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Rumen Fungi and Forage Fiber Degradation

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The role of anaerobic rumen fungi in in vitro forage fiber degradation was determined in a two forage × two inoculum source × five treatment factorial design. Forages used as substrates for rumen microorganisms were Coastal bermuda grass and alfalfa; inoculum sources were rumen fluid samples from a steer fed Coastal bermuda grass hay or alfalfa hay; treatments were whole rumen fluid (WRF), WRF plus streptomycin (0.2 mg/ml of rumen fluid) and penicillin (1.25 mg/ml of fluid), WRF plus cycloheximide (0.5 mg/ml of fluid), WRF plus streptomycin, penicillin, and cycloheximide, and McDougall buffer. Populations of fungi as shown by sporangial development were greater on bermuda grass leaves than on alfalfa leaflets regardless of inoculum source. However, endogenous fungal populations were greater from the alfalfa hay inoculum. Cycloheximide inhibited the fungi, whereas streptomycin and penicillin, which inhibit bacterial populations, resulted in an increase in numbers of sporangia in the alfalfa inoculum, suggesting an interaction between bacteria and fungi. Bacteria (i.e., WRF plus cycloheximide) were equal to the total population in degrading dry matter, neutral-detergent fiber (NDF), acid-detergent fiber (ADF), and cellulose for both inocula and both forages. Degradation of dry matter, NDF, ADF, and cellulose by anaerobic fungi (i.e., WRF plus streptomycin and penicillin) was less than that due to the total population or bacteria alone. However, NDF, ADF, and cellulose digestion was 1.3, 2.4, and 7.9 percentage units higher, respectively, for bermuda grass substrate with the alfalfa versus bermuda grass inoculum, suggesting a slight benefit by rumen fungi. No substantial loss of lignin (72% H_2SO_4 method) occurred due to fungal degradation. The most active fiber-digesting population in the rumen was the bacteria, even when streptomycin and penicillin treatment resulted in an increase in rumen fungi over untreated WRF. The development of large numbers of sporangia on fiber may not indicate a substantial role as digesters of forage.

The microbiology of the rumen is extremely complex due to the large numbers of organisms present, the shifting populations that result from changes in the diet of the host animal, and the interaction between microorganisms. Hungate (10) suggests that the diversity in the microbial population results from selection for maximum biochemical work as well as from the complexity of feedstuffs ingested by ruminants. The two major classes of rumen microorganisms are bacteria and ciliated protozoa. With a washed suspension, benefits in rumen fermentation were reported when both bacteria and protozoa exist in the system (18). Further, the rumen ciliate *Epidinium ecaudatum* has been shown to attach to damaged regions of fresh plant materials undergoing digestion in sheep rumen (7) and to associate with and degrade tissues in alfalfa stems (4) and mesophyll tissues of cool-season grass leaves (2). The rumen bacteria have been studied extensively and have been shown to be the primary degraders of fiber. Rumen protozoa are not essential for fiber digestion, and in the absence of protozoa often rumen bacteria numbers increase and maintain cellulose and fiber digestion (8).

Recently, anaerobic fungi have been shown to colonize plant fragments in the rumen of cattle and sheep fed fibrous diets and to have cellulolytic activity (6). Akin et al. (1) reported that rumen fungi are more prevalent in sheep fed sulfur-fertilized *Digitaria pentzii* than unfertilized forage and that the fungi preferentially colonize the lignified cells of blade sclerenchyma within 6 h of inoculation and cause extensive degradation by 24 h. In vitro studies for dry matter loss showed that rumen fungi, in the absence of bacteria, could remove 62% of the forage material (1). These data indicate that rumen fungi can be significant degraders of fiber and further establish a unique role for them in attacking lignocellulosic tissues which are resistant to rumen bacteria. Information is available on the role of anaerobic fungi as fiber degraders, but their role in comparison with the bacteria, particularly related to lignin degradation, requires further study. Therefore, the objectives of the present work were: (i) to determine the role of individual microbial groups in degrading fiber, using antibiotics to suppress growth of specific microbial populations; (ii) to quantitate fiber degradation by the rumen fungal population; and (iii) to investigate the relationship between colonization and fiber-degrading activity of rumen fungi, using inoculum sources from an animal on different diets.

MATERIALS AND METHODS

Substrate. Forages grown in well-managed fields were used and included Coastal bermuda grass (CBG) (*Cynodon dactylon* L. Pers.) and alfalfa (ALF) (*Medicago sativa* L.). CBG was harvested after a 9-week regrowth, and ALF was from the third cutting (50% bloom) near Athens, Ga. Plants were harvested ca. 10 cm from the ground and stored in plastic bags at −10°C until used. To test for sporangial development, we excised 2- to 5-mm matched (according to size) sections from the central portion of randomly selected, fresh-frozen leaf blades and leaflets. For in vitro dry matter digestibility (IVDMD) and chemical analyses, subsamples of whole forage were lyophilized in a Virtis model 40 REPP sublimer, and the dried samples were ground through a 1-mm screen in a Wiley mill.

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Microbial inoculum. A single cannulated steer was maintained on CBG hay or ALF hay fed ad libitum plus a supplement (2.3 kg/day) of oats (74%), cracked corn (10%), soybean meal (15%), trace minerals (0.5%), and defluorinated phosphate (0.5%). During the cross-over from feeding CBG hay to feeding ALF hay, a 10-day adaption period was used before sampling. Ingesta were removed from the rumen and squeezed through four layers of cheesecloth into a preheated (39°C) vacuum bottle. The strained rumen fluid was used in the preparation of inocula by dilution (1:2) with preheated (39°C) McDougall buffer (13) saturated with carbon dioxide.

In vitro digestion studies. To specifically define the contribution of bacteria and fungi to forage fiber degradation, we conducted a series of IVDMD experiments in the presence of antibiotics (i.e., treatments). Antibiotics against bacteria, i.e., penicillin (P) and streptomycin (S), were as those described by Joblin (11). These two were included in one solution as follows: 12.5 mg of penicillin G (1,600 U/mg; PEN-K; Sigma Chemical Co.) and 2 mg of streptomycin sulfate (650 U/mg; S-6501, Sigma) per ml of distilled water. To inhibit rumen fungi, we prepared cycloheximide (C) (C-6255, Sigma) with 5 mg per ml of distilled water. For use, 3 ml of an S plus P or C solution was used per 30 ml of rumen inoculum. Those tubes receiving only one antibiotic or none (i.e., whole rumen fluid plus McDougall buffer [WRF]) received distilled water to equal 6 ml of total liquid added to the 30-ml inoculum. IVDMD studies were conducted with 0.4 g of dry matter (DM) per tube for 48 h followed by incubation for 48 h with a solution of 0.1 N HCl-pepsin (2 g/liter). In addition, substrates were also incubated with McDougall buffer only and McDougall buffer followed by acid-pepsin as controls. IVDMD determinations were conducted in duplicate for each inoculum source with 15 tubes per treatment. An inoculum blank containing each antibiotic treatment was included to correct the indigestible dry matter remaining (IDMR) for inoculum residue. After incubations, all samples were lyophilized, and IVDMD was calculated based on DM recovery.

Chemical analyses. Lyophilized forages and IDMR were analyzed for neutral-detergent fiber (NDF), acid-detergent fiber (ADF), cellulose, and 72% H_2SO_4 lignin, as described by Goering and Van Soest (9). The IDMR from each treatment (i.e., 15 tubes) was combined, reground to pass a 1-mm screen, and analyzed for fiber components. Apparent digestibility coefficients (ADC) for fiber components were calculated based on their recovery in the IDMR.

Microscopic techniques. To evaluate rumen fungi, we counted sporangia attached to leaf blades and leaflets under a light microscope after they were stained for 30 to 60 s with lactophenol cotton blue (10 g of phenol, 10 ml of glycerin, 10 ml of lactic acid, 10 ml of distilled water, 20 to 50 mg of cotton blue) and washed twice with distilled water. Only sporangia which were attached to the plant material were counted. Relative numbers of sporangia on leaf surfaces were based on five randomly selected fields (×20 phase lens; area = 0.657 mm^2). Only the midrib surfaces of the blades were counted. Viable protozoa were counted after 20 h of incubation in the presence or absence of S-P-C. Protozoa were observed for motility under a ×20 phase lens.

Statistical analysis. Data were treated by analyses of variance for a 2 × 2 × 5 factorial design with in vitro replication nested within inoculum source, using the statistical analysis system described by Barr et al. (3). Differences between means were determined by using Scheffe's multiple comparison procedure as described by Kleinbaum and Kupper (12).

TABLE 1. Numbers of rumen fungal sporangia associated with leaf blades after incubation with rumen fluid and antibiotics

Treatment	Inoculum	ALF leaflet		CBG leaf	
		Cut edge[a]	Surface[b]	Cut edge[a]	Surface[b]
No antibiotics	ALF	1.8	0	9.3	0.7
S, P	ALF	0.3	0.7	99.5	5.4
C	ALF	0	0	0	0
No antibiotics	CBG	0	0	7.0	0.3
S, P	CBG	2.0	0.1	2.0	0.2
C	CBG	0	0	0	0

[a] Sporangia on cut edge (per leaf or leaflet), average of two experiments.
[b] Average sporangia number under microscopic field (area = 0.657 mm^2) for five sites per blade.

RESULTS

The forages were typical for 50% full bloom ALF and mature CBG. CBG was higher in NDF, ADF, and cellulose than was ALF (67.7, 34.4, and 29.3%, versus 32.8, 25.2, and 20.1%, respectively). The H_2SO_4 lignin values of both forages were identical (i.e., 5.1%). Although the gravimetric lignin values were equal, the degree of lignification between the forages differed due to the amount of cell wall fiber. These differences provide a comparative determination of the role microbial groups play in degrading fiber components, especially lignin and lignocellulose.

The numbers of rumen fungal sporangia associated with leaf blades after incubation with rumen fluid in the presence of antibiotics are shown in Table 1. The development of fungi on plants as shown by sporangia was greater on CBG leaves than on ALF leaflets, regardless of the inoculum source. The ALF inoculum stimulated the fungal populations on CBG leaves, whereas no stimulation occurred with CBG inoculum and numbers were relatively low. In addition, the number of sporangia was always greater on the cut edge of the leaf blade than the surface, regardless of the inoculum source and forage. C treatment totally inhibited the fungal population, whereas S and P treatment resulted in an increase in sporangia numbers on CBG leaves with an ALF inoculum.

To determine the percentage of DM lost due only to microbial degradation, we corrected the total number of milligrams digested during incubation for DM losses from treatment with buffer and the additional losses from treatment with acid-pepsin. Therefore, the data in Table 2 represent the percentage of DM and fiber loss attributed to microbial degradation. The DM digestion was equal for WRF or C treatment and greater than that with S-P in both forages. However, the greater DM loss of ALF with S-P-C treatment resulted in a substrate × treatment interaction ($P < 0.02$). Digestion of NDF when averaged across treatments and substrates was higher ($P < 0.01$) with an ALF inoculum. The ADC were higher in ALF NDF than in CBG NDF in the presence of WRF, C, or S-P-C, which resulted in a substrate × treatment interaction ($P < 0.01$). In addition, the S-P treatment resulted in lower digestibilities than either WRF or C treatment. The ADC for ADF reflected a substrate × treatment ($P < 0.02$) and a substrate × inoculum ($P < 0.03$) interaction. The ADC for ADF when averaged across treatments in ALF and CBG were equal with an ALF inoculum, but digestion of ALF ADF was greater ($P < 0.05$) with a CBG inoculum. The substrate × treatment interaction was due to higher ADF digestion in ALF than in CBG in the

TABLE 2. ADC of fiber components due to microbial degradation of CBG and ALF forages

Component	Inoculum	Substrate	ADC (%) with the following treatment:				SEM
			WRF	S-P	C	S-P-C	
DM	ALF	ALF	19.2[a]	16.7[a]	17.3[a]	5.5[b]	1.4
	ALF	CBG	21.6[a]	14.7[b]	19.8[a]	0.0[c]	
	CBG	ALF	16.3[a]	11.4[b]	15.3[a,b]	5.2[c]	
	CBG	CBG	21.7[a]	14.3[b]	18.6[a]	0.0[c]	
NDF	ALF	ALF	27.7[a]	14.7[b]	26.7[a]	11.2[b]	1.4
	ALF	CBG	18.0[a]	9.7[b]	15.0[a]	0.0[c]	
	CBG	ALF	25.5[a]	9.9[b]	26.5[a]	3.9[c]	
	CBG	CBG	19.0[a]	8.4[b]	15.2[a]	0.0[c]	
ADF	ALF	ALF	20.1[a]	16.7[b]	28.1[c]	0.0[d]	2.7
	ALF	CBG	26.2[a]	15.1[b]	23.8[a]	0.0[c]	
	CBG	ALF	26.9[a]	16.7[b]	35.8[c]	0.0[d]	
	CBG	CBG	24.1[a]	12.7[b]	19.4[a]	0.0[c]	
Cellulose	ALF	ALF	36.4[a]	26.8[b]	45.8[c]	0.0[d]	2.8
	ALF	CBG	35.7[a]	21.9[b]	31.9[a]	0.0[c]	
	CBG	ALF	41.2[a]	18.7[b]	41.3[a]	0.0[c]	
	CBG	CBG	32.9[a]	14.0[b]	27.8[c]	0.0[d]	

[a,b,c,d] Means within rows within component with unlike superscripts differ ($P < 0.05$).

presence of C; however, digestion with S-P treatment in both forages was less ($P < 0.05$) than that obtained with WRF or C. The ADC for cellulose also reflected a substrate × treatment interaction ($P < 0.04$) due to the higher cellulose digestion in an ALF substrate with WRF, S-P, or C. Treatment with S-P resulted in lower ($P < 0.05$) cellulose digestion in both substrates when compared with WRF or C. No digestion of lignin occurred with any treatment.

Rumen protozoa were evaluated for motility in the presence of S-P-C and compared with activity in the absence of antibiotics. After 24 h, S-P-C showed essentially no effect on the motility of the holotrichs. The motility of entodiniomorph protozoa incubated with S-P-C was 24 to 85% and 8 to 132% of similar incubations without antibiotics with orchard grass and ALF, respectively. The small loss in DM and NDF with S-P-C (Table 2) could occur due to the protozoa, but their activity was usually small in comparison with that of fungi.

DISCUSSION

The importance of rumen fungi cannot be assessed by enumeration of the stage of their life cycle, particularly the zoospore stage (5, 6). To assess the fungal stage related to fiber digestion, we counted sporangia associated with leaf blades of ALF and CBG (Table 1). The sporangia were greater on the more fibrous substrate (i.e., CBG) and on the cut edge of the leaf regardless of inoculum source, indicating a lack of association with ALF leaflets. These data are in agreement with those of Bauchop (6), who reported that the major route of fungal invasion was via damaged tissues and that leaves of grasses were more heavily colonized than low-fiber substrates. In addition, Bauchop (5) reported that zoospores or sporangia were not detected microscopically in rumen contents of sheep continuously grazing pastures or pure stands of young clover (i.e., low-fiber diets), whereas the highest populations were found with fibrous, stalky diets. In our experiments, the fungal populations were stimulated with the ALF diet to a much greater extent than that observed with the CBG diet. Both the ALF and CBG may be considered more fibrous diets than continuously grazed (immature) pasture or pure stands of young clover, but CBG hay is a more fibrous forage than ALF hay (i.e., NDF = 69.7% versus 32.8%, respectively). Therefore, the general statement that the most fibrous diets appear to support the greatest population of fungi was not valid in comparing these two forages and indicates that factors other than fiber amount affect fungal development.

Antibiotics provided a way to manipulate the various microbial groups to evaluate fungal contribution to fiber digestion in the rumen. Inclusion of S and P in an ALF inoculum resulted in higher numbers of sporangia and hyphal overgrowth (Akin, unpublished data) than did treatment without antibiotics (i.e., WRF). These differences in growth and numbers of sporangia in the absence of bacteria suggest a biological interaction between the bacteria and fungi. A similar interaction had been observed in other studies with a CBG inoculum (Akin, unpublished data), but was not observed with CBG herein. However, the nature of this interaction has not been resolved. With ALF inoculum and treatment with S and P, the higher number of sporangia should allow a delineation of the near-maximum ability of fungi to degrade forage fiber. Despite this high number of sporangia in an ALF inoculum, digestion of fiber components was always less than that with WRF or C (Table 2). These data indicate that the fungal population was not as active in fiber digestion as the bacteria in this system. In these studies in which numbers of rumen fungi were increased with antibiotics (ALF inoculum) and in studies in which they were not increased (CBG inoculum), fiber digestion was less with S and P; digestion was greater by a few percentage units with an ALF inoculum.

Rumen fungi have been shown to attack and degrade tissues containing lignin, which are more resistant to rumen bacteria (1). Further, some removal of lignin from grass fiber has been reported by anaerobic fungi from horse cecum (16). However, in our study, evaluation of digestion of ADF and residual lignin present in IDMR did not indicate a major role for these rumen fungi as utilizers of lignin or degraders of lignified fiber. The only link between an increased fungal population and fiber digestibility occurred for ADF and cellulose (Table 2). This link, although not statistically significant, indicated that an ALF inoculum resulted in a 2.4 and 7.9 percentage unit increase in the digestion of ADF and cellulose, respectively, with S and P over that with a CBG inoculum, possibly due to variations in rumen fungal activity.

Earlier work from Australia (1) clearly indicated a major role for rumen fungi in degrading and weakening grass fiber (including lignified tissues) that contributed to substantial increases in forage consumption by ruminants. It is likely that fungal populations vary in their ability to attack and degrade fiber and that the population observed herein apparently lacks the potential for substrate digestion as in other systems. Indeed, Bauchop (6) has suggested that fungal populations adapted to different diets could vary in invasiveness, and Orpin (16) has shown variation in substrate digestion by different isolates of anaerobic fungi. Although factors influencing fungal development and fiber-degrading activity are not well established, it has been shown that sulfur fertilization of plants grown in deficient areas results in an active rumen fungal population with or without bacteria (1). However, other work with gnotobiotic cultures has shown that cellulolytic activity by the rumen fungus *Neocallimastix frontalis* is enhanced in the presence of methanogenic rumen

bacteria (14). The exact factors in ALF responsible for stimulation of fungal development are not known, but under these experimental conditions it does not appear that enhanced fiber-degrading activity is significantly correlated with enhanced sporangial development. Orpin (15) has reported data in which higher population densities of *N. frontalis* resulted in lower DM digestibilities.

C totally inhibited sporangial development on leaf blades, but digestion of fiber components usually was as high or higher than in incubations without antibiotics (Table 2). These data indicate that the rumen bacteria are the major microbial group responsible for fiber digestion in this system regardless of inoculum source.

Despite the fact that C inhibits protein synthesis (17), often protozoa, and especially the holotrichs, maintained some activity in the presence of this antibiotic. Therefore, these protozoa, although not the major species usually reported to be involved with fiber digestion (2, 5), may have degraded the more soluble carbohydrate components in the DM and NDF fractions of ALF and accounted for the small loss in the presence of S-P-C (Table 2). In addition, with an ALF inoculum, digestion of ALF DM was not different with WRF, S-P, or C. These data also suggest a biological interaction of protozoa and fungi which resulted in a loss of substantial amounts of components from ALF but not from CBG. However, the nature of this interaction has not been resolved. No protozoal digestion was observed with ADF or cellulose from either substrate, which is in agreement with studies showing the inability of protozoa to degrade the more-resistant fiber (2) and supports the conclusion that the slight increase in ADF and cellulose digestibility with an ALF inoculum and S-P treatment (Table 2) was due to the fungi.

In summary, rumen bacteria appeared to be the most active fiber degraders. Rumen sporangial development on CBG leaves was stimulated by ALF in the diet, but higher numbers did not result in substantial increases in fiber digestion. Substrate digestion by rumen fungi (i.e., WRF plus S and P) was less than that of rumen fluid without antibiotics (i.e., WRF) or with WRF plus C. The rumen fungi did not degrade lignin or lignified tissue to any significant extent in these studies.

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