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In Vitro Stimulation of Forage Fiber Degradation by Ruminal Microorganisms with *Aspergillus oryzae* Fermentation Extract

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Aspergillus oryzae fermentation extract (Amaferm) was evaluated for its ability to influence degradation of brome grass and switchgrass fiber fractions by mixed ruminal microorganisms in vitro. Addition of Amaferm at a concentration of 0.067 mg/ml, which is approximately the concentration found in the rumen ecosystem (0.06 mg/ml), increased the degradation of brome grass neutral detergent fiber (NDF) by 28% after fermentation for 12 h ($P < 0.01$), but had no effect after fermentation for 24 or 48 h. The levels of degradation of both the cellulose and hemicellulose fractions were increased after fermentation for 12 h ($P < 0.01$). Additions of 0.08 and 8% (vol/vol) Amaferm filtrate (12.5 g/100 ml) stimulated degradation of switchgrass NDF by 12 and 24% ($P < 0.01$), respectively, after fermentation for 12 h; when 80% filtrate was added, degradation was decreased by 38%. The concentrations of total anaerobes in culture tubes containing 80% filtrate were 5 times greater than the concentrations in the controls; however, the concentrations of cellulolytic organisms were 3.5 times lower than the concentrations in the controls ($P < 0.05$). These results suggested that the filtrate contained high concentrations of soluble substrate which did not allow the cellulolytic organisms to compete well with other populations. The remaining concentrations of esterified *p*-coumaric and ferulic acids were lower at 12 h in NDF residues obtained from fermentation mixtures supplemented with Amaferm. Because the total anaerobes were not inhibited in fermentation mixtures containing Amaferm, antibiotics are unlikely to be involved as a mode of action for increasing NDF degradation. The possibility that Amaferm contains enzymes (possibly esterases) that may play a role in stimulating the rate of fiber degradation by mixed ruminal microorganisms by removal of plant cell wall phenolic acid esters is discussed.

The effect of feeding microbial cultures or extracts of microbial cultures to ruminant animals has been reviewed recently (16). Interest in such studies originates from concern about feeding subtherapeutic levels of antibiotics to animals and the potential adverse affects generated by these compounds. *Aspergillus oryzae* fermentation extract and *Saccharomyces cerevisiae* cultures have most commonly been fed to animals to promote desired responses, such as increased weight gain, milk production, or total tract digestibility of feed components. Enhancement of these responses has been mixed (5, 8, 30, 31). Some data have indicated that significant improvements in the digestibility of fiber fractions can be achieved when *A. oryzae* extract is fed to ruminants (7, 26); this coincides with an increase in the number of cellulolytic bacteria in the rumen (30).

Data on the effects of these supplements from in vitro studies performed with mixed ruminal microorganisms have also been inconclusive (16). However, the results of several studies have indicated that the concentrations of cellulolytic bacteria increase when in vitro samples are supplemented with *A. oryzae* extract (18, 19). Newbold et al. (19) also concluded that increases in the levels of fiber digestion by animals fed *A. oryzae* extract are most likely due to stimulation of bacterial activity rather than fungal or protozoan activity in the rumen. Martin and Nisbet (16) demonstrated that *A. oryzae* extract increased the growth of and uptake of lactate by *Selenomonas ruminantium*. This extract appears to provide soluble factors, including malate, that stimulate lactate utilization by *S. ruminantium*, which in turn may stabilize the rumen environment (31).

The objective of our study was to evaluate the effect of *A. oryzae* fermentation extract on the ability of mixed ruminal microorganisms to degrade cell wall components of brome grass and switchgrass in vitro. Our results indicate that this extract stimulates forage fiber degradation in a time-dependent manner.

MATERIALS AND METHODS

Amaferm supplement. *A. oryzae* fermentation extract (Amaferm) was supplied by BioZyme Enterprises, Inc., St. Joseph, Mo. Amaferm was used in various studies as it was received. This product consists of *A. oryzae* fermentation extract dried on an insoluble carrier (wheat bran and wheat middlings). Amaferm was added to in vitro digestion tubes without being heat sterilized. No degradation of forage cell walls took place in control tubes containing cell walls, Amaferm, and sterile rumen fluid (20%, vol/vol) that were incubated for 72 h. In other studies, a filtrate of Amaferm was prepared by using a procedure similar to the procedure of Nisbet and Martin (21). Amaferm (12.5 g/100 ml of McDougall's buffer [17]) was mixed for 1 h with a magnetic stirrer, vacuum filtered through a Whatman no. 1 filter (Whatman Lab Sales, Hillsboro, Oreg.), filter sterilized, and gassed with O_2-free CO_2. For unknown reasons a precipitate

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developed in the filtrate after 3 to 4 days; thus, the filtrate was prepared fresh for each experiment. The pH of the filtrate before it was gassed with CO_2 was 6.96 and after it was gassed was 5.53, which may explain the precipitate. The filtrate was autoclaved at 121°C for 15 min in one experiment to determine the effect of heat on the product.

In vitro grass cell wall degradation. The effect of Amaferm on in vitro degradation of brome grass and switchgrass cell wall components (cell walls, cellulose, hemicellulose) by mixed ruminal organisms was determined at 0, 12, 24, and 48 h. These grasses were selected because they represent two major taxa of forages normally fed to ruminants and are known to differ in cell wall chemistry (15). The forages were ground and passed through a 1-mm-pore-size screen prior to being used to make cell wall preparations. They were boiled for 1 h with neutral detergent, and the insoluble residues (cell walls) were extensively washed to remove the detergent before drying (27).

Three 0.5-g samples of each substrate at each time period were placed in 50-ml plastic culture tubes with screw caps. Each tube received 24 ml of buffer (17) and 6 ml of rumen fluid which was blended and strained through two layers of cheesecloth, and Amaferm was added to some of the tubes. The rumen fluid was obtained from a steer fed 70% corn silage, 20% alfalfa hay, 9% soybean meal, trace minerals, and vitamins (13% crude protein diet). The samples were vigorously shaken at 0800 and 1600 h daily. As each digestion period was completed, 1 ml of 5% sodium azide was added to the tube to stop fermentation. The concentrations of the volatile fatty acids in in vitro fermentation cultures were determined at each time interval tested (0, 12, 24, and 48 h). The fermented cell wall samples were frozen and later analyzed for fiber content by the sequential detergent system (27, 28). Sulfuric acid (72%) was used to solubilize cellulose and isolate crude lignin plus ash.

The total cell wall preparation was defined as the neutral detergent fiber (NDF). Hemicellulose and cellulose contents were calculated from differences in weight as follows: the NDF content minus the acid detergent fiber content equaled the hemicellulose content, and the acid detergent fiber content minus the acid detergent lignin content equaled the cellulose content. The level of degradation was calculated by determining the amount of a component that disappeared during fermentation and comparing this value with the initial concentration. A correction was made for the addition of the components in the inoculum. Each result given below is the mean of the values from three replicate tubes.

Microbiological analyses. The Hungate anaerobic culture method as described by Bryant (4) was used for microbiological analyses. The total concentrations of viable and cellulolytic bacteria were determined from duplicate in vitro tubes different from the tubes used to analyze cell wall degradation. The medium used to determine total viable bacterium concentrations from duplicate in vitro tubes contained the following components (per 100 ml): 30.0 ml of clarified ruminal fluid, 0.03 g of glucose, 0.03 g of cellobiose, 0.03 g of maltose, 0.03 g of starch, 0.03 g of xylose, 0.03 g of glycerol, 0.2 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 0.0001 g of resazurin, 5 ml of mineral S2 (23), and 1.75 g of purified agar (BBL). Sodium carbonate (0.4%) and cysteine hydrochloride (0.05%) were added as sterile anaerobic solutions after the medium was autoclaved (4). Roll tubes (four replicates) were incubated at 37°C, and colonies were counted after 7 days. The cellulose agar roll tube medium contained (per 100 ml) 15 ml of clarified, preincubated ruminal fluid, 0.2 g of Trypticase, 0.05 g of yeast extract, 5 ml of mineral S2, 0.2 g of cellulose (Whatman no. 1 filter paper ball milled with flint pebbles for 18 h), 0.0001 g of resazurin, 0.4 g of Na_2CO_3, 0.05 g of cysteine hydrochloride, and 0.07 g of purified agar. Four replicates of these tubes were incubated for 2 weeks before zones of clearing were counted.

Ferulic and *p*-coumaric acid analyses. Esterified ferulic and *p*-coumaric acids were extracted with alkali as previously described (15). Each acidified sample solution was loaded on a C_{18} solid-phase extraction column (Supelco, Inc., Bellefonte, Pa.), the column was washed with 2 ml of an NaOH-phosphoric acid solution (pH 2.6), and the cinnamic acids were eluted with two 2.5-ml 50% methanol washes. The eluted samples were brought to a final volume of 10 ml, filtered through a 0.2-μm filter, and stored at −20°C until they were analyzed. All extractions were performed in duplicate.

Identification and quantification of phenolic acids were done by liquid chromatography. A gradient autoanalytical system (Gilson Medical Electronics, Inc., Middleton, Wis.) equipped with a programmable Gilson model 116 dual-wavelength UV detector was utilized. Samples (20 μl) were injected and separated by isocratic elution through a Spherisorb-ODS, C_{18}, 5-μm-inner-diameter column (Supelco). The solvent (97.7% water, 0.3% glacial acetic acid, 2% butanol) was pumped at a rate of 3 ml/min. The column temperature was maintained at 50°C. *p*-Coumaric and ferulic acids were quantified by measuring A_{320}.

Statistics. Fiber digestibility data were analyzed by using a completely randomized design. Means were compared by the F-protected ($P < 0.05$) least-significant-difference method using SAS (24).

RESULTS

Our initial experiments were performed to determine whether Amaferm affected degradation of plant cell wall components when it was added to an in vitro system at concentrations which approximated those in the rumen. Assuming that a 50-liter rumen received 3 g per day, which is the manufacturer's recommended feeding level for a lactating cow, the equivalent amount in a 30-ml working volume culture tube was 1.8 mg. This value was not adjusted for the fivefold dilution of microbial mass in our in vitro fermentation mixtures (6 ml of rumen fluid in 24 ml of buffer) compared with a rumen. Amaferm was added at a concentration of 2 mg/30 ml (0.067 mg/ml) and also at a higher concentration, 6 mg/30 ml (0.2 mg/ml). Table 1 shows that these concentrations increased the degradation of brome grass NDF compared with the control after fermentation for 12 h but not after fermentation for 24 or 48 h. The levels of the increases were 28 and 33% ($P < 0.01$) for the 0.067- and 0.2-mg/ml concentrations, respectively. The levels of degradation of the hemicellulose and cellulose fractions of NDF were also increased ($P < 0.01$) by the addition of Amaferm (Fig. 1).

The concentration of branched-chain acids in a 20% ruminal fluid inoculum can at times be limiting for in vitro fermentation (9). The concentrations of isobutyric and isovaleric acids in our zero time culture tubes (20% ruminal inoculum) were 0.25 and 0.30 mM, respectively. These values are well above the minimum concentration (0.15 mM) that had a stimulatory effect on total cell wall digestion by isolated bacteria (9). The concentrations of total or individual acids did not differ whether Amaferm was added to the culture tubes or not (data not shown).

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TABLE 1. Influence of Amaferm on in vitro degradation of brome grass NDF and remaining concentrations of esterified *p*-coumaric and ferulic acids

Amaferm concn (mg/ml)	Time (h)	% NDF degradation	Esterified monomer concn (mg/g)[a]	
			p-Coumaric acid	Ferulic acid
0	12	8.1[b]	3.15 ± 0.18	2.42 ± 0.12
0.067	12	11.3[c]	2.73 ± 0.11	2.11 ± 0.04
0.2	12	12.1[c]	2.57 ± 0.11	2.09 ± 0.06
0	24	23.0	2.75 ± 0.06	2.09 ± 0.01
0.067	24	22.8	2.76 ± 0.05	2.14 ± 0.03
0.2	24	22.9	2.72 ± 0.09	2.13 ± 0.05
0	48	30.7	2.86 ± 0.09	2.04 ± 0.14
0.067	48	30.4	2.77 ± 0.22	2.05 ± 0.14
0.2	48	30.3	2.61 ± 0.02	2.00 ± 0.03
SE		0.6		

[a] The concentrations of p-coumaric and ferulic acids in brome grass NDF before autoclaving were 3.30 ± 0.18 and 2.70 ± 0.17 mg/g, respectively; after autoclaving, the concentrations were 3.06 ± 0.04 and 2.52 ± 0.02 mg/g, respectively. The means ± standard errors are the averages of two analyses.

[b,c] Values with different superscripts are significantly different ($P < 0.01$).

To verify the finding of Nisbet and Martin (21) that an Amaferm filtrate which retained activity could be prepared, we prepared a filtrate to examine its ability to increase the amount of switchgrass NDF degraded (Fig. 2). After fermentation for 12 h, with the exception of a concentration of 0.0008%, increasing amounts of NDF were degraded with increasing levels of filtrate up to a concentration of 8%. At a filtrate concentration of 80%, there was a decrease ($P < 0.01$) in the amount of NDF that was degraded; 0.08 and 8% filtrate increased the amount of NDF that was degraded after fermentation for 12 h by 12 and 24% ($P < 0.01$), respectively, while 80% filtrate decreased the amount of NDF that was degraded by 38%. At 24 h most levels of Amaferm filtrate had little effect; the only exception was a filtrate concentration of 80%, which reduced the level of degradation by 47%.

To determine whether the component in Amaferm which increased NDF degradation was heat stable, we autoclaved the filtrate and compared the resulting preparation with filter-sterilized filtrate additions. At the two concentrations that we examined, 8 and 80%, the heat-sterilized filtrate gave the same responses as the filter-sterilized filtrate (Fig. 3). Concentrations of 8% increased the levels of NDF degradation by an average of 21% ($P < 0.01$), while concentrations of 80% decreased the levels of degradation by 64%.

The numbers of total viable and cellulolytic bacteria were determined (Table 2) from duplicate in vitro tubes in the experiment described above. When 8% filtrate was added, the total viable bacterial counts increased threefold (from 8.3×10^9 to 25.7×10^9 or 25.3×10^9 cells per ml) ($P < 0.05$); when 80% filtrate was added, the counts increased approximately fivefold ($P < 0.05$) for both the filter-sterilized and autoclaved filtrates (to 41.8×10^9 cells per ml). The cellulolytic bacterial counts increased 1.4- to 1.8-fold ($P < 0.05$) when 8% filtrate was added and decreased 3.5-fold ($P < 0.05$) when 80% filter-sterilized filtrate was added. There was little difference in the numbers of cellulolytic bacteria between the control preparation and the preparation containing 80% Amaferm filtrate when the filtrate was autoclaved.

DISCUSSION

Our data indicate that Amaferm, both in the dry product form and as a liquid filtrate, can enhance the rate of in vitro degradation of brome grass or switchgrass fiber fractions by mixed ruminal microorganisms (Table 1 and Fig. 2). The in vitro concentration of Amaferm used in this study (0.067 mg/ml) is approximately the concentration which microorganisms may be exposed to in the bovine rumen. Stimulation of the rate, but not the extent, of forage fiber degradation confirms the results of Fondevila et al. (6), who found that

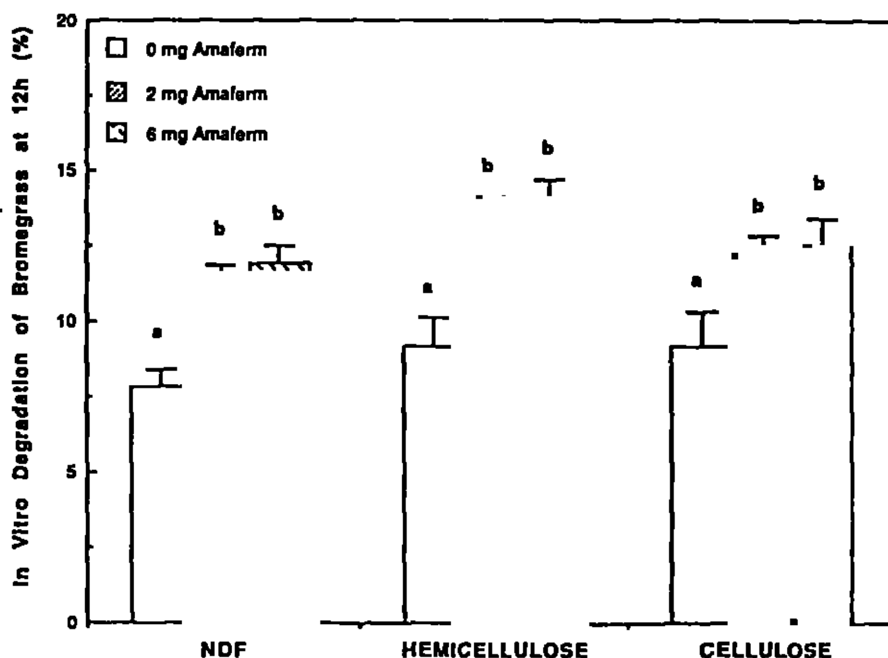

FIG. 1. Influence of Amaferm on in vitro degradation of brome grass fiber fractions. The letters at the tops of the bars indicate statistical significance; means with different letters are significantly different ($P < 0.01$).

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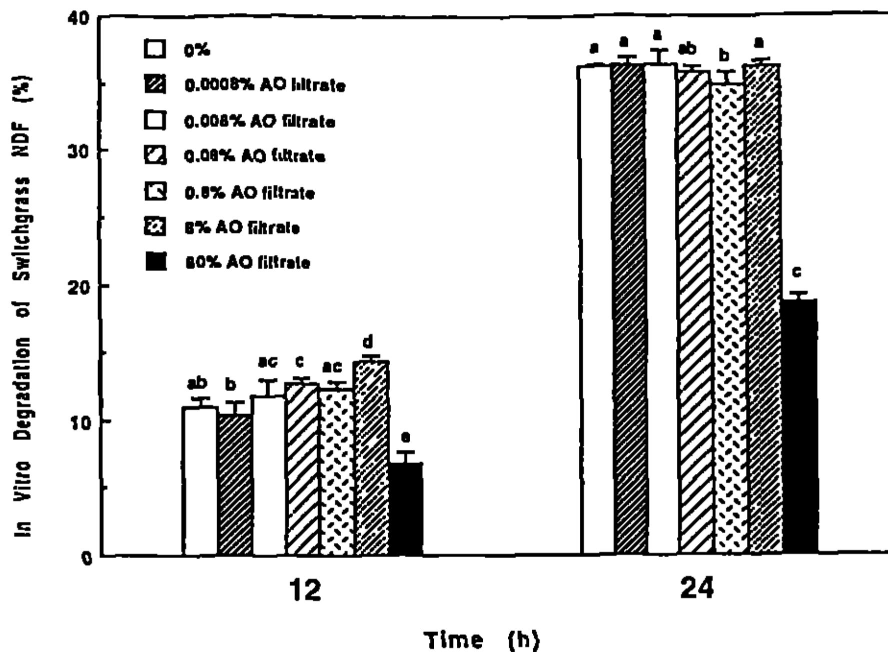

FIG. 2. Influence of Amaferm filtrate on in vitro degradation of switchgrass NDF after fermentation for 12 and 24 h. Amaferm (12.5 g/100 ml) was extracted in McDougall's buffer and filtered. Mixing 24 ml of the filtrate plus 6 ml of rumen fluid inoculum resulted in an Amaferm (AO) filtrate concentration of 80% (vol/vol). The letters at the tops of the bars indicate statistical significance; means with different letters are significantly different ($P < 0.01$).

Amaferm increased the rate of straw degradation in sheep but did not alter the final extent of degradation.

Figure 2 shows that a very high concentration of Amaferm (8% Amaferm filtrate, compared with the rumen concentration of 0.067 mg/ml) can be added to an in vitro digestion system without an inhibitory response being observed. Only when the 80% filtrate was added was the level of NDF degradation significantly reduced (38%). Because there was no inhibitory fermentation response observed with 8% filtrate, we concluded that an agent such as an antibiotic (much like feeding subtherapeutic levels of antibiotics to animals for growth efficiency) can be excluded as a factor which may explain the enhanced rate of fiber degradation by Amaferm. This conclusion is further supported by our observation of gas pressure in the in vitro tubes when they were opened for analysis. The tubes containing the 80% filtrate had considerably higher gas pressure (greater microbial activity) than the tubes containing less Amaferm. Our conclusion is that the 80% filtrate contained a significant amount of soluble

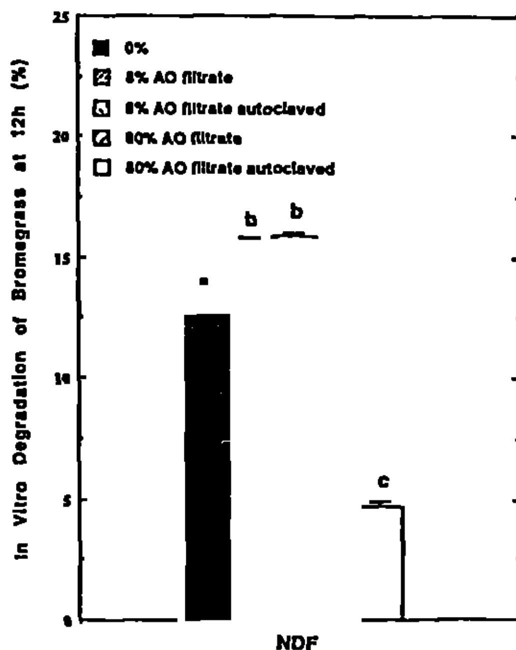

FIG. 3. Influence of heat- and filter-sterilized Amaferm filtrate on in vitro degradation of brome grass NDF after fermentation for 12 h. See Table 2, footnote *a*, for an explanation of Amaferm (AO) concentrations. The letters at the tops of the bars indicate statistical significance; means with different letters are significantly different ($P < 0.05$).

TABLE 2. Influence of Amaferm filtrate on the total viable and cellulolytic bacterial counts in 12-h in vitro digestion tubes containing brome grass NDF

Amaferm concn (%)[a]	Total bacteria (cells/ml, 10^9)	Cellulolytic bacteria (cells/ml, 10^6)
0	8.3[b]	9.7[b]
8	25.7[c]	18.0[c]
80	40.3[c]	2.8[d]
8 (autoclaved)	25.3[c]	13.5[c]
80 (autoclaved)	41.8[c]	8.3[b]
SE	5.3	2.5

[a] Amaferm (12.5 g/100 ml) was extracted in McDougall's buffer and filtered; mixing 24 ml of the filtrate plus 6 ml of rumen fluid inoculum resulted in a filtrate concentration of 80% (vol/vol). The autoclaved filtrates were autoclaved before they were added to the in vitro digestion tubes.

[b,c,d] Values in the same column with different superscripts are significantly different ($P < 0.05$).

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substrate which inhibited the degradation of insoluble NDF substrate. This is supported by the data in Table 2. The concentration of total viable bacteria found in tubes supplemented with 80% filtrate increased 5-fold, while the concentration of cellulolytic bacteria decreased 3.5-fold. Because of the possibility that significant substrate was present in the tubes containing 8 and 80% filtrate, which would have masked any trace element effect, we were not able to conclude whether the component which stimulated NDF degradation at concentrations of 0.067 and 0.2 mg/ml is heat sensitive. We did observe stimulation of cell wall degradation when 8% autoclaved filtrate was added. Newbold et al. (18) recently concluded that the action of Amaferm on rumen fermentation depends on a heat-labile component. The stimulation of NDF degradation that we observed with Amaferm levels of 0.067 and 0.2 mg/ml was due to a trace component. However, the stimulation that we observed when 8% Amaferm filtrate was added was probably due to soluble substrates being rapidly fermented by organisms, including the cellulolytic population, whose numbers increased compared with control sample levels; thus, greater amounts of NDF were degraded. Our results suggest that other studies (1, 16, 21) in which Amaferm filtrates were used at concentrations of 2 to 5% may not have used physiological concentrations (0.06 mg/ml) similar to the concentrations to which microorganisms in the rumen ecosystem are exposed; thus, such results may not help explain what happens in the in situ environment.

Aspergillus species are known to produce a wide variety of polysaccharidase enzymes (cellulases, hemicellulases) which could influence plant cell wall degradation (22). The high fibrilytic enzyme content of *A. oryzae* is still likely the primary mode of action for improved fiber breakdown in the rumen (20). *A. oryzae* is an aerobic organism and is not expected to multiply in the rumen (6). Whether it is viable for any period of time is unknown. Recently, an esterase has been purified from *A. oryzae* which liberates ferulic, *p*-coumaric, and acetic acids from wheat straw (25). The phenolic acids, ferulic and *p*-coumaric, are covalently bound to cell wall polysaccharides and may act as cross-linking agents between lignin and hemicellulose (10, 12). The extent of cross-linking by ferulic and *p*-coumaric acid dimers and the esterification of feruloyl and *p*-coumaroyl groups to arabinoxylans have been shown to limit plant cell wall biodegradation (10, 14). Ferulic acid esters of arabinoxylan are thought to limit the rate of fiber degradation, but are not thought to limit the extent of degradation because of naturally occurring ruminal esterases (13). We have previously hypothesized that bacterial species capable of metabolizing phenolic monomers may act as catalysts for forage fiber breakdown by increasing microbial access to cell wall polysaccharides (29). Similarly, high concentrations of both feruloyl and *p*-coumaroyl esterases in rumen anaerobic fungi, which can be 10-fold greater than the concentrations in rumen bacteria (2), may provide a unique advantage to these microorganisms for the biodegradation of phenolic acid-containing and phenolic acid cross-linked arabinoxylans (3). This in turn could render the polysaccharides in cell walls more available to degradation. Once the aerobic fungus *A. oryzae* is exposed to an anaerobic environment, it may simply lyse and release esterase and (potentially) other enzymes which enhance the rate of plant cell wall breakdown but not necessarily the extent of breakdown.

With this mechanism in mind (i.e., the possibility that *A. oryzae* produces esterases which enhance cell wall degradation), we analyzed the residual concentrations of esterified *p*-coumaric and ferulic acids in brome grass NDF fermentation preparations that contained and lacked Amaferm (Table 1). After fermentation for 12 h an increase in the level of NDF degradation with Amaferm was observed along with decreases in the remaining esterified *p*-coumaric and ferulic acid concentrations. After fermentation for 24 and 48 h, no differences in the levels of NDF degradation with or without Amaferm were observed, and little difference was observed in the remaining *p*-coumaric and ferulic acid concentrations. This suggests that esterase enzymes from Amaferm may play a role in the degradation of brome grass fiber components. A preliminary measurement of the feruloyl esterase activity (11) of Amaferm at 25°C gave a value of 0.01 μmol/h/mg of protein. This activity should be considered the minimal activity because it was based on the total protein extracted from Amaferm and it was the activity at 25°C instead of the activity at the temperature of the rumen. Further studies are needed to determine the survival of *A. oryzae* in the vicinity of the rumen wall, where it may be able to scavenge some oxygen for short-term growth. Esterase activity in pure cultures of *A. oryzae* under anaerobic conditions should also be measured.

In summary, two important conclusions can be drawn from our results. First, *A. oryzae* fermentation extract stimulates, by an as-yet-unknown mechanism, the rate but not the extent of in vitro degradation of grass forages by mixed ruminal microorganisms. Second, caution should be exercised in drawing conclusions from studies in which substrate levels of fermentation extract are added, because the extract contains high levels of soluble components which are fermented; this is likely to mask the true trace element effect in the extract, such as the effect that the ruminal ecosystem experiences when 3 g of extract per 50 liters is added.

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