria. In: The rumen and its microbes (Ed. R. E. Hungate). Academic Press, New York and London, p. 8.

Kneifel, H., W. A. Konig, W. Loeffler and R. Muller. 1977. Ophiocordin, an antifungal antibiotic of Cordyceps ophioglossoides. Arch. Microbiol. 113:121-130.

Koh, J. H., H. J. Suh and T. S. Ahn. 2003. Hot-water extract from mycelia of Cordyceps sinensis as a substitute for antibiotic growth promoters. Biotechnol. Lett. 25:585-590.

Koh, J. H., K. W. Yu, H. J. Suh, Y. M. Choi and T. S. Ahn. 2002. Activation of macrophages and the intestinal immune system by orally administered decoction from cultured mycelium of Cordyceps sinensis. Biosci. Biotechnol. Biochem. 66:407-411.

Li, S. P., F. Q. Yang and K. W. K. Tsim. 2006. Quality control of Cordyceps sinensis, a valued traditional Chinese medicine. J. Pharm. Biomed. Anal. 41:1571-1584.

Menke, K. H. and H. Steingass. 1988. Estimation of the energetic feed value obtained from chemical analysis and gas production using rumen fluid. Anim. Res. Dev. 28:7-55.

Miller, J. L., R. Blum, W. E. Glennon and A. L. Burton. 1960. Measurement of carboxymethyl cellulase activity. Anal. Biochem. 1:127-132.

Nakamura, K., Y. Yamaguchi, S. Kagota, Y. M. Kwon, K. Shinozuka and M. Kunitomo. 1999. Inhibitory effect of Cordyceps sinensis on spontaneous liver metastasis of Lewis lung carcinoma and B16 melanoma cells in syngeneic mice. Jpn. J. Pharmacol. 79:335-341.

Orpin, C. G. and K. N. Joblin. 1997. The rumen anaerobic fungi. In: The Rumen Microbial Ecosystem (Ed. P. N. Hobson and C. S. Stewart). Chapman and Hall, London, UK, pp. 140-195.

SAS Institute. 1996. SAS User’s Guide: Statistics. SAS Institute, Inc., Cary, NC, USA.

Williams, P. E. V., C. J. Newbold, A. Walker and R. J. Wallace. 1990. Rumen probiotics: The effects of including yeast culture (Saccharomyces cerevisiae plus growth medium) in the diet for sheep fed continuously or in meal fed steers. J. Dairy Sci. 72 (Suppl. 1):521 (Abstract).

Yeo, J. M., S. J. Lee, S. M. Lee, S. H. Shin, S. H. Lee, J. K. Ha, W. Y. Kim and S. S. Lee. 2009. Effects of Cordyceps militaris mycelia on in vitro rumen microbial fermentation. Asian-Aust. J. Anim. Sci. 22:201-205.

Yeon, S. H., J. R. Kim and Y. J. Ahn. 2007. Comparison of growth-inhibiting activities of Cordyceps militaris and Paecilomyces japonica cultured on Bombyx mori pupae towards human gastrointestinal bacteria. J. Sci. Food Agric. 87:54-59.

Yu, K. W., K. M. Kim and H. J. Suh. 2003. Pharmacological activities of stromata of Cordyceps scarabaeoidea. Phytother Res. 17:244-249.

Yu, R., L. Song, Y. Shao, W. Bin, L. Wang, H. Shang, Y. Wo, W. Ye and X. Yao. 2004a. Isolation and biological properties of polysaccharide CPS-1 from cultured Cordyceps militaris. Fitoterapia 75:465-472.

Yu, R., L. Wang, H. Zhang, C. Zhou and Y. Zhao. 2004b. Isolation, purification and identification of polysaccharides from cultured Cordyceps militaris. Fitoterapia 75:662-666.