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

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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 celluloytic 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. Carboxylmethyl 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)

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

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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 O_2-free CO_2, 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 K_2HPO_4, 240 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4,000 mg of Na_2CO_3, and 600 mg of cysteine hydrochloride in l,000 ml of O_2-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 carboxylmethyl cellulase (CMCase) analysis, 0.5 ml of the supernatant was mixed with 0.5 ml of 1.0% carboxylmethyl 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 cellulolytic 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.

RESULTLS 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 0.25 g/L 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

Table 1. Effects of supplement levels (g/L) of *Cordyceps militaris* on microbial populations *in vitro*

	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
Bacterial, cfu[1] $\times 10^9$	13.0^{c}	14.0^{c}	15.3^{c}	19.5^{ab}	21.5^{a}	15.8^{bc}	2.60	0.003	0.001	0.032
Cellulolytics, cfu$\times 10^7$	12.3^{c}	16.9^{c}	22.0^{bc}	32.3^{ab}	35.3^{a}	22.3^{bc}	8.16	0.006	0.003	0.234
Fungi, tfu[2] $\times 10^3$	76.8^{a}	48.3^{b}	46.3^{b}	13.0^{c}	13.3^{c}	3.0^{c}	10.64	<0.001	0.004	0.335

SEM = Standard error of the mean. $^{a, b, c, d}$ Means within a row with different superscripts differ significantly ($p<0.05$).
[1] cfu = Cell forming unit. [2] tfu = Thallus forming unit.

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). Cordycepin, one of the markers for quality control of *Cordyceps* is known to be responsible for the antifungal and antibacterial activity (Kneifel et al., 1977; Yeon et al., 2007). However, the antibacterial activity of *Cordyceps* would seem very unlikely to be applied to cellulolytic bacteria in the rumen since the addition of *C. militaris* mycelia to the culture of mixed rumen microorganisms substantially increased the number of cellulolytic bacteria in the present study, suggesting that *Cordyceps* might have a selective antibacterial effect. Indeed, it has been shown that *Cordyceps* decreased harmful intestinal bacteria without adverse effects on 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 solublize 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 addition 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

Table 2. Effects of supplement levels (g/L) of *Cordyceps militaris* on carboxymethyl cellulase activities (U/m h^{-1}) in the supernatant of the medium *in vitro*

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^{ab}	6.04^{ab}	6.26^{ab}	6.50^{a}	4.84^{c}	5.55^{b}	0.38	0.011	0.125	0.004
9	6.78^{b}	7.85^{b}	7.67^{b}	9.52^{a}	6.67^{b}	7.98^{b}	0.76	0.443	0.049	0.029
12	7.26^{c}	8.59^{bc}	8.56^{bc}	10.06^{a}	9.73^{ab}	9.70^{ab}	0.76	0.001	0.026	0.701
24	8.80^{d}	9.69^{bcd}	9.31^{cd}	11.62^{a}	11.18^{ab}	10.57^{abc}	0.86	0.004	0.025	0.326
36	9.11^{b}	9.83^{b}	10.44^{b}	12.81^{a}	12.29^{a}	10.43^{b}	0.88	0.005	<0.001	0.184
48	9.08^{c}	9.86^{c}	10.89^{bc}	14.39^{a}	12.68^{ab}	10.57^{bc}	1.34	0.023	0.002	0.402
72	8.77^{d}	9.78^{cd}	10.29^{c}	13.43^{a}	12.00^{b}	10.50^{c}	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	4.38^{b}	5.10^{b}	5.44^{b}	6.02^{b}	7.84^{a}	5.68^{b}	0.88	0.005	0.020	0.068
6	4.63^{c}	5.19^{bc}	5.97^{bc}	6.85^{ab}	7.88^{a}	6.29^{abc}	0.92	0.003	0.014	0.243
9	8.04^{b}	10.54^{b}	13.49^{b}	22.93^{a}	24.23^{a}	29.15^{a}	4.20	<0.001	0.332	0.980
12	8.89^{b}	10.94^{b}	13.89^{b}	29.58^{a}	28.86^{a}	30.19^{a}	3.58	<0.001	0.009	0.209
24	9.93^{b}	12.04^{b}	13.39^{b}	29.28^{a}	31.95^{a}	31.95^{a}	1.99	<0.001	<0.001	0.002
36	12.57^{c}	14.75^{c}	14.50^{c}	30.57^{b}	36.64^{a}	35.30^{a}	2.44	<0.001	0.009	<0.001
48	10.73^{b}	12.84^{b}	14.19^{b}	30.08^{a}	32.75^{a}	32.75^{a}	1.99	<0.001	<0.001	0.002
72	12.64^{d}	18.50^{c}	23.86^{b}	32.04^{a}	34.09^{a}	33.24^{a}	2.88	<0.001	<0.001	0.849

SEM = Standard error of the mean. $^{a, b, c}$ 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	2.30^{d}	2.44^{cd}	2.66^{bcd}	3.01^{abc}	3.21^{ab}	3.39^{a}	0.35	<0.001	0.488	0.988
6	2.82^{b}	2.82^{b}	3.07^{b}	3.29^{b}	4.26^{a}	3.55^{b}	0.39	<0.001	0.179	0.039
9	3.24^{b}	3.37^{b}	3.79^{b}	3.99^{ab}	4.86^{a}	3.96^{ab}	0.55	0.011	0.077	0.184
12	3.53^{b}	3.66^{b}	4.11^{b}	4.26^{b}	5.46^{a}	4.58^{ab}	0.57	0.002	0.150	0.145
24	3.70^{c}	3.90^{bc}	4.41^{bc}	4.61^{ab}	5.30^{a}	4.59^{ab}	0.43	0.002	0.025	0.282
36	4.10^{b}	4.19^{b}	4.50^{b}	4.79^{b}	5.51^{a}	4.64^{b}	0.38	0.003	0.022	0.054
48	4.29^{c}	4.51^{bc}	4.73^{bc}	4.98^{bc}	5.92^{a}	5.36^{ab}	0.46	<0.001	0.224	0.172
72	4.39^{c}	4.58^{bc}	4.78^{bc}	5.02^{bc}	5.79^{a}	5.15^{b}	0.35	<0.001	0.065	0.084

SEM = Standard error of the mean. $^{a, b, c}$ 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 were shown to be linear (r^2 = 0.82, 0.85 and 0.58 for CMCase, avicelase and xylanase, respectively; $p<0.001$).

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. The results of the previous (Yeo et al., 2009) and present studies suggest that *C. militaris* mycelia may be a useful feed additive in enhancing feedstuff utilization by increasing 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.

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