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Antibiosis between Ruminal Bacteria and Ruminal Fungi†

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Cellulose digestion, bacterial numbers, and fungal numbers were monitored over time in vitro by using a purified cellulose medium with and without antibiotics (penicillin and streptomycin). All fermentations were inoculated with a 1:10 dilution of whole rumen contents (WRC). Without antibiotics, cellulose digestion was higher ($P < 0.01$) at 24, 30, 48, and 72 h; fungi had almost disappeared by 24 h, while bacterial concentrations increased over 100-fold in 24 h and then decreased gradually up to 72 h. In those fermentations with added antibiotics, fungal concentrations increased 4-fold by 30 h and up to 42-fold at 72 h; bacterial concentrations were markedly reduced by 24 h and remained low through 72 h. Similar results were obtained with ground alfalfa as a substrate. In further studies, the in vitro fermentation of purified cellulose without antibiotics was stopped after 18 to 20 h, and the microbial population was killed by autoclaving. Antibiotics were added to half of the tubes, and all tubes were reinoculated with WRC. After 72 h, extensive cellulose digestion had occurred in those tubes without antibiotics, as compared to very low cellulose digestion with added antibiotics. The extent of this inhibition was found to increase in proportion to the length of the initial fermentation period, suggesting the production of a heat-stable inhibitory factor or factors. The inhibitory activity was present in rumen fluid, could be extracted from lyophilized rumen fluid (LRF) with water, and was stable in response to proteolytic enzymes. In addition, the water-extracted residue of LRF was found to contain growth factor activity for rumen fungi in vitro.

The flagellated microorganisms observed in rumen contents by Liebetanz in 1910 (36) and Braune in 1913 (13) were originally believed to be flagellate protozoa. However, in a series of classic studies, Orpin (39–45) determined that these organisms were actually flagellated zoospores of anaerobic fungi. Although concentrations of the fungi are relatively low in comparison to those of the bacteria and ciliate protozoa, they possess a wide range of enzymes which are capable of hydrolyzing most of the structural polysaccharides occurring in plant cell walls (21, 26, 35, 48, 53, 55). The fungi also appear to be superior to the rumen bacteria in their ability to break down and degrade the structural barriers in plant material (2). They are able to weaken and partially or fully degrade the more recalcitrant plant tissues as well as penetrate the cuticle barrier (3, 5, 34). When fungi were removed from the rumen, both feed intake and fiber digestibility were decreased; however, total viable bacteria, cellulolytic bacteria, or ciliate protozoal concentrations were not affected (24). Based on in vitro studies with rumen fluid, using antibiotics and a fungicide to selectively culture either the bacteria or fungi, Akin and coworkers (1, 56) concluded that the bacteria were the most active fiber-digesting organisms, even though fungal numbers were increased in the antibiotic-treated cultures.

A number of studies have been conducted on the interrelationships between the fungi and rumen bacteria and protozoa. The fungi form quite stable cocultures with rumen methanogenic bacteria as a result of their high production of hydrogen (23, 47). In general, these cocultures produce an increased amount of fungal biomass (9) and exhibit an increase in both the rate and extent of cellulose degradation (7, 10, 33). On the other hand, when combined in coculture with the cellulolytic ruminococci, their cellulolytic activity appeared to be inhibited (11, 28, 50). In contrast, *Fibrobacter succinogenes* appears to have little if any effect on the activity of the fungi (11, 50). Coincubations of protozoa with fungi have shown that the protozoa are able to both ingest and digest fungi (47). Morgavi et al. (37) found considerable chitinase activity in samples of mixed rumen protozoa, which would account for their predatory activity on the rumen fungi (30, 54). No effects were noted when a washed preparation of small protozoa (over 95% *Dasytricha ruminantium*) was incubated with the rumen fungus *Neocallimastix frontalis*. However, in a similar experiment using medium-sized protozoa (both holotrichs and entodiniomorphs), fungal digestion was markedly inhibited (23).

In spite of the unique abilities of the fungi to attack and degrade the more resistant plant cell walls, their role and importance in the overall rumen fermentation remain as major questions to be answered. Their rates of growth and digestion of plant polysaccharides appear to be somewhat slower than those of the bacteria, perhaps as a result of their more complex life cycle (45, 47). Some type of bacterial inhibition might also be postulated, based on the decreased cellulolytic activity which occurs in coculture with ruminococci (11, 50; A. J. Richardson, C. S. Stewart, G. P. Campbell, A. B. Wilson, and K. N. Joblin, Proc. XIV Int. Congr. Microbiol., abstr. PG2-24, p. 233, 1986) and the fact that fungal colonies are not detected in roll tubes inoculated with rumen contents if antibiotics are not added to the medium (29). The present study was undertaken to enumerate bacterial and fungal numbers after the in vitro fermentation of cellulose by whole rumen contents in the presence and absence of antibiotics. Both purified cellulose and intact forage were used as substrates.

MATERIALS AND METHODS

Media and culture. Unless noted otherwise, the basal purified cellulose medium was used in all experiments and contained the following ingredients per 100 ml: 15 ml each of mineral solutions I and II of Bryant and Burkey (15), 0.1 ml of

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TABLE 1. Cellulose digestion and changes in bacterial and fungal concentrations during the fermentation of purified cellulose by rumen contents without (control) and with added antibiotics[a]

Time (h)	Cellulose digestion (%)[b]		No. of bacteria/ml (10^7)[c]		No. of fungi/ml (10^2)[d]	
	Control	Antibiotics	Control	Antibiotics	Control	Antibiotics
0	0	0	9.4 ± 3.1	9.4 ± 3.1	11.9 ± 4.1	11.9 ± 4.1
24	37.5 ± 4.6[e]	1.2 ± 0.7	1,000 ± 489	0.0004 ± 0.0001	0.01 ± 0.01	15.5 ± 9.7
30	51.2 ± 8.7	3.1 ± 1.0	451 ± 231	0.0023 ± 0.0011	0.02 ± 0.01	50.3 ± 33.4
48	56.8 ± 8.3	16.6 ± 1.9	290 ± 214	0.0044 ± 0.0010	0	229.8 ± 86.1
72	70.0 ± 1.7	47.2 ± 0.8	38.2 ± 4.8	0.0029 ± 0.0014	0	510.0 ± 80.0

[a] Penicillin and streptomycin.
[b] At all time periods, cellulose digestion in the control was higher than with antibiotics ($P < 0.02$).
[c] Bacterial concentrations higher in the control tubes at 24 h ($P < 0.13$), 30 h ($P < 0.15$), at 48 h ($P < 0.27$), and 72 h ($P < 0.01$).
[d] Fungal concentrations higher in the antibiotic tubes at 24 h ($P < 0.21$), 30 h ($P < 0.23$), and 48 and 72 h ($P < 0.01$).
[e] Mean ± standard error.

a 0.1% resazurin solution, 0.2 g of Trypticase, 0.05 g of yeast extract, 0.45 ml of the volatile fatty acid mix of Caldwell and Bryant (16), 33.33 ml of a 6% solution of 24-h ball-milled cellulose (Sigmacell-20; Sigma, St. Louis, Mo.), 0.1 g of glucose, 20 ml of rumen fluid (RF [the supernatant obtained from centrifugation at 1,000 × *g* for 10 min]), 3.33 ml of 12% Na_2CO_3, 1.67 ml of 3% cysteine hydrochloride, and 4.0 ml of distilled water. Thirteen-milliliter aliquots were tubed under O_2-free CO_2 into culture tubes (16 by 150 mm), closed with rubber stoppers, and autoclaved in racks at 121°C for 20 min (18). An additional 1 ml of either sterile distilled water or antibiotic solution was added at the time of inoculation, bringing the volume to 14 ml.

For the alfalfa forage medium, the ball-milled cellulose and glucose were omitted and 5 g of third-cutting alfalfa hay (ground to pass through a 0.5-mm-pore-diameter screen) were added. The distilled water was increased to 44.5 ml.

In all fermentations where fresh RF, lyophilized RF (LRF), or any fractions prepared from them were tested, they were added before autoclaving the medium, unless otherwise designated.

The antibiotic stock solution contained 32,000 U of penicillin G (Sigma, St. Louis, Mo.) and 2,080 U of streptomycin sulfate (U.S. Biochemical Corp., Cleveland, Ohio) per ml. One milliliter of the stock solution added to the fermentation tube (total volume of 16 ml) gave a final concentration of 2,000 U of penicillin and 130 U of streptomycin per ml. Cycloheximide (U.S. Biochemical Corp.) was used in several experiments at a final concentration of 0.5 mg per ml to inhibit fungal growth. The antibiotics and fungicide were dissolved in distilled water previously gassed with O_2-free CO_2, sterilized by passage through a 0.2-μm-pore-diameter polysulfone membrane filter (Gelman Sciences, Ann Arbor, Mich.), and added anaerobically and aseptically to the individual tubes prior to inoculation.

LRF was prepared as described previously (19). Water extraction of LRF was carried out by adding 5.0 ml of distilled water to 250 mg of LRF in a culture tube (20 by 150 mm), shaking for 1 h at room temperature, centrifugation at 17,500 × *g* for 20 min, and decanting the supernatant.

Inoculum. Rumen contents were obtained through a permanent fistula from a steer fed a diet of grass hay. The sample was taken just before the morning feeding. Twenty grams of rumen ingesta was diluted 1:10 with the anaerobic dilution solution of Bryant and Burkey (15), and the mixture was agitated for 3 min under a vigorous stream of O_2-free CO_2. Fermentation tubes were inoculated with 2 ml of the 1:10 dilution.

Bacterial and fungal assays. Total bacterial and fungal concentrations were determined by using most-probable-number (MPN) assays as described by Dehority et al. (20) and Obispo and Dehority (38). For all experiments, the basal medium of Obispo and Dehority (38) was used without any additions to determine bacterial concentrations, with antibiotics to enumerate fungi, or with cycloheximide to determine the possible fungal contribution to total cellulose digestion.

Chemical analyses. Purified cellulose digestion was measured by the procedure previously described by Hiltner and Dehority (27). Essentially this involves centrifugation of the insoluble material, digestion with acid-detergent-fiber solution for 1 h at 100°C, washing with hot water by centrifugation, and weighing the residue. Digestion of cellulose from alfalfa was determined by using the Crampton-Maynard procedure (17). Glucose utilization was determined colorimetrically at 520 mμ with the orcinol reaction (14).

Experimental protocol. The designs of the major experiments (results shown in Tables 1 to 4) are given below.

(i) Bacterial and fungal growth related to purified cellulose digestion (Table 1). Using the purified cellulose broth medium, duplicate tubes, with and without antibiotics, were inoculated with rumen contents and allowed to ferment for 0, 24, 30, 48, and 72 h. The tubes were then analyzed for cellulose digestion, bacterial concentrations, and fungal concentrations. Each time period was replicated twice, so there are 8 values behind the 0-h data and four values behind each of the other time periods.

(ii) Bacterial and fungal growth related to digestion of cellulose from ground alfalfa (Table 2). The same format was used as in the first experiment, except ground alfalfa was used as the substrate and samples were taken after fermentation for 0, 30, and 72 h. Three replicates were conducted, with duplicate tubes at each time period.

(iii) Cellulose digestion in sterilized medium after an initial 18-h fermentation (Table 3). Tubes containing purified cellulose medium were inoculated and incubated for 0 h (4 tubes) or 18 h (8 tubes). At the end of their respective fermentations, all tubes were autoclaved for 20 min at 121°C. Using duplicate tubes, additions were then made as follows: 0-h tubes, none or antibiotics; 18-h fermentation tubes, none, antibiotics, cycloheximide, or antibiotics plus cycloheximide. All tubes were then inoculated again, allowed to ferment for 72 h, and analyzed for residual cellulose.

(iv) Effect of initial fermentation time on inhibitor production (Table 4). Sixteen tubes of purified cellulose medium were inoculated and allowed to ferment, with four tubes being removed and autoclaved (20 min at 121°C) at 0, 5, 10, and 20 h. For each set of four tubes, no additions were made to two tubes and antibiotics were added to the other two. All tubes were inoculated, allowed to ferment for an additional 72 h, and analyzed for residual cellulose.

Effect of proteolytic enzymes. The water extract of LRF was incubated for 1 h at 38°C with an equal volume of 0.01 M phosphate buffer containing 1 mg (per ml) of either a nonspecific protease from *Streptomyces griseus* or trypsin (Sigma). At the same time, solutions of casein and bovine albumin (1 mg/ml) were also treated with the enzyme solutions (control and boiled), and the activity of the enzymes was determined by estimating hydrolysis of the proteins based on the presence of a precipitate following the addition of trichloroacetic acid.

Statistical analysis. Data were analyzed with the *t* test and paired *t* test (49).

RESULTS

Bacterial and fungal growth related to purified cellulose digestion. This experiment was specifically designed to monitor bacterial and fungal concentrations over time and attempt to relate them to cellulose digestion. Table 1 shows that 37.5% cellulose digestion occurred in the control fermentation tubes at 24 h, with values increasing to 51, 57, and 70% at 30, 48, and 72 h, respectively. In contrast, cellulose digestion in those tubes with the added antibiotics was 1, 3, 17, and 47% at the same time periods. Bacterial concentrations in the control fermentation tubes increased markedly in the first 24 h and then gradually declined up to 72 h, almost decreasing back to their starting level. Although the bacteria were not completely eliminated when antibiotics were added, concentrations fell to barely detectable levels by 24 h and remained there throughout the 72-h fermentation. Fungal concentrations in the control tubes essentially decreased to zero within the first 24 h. However, with added antibiotics, fungal concentrations remained at or slightly above their starting value at 24 h, increased about fivefold by 30 h, and continued to increase at both 48 and 72 h.

Bacterial and fungal growth related to digestion of cellulose from ground alfalfa. A similar set of fermentations and analyses were conducted with immature alfalfa as a substrate (Table 2); however, fermentations were carried out only for 30 and

TABLE 2. Cellulose digestion and changes in bacterial and fungal concentrations during the fermentation of alfalfa by rumen contents without (control) and with added antibiotics[a]

Time (h)	Cellulose digestion (%)[b]		No. of bacteria/ml (10^7)[c]		No. of fungi/ml (10^2)[d]	
	Control	Antibiotics	Control	Antibiotics	Control	Antibiotics
0	0	0	8.6 ± 1.9	8.6 ± 1.9	14.1 ± 5.2	14.1 ± 5.2
30	40.3 ± 1.6[e]	8.7 ± 1.8	757 ± 348	2.8 ± 2.5	8.0 ± 7.0	39.3 ± 13.0
72	48.8 ± 2.4	32.8 ± 3.4	20.4 ± 7.9	0.2 ± 0.2	0.007 ± 0.007	11.1 ± 7.2

[a] Penicillin and streptomycin.
[b] At both 30 and 72 h, cellulose digestion in the control was higher than with antibiotics ($P < 0.01$).
[c] Bacterial concentrations higher in the control tubes at 30 h ($P < 0.08$) and 72 h ($P < 0.05$).
[d] Fungal concentrations higher in the antibiotic tubes at 30 h ($P < 0.07$) and 72 h ($P < 0.18$).
[e] Mean ± standard error.

72 h. Marked cellulose digestion occurred by 30 h in the control tubes without antibiotics as compared to minimal cellulose digestion in the antibiotic tubes. However, considerable cellulose digestion took place in the antibiotic-containing tubes between 30 and 72 h. Without antibiotics, bacterial concentrations increased nearly 100-fold in 30 h and then fell back almost to their initial starting level by 72 h. With antibiotics, bacterial concentrations steadily decreased from 0 to 72 h. Fungal concentrations in the controls decreased from approximately 1,400 per ml at the start to 800 and 0.7 per ml at 30 and 72 h, respectively. With antibiotics, fungal concentrations increased at 30 h, but fell back to initial levels by 72 h. This decrease in fungal concentrations between 30 and 72 h, when most of the cellulose digestion took place, was somewhat unexpected, but fairly consistent across all three replicates.

Possible production of a fungal inhibitor(s) by the rumen bacteria. The two previous sets of experiments clearly demonstrate the rapid growth of bacteria compared to that of the fungi and further suggest that the bacteria somehow inhibit growth of the fungi. In order to study this apparent inhibition further, six control fermentation tubes were incubated for 20 h. Two tubes were removed, and pH measurements of the medium were taken, followed by analysis for residual cellulose. In two of the remaining four tubes, enough sterile Na_2CO_3 was added to raise the pH from approximately 6.1 to 6.6, and antibiotics were added to all tubes. The tubes were then incubated for an additional 72 h. In two experiments, cellulose digestion averaged 38.6% ± 2.5% in the first 20 h. Total digestion after the additional 72-h fermentation was 50.2% ± 6.2% in the pH-adjusted tubes and 63.2% ± 1.2% in the nonadjusted tubes. However, no fungi were detected in any of the tubes, suggesting that the second fermentation was probably bacterial and not fungal. Apparently bacterial numbers had increased enough in 20 h (Table 1) that the level of antibiotics was not adequate to totally inhibit bacterial growth. However, when antibiotic levels were increased in separate experiments, fungal growth was also inhibited. Thus, the only procedure available to study the negative effect of bacterial fermentation on fungal growth appeared to be sterilization of the control culture after the initial fermentation period. Antibiotics could then be added, and the tube could be reinoculated with rumen contents.

Attempts to sterilize the fermentation tubes by oxidation, using the procedure described by Fondevila and Dehority (22), were unsuccessful, even though pure oxygen was used in place of air. Essentially the tubes seemed to rapidly reduce again after they had been gassed with oxygen, after the stopper had been replaced with a cotton plug, and after they had been placed back into the incubator. In addition, the normal color change for resazurin was affected (i.e., instead of changing from colorless to pink in the oxidized state, the medium turned a darkish brown). Thus, we used heat to sterilize the tubes after the initial fermentation (autoclaving at 121°C for 20 min).

Cellulose digestion in sterilized medium after an initial 18-h fermentation. In addition to looking for the production of an inhibitor by the bacteria, a primary concern in this experiment was the possibility that heat might destroy any inhibitory factor(s) that was produced. The results of these fermentations are shown in Table 3. After the initial 20-h fermentation, 39.1 mg (20%) of the cellulose had been digested, and the medium pH was about 6.5 (no pH adjustments were made). All tubes were autoclaved, inoculated, and incubated for an additional 72 h. In the control and antibiotic tubes without an initial fermentation (0 h), 112.6 mg (59.1%) and 95.4 mg (50.7%) of the cellulose were digested, respectively. In those tubes which had an 18-h initial fermentation, 73.8 mg of cellulose was digested in the second fermentation without any additions (control), which when added to the 39.1 mg which had already been digested, gave a total of 112.9 mg (59.2%) of cellulose digested. This indicates that autoclaving and reinoculation had no apparent negative effects on bacterial digestion of cellulose. However, only 21.4 mg of cellulose was digested in the tubes with antibiotics (fungal digestion), giving an overall total of only 31.4% cellulose digested. With added cycloheximide (bacterial digestion), 68.8 mg of cellulose was digested (56.6% of total cellulose). As expected, no additional digestion of cellu-

TABLE 3. Digestion of purified cellulose by rumen contents in media without additions or with antibiotics, cycloheximide, or both after an initial 0- or 18-h fermentation and autoclaving

Initial incubation (h)[a]	Amt of cellulose digested in initial incubation (mg)	Amt of cellulose digested (mg) in second fermentation (72 h)[b]			
		Control	Antibiotics	Cycloheximide	Antibiotics + cycloheximide
0	0	112.6 ± 4.2 (59.1)[c]	95.4 ± 16.0 (50.7)		
18	39.1 ± 10.8 (20.0)	73.8 ± 10.0A (59.2)	21.4 ± 4.4B (31.4)	68.8 ± 8.1A (56.6)	0

[a] All tubes were autoclaved for 20 min at 121°C after the initial incubation time and then inoculated again with rumen contents for the second fermentation.
[b] Means in the same row without a common letter differ ($P < 0.01$).
[c] Total percent of cellulose digested in all incubations, single or combined where applicable.

TABLE 4. Digestion of purified cellulose by rumen contents in media without (control) or with added antibiotics after an initial 0-, 5-, 10-, or 20-h incubation and autoclaving

Initial incubation (h)[a]	Amt of cellulose digested in initial incubation (mg)	Amt of cellulose digested (mg) in second fermentation (72 h)[b]	
		Control	Antibiotics
0	0	67.5 ± 3.6 (34.5)[c]	53.0 ± 8.5 (27.0)
5	6.1 ± 5.5 (3.0)	69.2 ± 2.8A (38.5)	37.6 ± 7.4B (22.1)
10	16.2 ± 5.6 (8.1)	52.8 ± 6.4A (35.3)	6.0 ± 3.4B (11.1)
20	63.4 ± 3.8 (32.5)	16.3 ± 4.1C (40.8)	0.1 ± 0.1D (32.5)

[a] All tubes were autoclaved for 20 min at 121°C after the initial incubation time and then inoculated again with rumen contents for the second fermentation.
[b] Means in the same row without a common superscript differ ($P < 0.01$ for A and B; $P < 0.02$ for C and D).
[c] Total percentage of cellulose digested in all incubations, single or combined where applicable.

TABLE 5. Effect of adding either liquid RF or LRF to media with and without antibiotics on the in vitro digestion of purified cellulose by rumen contents[a]

Type of RF	% in media	Cellulose digestion (%)[b]	
		Control	Antibiotics[c]
Liquid (fresh)	0	79.7 ± 3.3[d]	51.9 ± 3.7A
	30	72.7 ± 4.8	20.8 ± 3.6B
	60	72.6 ± 4.7	21.7 ± 2.8B
Lyophilized	0	56.8 ± 1.5	45.9 ± 2.1A
	60[e]	60.3 ± 1.7	28.3 ± 3.0B

[a] Each value for liquid RF is based on 10 fermentation tubes (five replicates in duplicate). Each value for LRF is based on eight fermentation tubes (four replicates in duplicate).
[b] For each type of RF, means in the same column without a common letter differ ($P < 0.01$).
[c] Penicillin and streptomycin.
[d] Mean ± standard error.
[e] The weight of LRF was equivalent to 60 ml of liquid RF.

lose occurred when both antibiotics and cycloheximide were added before the second fermentation. For the tubes preincubated for 18 h, the amounts of cellulose digested in the control and cycloheximide tubes (bacterial digestion) were not different ($P > 0.72$); however, both were greater ($P < 0.01$) than those in the antibiotic tubes (fungal digestion).

After an initial 18-h fermentation and autoclaving, 0.81 mg of glucose per ml was added to tubes with and without antibiotics before the second fermentation. With added glucose, a slight decrease in cellulose digestion occurred in tubes without antibiotics; however, addition of glucose had no effect on cellulose digestion in the antibiotic tubes (data not shown). The possible effect of soluble carbohydrates on fungal growth was studied further by comparing fungal concentrations determined in three different MPN media. The control medium contained 0.1% (each) glucose, cellobiose, maltose, and xylose plus 0.75% cellulose; the second MPN medium was the same composition with 20% RF added; and the third medium was the same as the control, but minus the soluble sugars. Based on six replicates, no significant differences were found in the concentration of fungi in rumen contents (data not shown). Thus, lack of a soluble energy source for encystment of zoospores does not appear to be a factor in the observed inhibition.

Effect of initial fermentation time on inhibitor production. The above experiments suggest that the inhibition of fungal growth is caused by bacterial production of one or more inhibitory compounds. If true, then the extent of inhibition should be related to the length of the initial fermentation. This was investigated, and the results are shown in Table 4. As expected, cellulose digestion increased with time of the initial fermentation, increasing from 6.1 mg in 5 h to 63.4 mg by 20 h. During the second fermentation, the amounts of cellulose digested in the control tubes (bacterial digestion) were not different between the 0- and 5-h initial fermentations ($P > 0.72$), decreased slightly between 5 and 10 h ($P < 0.06$), and fell markedly between 10 and 20 h ($P < 0.01$). In the antibiotic tubes (fungal digestion), the amount of cellulose digested decreased from 53 mg at 0 h to 37.6 mg ($P < 0.21$) after the initial 5-h fermentation. Between the 5- and 10-h initial fermentations, the amount of cellulose digested decreased markedly, i.e., falling from 37.6 mg to 6.0 mg ($P < 0.01$). A further decrease was observed after 20 h, from 6.0 mg down to 0.1 mg ($P < 0.14$). Differences between the control and antibiotic tubes were significant at 5 and 10 h ($P < 0.01$) and 20 h ($P < 0.02$). When the total percent cellulose digested in both fermentations was calculated, there were no differences with time in the control fermentations ($P > 0.34$). However, the total percent cellulose digestion in the antibiotic tubes after the 10-h initial fermentation was lower than those of both the 5-h ($P < 0.15$) and 20-h ($P < 0.01$) fermentations.

Inhibition of fungal growth by RF. If a mixed culture of rumen bacteria growing in vitro on purified cellulose or alfalfa produces a factor or factors which are inhibitory to rumen fungi, this factor should be present in RF. Thus, several levels of RF were incorporated into the basal in vitro medium, and cellulose digestion by rumen contents was determined with and without antibiotics. The inclusion of 30 or 60% RF in the medium had no effect on bacterial cellulose digestion in the control tubes (top portion of Table 5). In contrast, addition of RF drastically reduced cellulose digestion ($P < 0.01$) in those tubes containing antibiotics (fungal digestion). There were no differences in the extent of inhibition between the 30 and 60% levels of added RF. When RF was centrifuged at 17,500 × *g* for 20 min, addition of the supernatant equivalent to 70% RF in the medium decreased cellulose digestion compared to that in the zero RF control ($P < 0.05$). The precipitate was not different from the control ($P > 0.30$).

A similar set of experiments were run by using LRF at a level equivalent to 60% RF. These data are shown at the bottom of Table 5, and as with fresh RF, no change in cellulose digestion occurred in the control tubes, while a decrease was observed with added LRF ($P < 0.01$). Since LRF gave the same response as fresh RF, it was used in all further studies, because large amounts could be lyophilized at one time and used in several experiments and volume problems in medium preparation were avoided.

The inhibitory activity present in LRF was extracted with water and not inactivated by autoclaving (Table 6). An unexpected result in two of the three experiments was an increase in cellulose digestion over the levels of the controls in those antibiotic-containing tubes containing the water-extracted residue of LRF ($P < 0.05$). Further studies confirmed this fungal growth-promoting activity in the water-extracted residue of LRF and showed that it could not be extracted with water, even by autoclaving in water for 20 min at 121°C (Table 7).

To study the nature of the inhibitory factor(s) in RF, antibiotic-containing fermentations were conducted by using a soluble substrate (glucose) instead of cellulose. Depression of pH and visible observation of fungal growth suggested a lack of inhibition with the soluble substrate (data not shown). In a second set of experiments, with glucose utilization as a measure of activity, glucose disappearance in the control tubes was

TABLE 6. Water solubility of the inhibitory activity in LRF[a]

Addition to antibiotic-containing medium	Cellulose digestion (%) after incubation period[b,c]		
	Trial 1		Trial 2 (7 days)
	5 days	14 days	
None	31.2 ± 2.5A	37.8 ± 2.9B	41.0 ± 5.3B
LRF[d]	11.4 ± 1.3BC	3.2 ± 2.1DE	ND[e]
Water insoluble	43.8 ± 4.3E	45.2 ± 2.5B	58.6 ± 3.6E
Water soluble	2.6 ± 1.7F	3.0 ± 1.9E	13.7 ± 1.6F

[a] Each value is based on six fermentation tubes (three replicates in duplicate).
[b] Means in the same row without a common letter differ at $P < 0.01$ for C and D and means in the same column without a common letter differ at $P < 0.05$ for A, B, E, and F.
[c] Mean ± standard error.
[d] LRF added at a level equivalent to 100% rumen fluid in the medium.
[e] ND, not determined.

59.9% ± 2.3% compared to 61.3% ± 4.2% with the water extract of LRF added ($P > 0.76$). When the water extract of LRF was treated with two different proteolytic enzymes, there was no loss of inhibitory activity (Table 8). The activity of the enzymes was verified by determining their ability to hydrolyze casein and albumin.

DISCUSSION

The present results clearly demonstrate that growth of the rumen fungi is markedly inhibited in cocultures with rumen bacteria (Tables 1 and 2). However, if antibiotics are added to the fermentation to inhibit bacterial growth, the fungi will proliferate and digest purified cellulose as well as the cellulose from ground alfalfa. Both the rate of growth and rate and extent of cellulose digestion are lower for the fungi. Babel (6) suggested the term antibiosis for this type of relationship, which he described as "an antagonistic association between two microorganisms to the detriment of one of them." This would appear to correctly describe the relationship observed between the bacteria and fungi in this study.

Possible factors which might contribute to this antibiosis are that rapid bacterial growth decreases pH, which in turn inhibits flagellate growth and germination (25, 42, 46); there is not enough soluble energy in the medium for encystment and germination of the zoospores (46); or the bacteria produce an inhibitory factor or factors. The results of this study appear to eliminate low pH and lack of soluble substrates as potential causes of the inhibition observed between rumen bacteria and fungi and support the explanation of inhibitor production. In addition to some preliminary studies by Akin and Windham (4), which suggested that rumen bacteria could inhibit fungal growth and activity, investigators have noted that some species of fibrolytic rumen bacteria can strongly inhibit the fungi (47). Most, but not all, strains of *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Butyrivibrio fibrisolvens* will inhibit the fungi in coculture; however, this was also found to vary between species of fungi (8, 11, 28, 32, 47, 50). In contrast, the strongly cellulolytic species *F. succinogenes* has little if any effect on the fungi (8, 11, 28, 50). This difference between species and strains appears to rule out fermentation products as the source of the inhibition. Joblin and Naylor (31) tested the effects of bacterial fermentation products on cellulose digestion by *N. frontalis* and concluded that it was unlikely that they would cause the extensive inhibition occurring in the bacterial and fungal cocultures. The data shown in Table 4 also support this conclusion, since marked inhibition of cellulose digestion occurred in the tubes containing antibiotics, despite a very limited digestion in the 5- and 10-h initial fermentations.

TABLE 7. Fungal growth factor activity in the water-extracted residue of LRF

Addition to antibiotic-containing medium	Cellulose digestion (%)[a]
None	35.4 ± 4.7A
Water-extracted residue of LRF (WE-LRF)[b]	53.9 ± 5.5B
Residue of WE-LRF reextracted with heat[c]	46.6 ± 7.2B
Water extract from heat-extracted WE-LRF[d]	30.6 ± 3.0A

[a] Each value is based on eight fermentation tubes (four replicates in duplicate). Values are means ± standard errors. Means in the column without a common letter differ at $P < 0.05$.
[b] Added at a level equal to 100% RF in the medium.
[c] Autoclaved in water for 20 min at 121°C, and residue separated by centrifugation.
[d] Water extract obtained after autoclaving.

TABLE 8. Effect of proteolytic enzymes on inhibitory activity in the water extract of LRF

Addition to antibiotic-containing medium	Cellulose digestion (%)[a]
None	26.4 ± 2.9A
Water extract of LRF	7.3 ± 2.4B
Water extract of LRF plus protease[b]	10.7 ± 4.2B
Water extract of LRF plus trypsin	12.1 ± 4.1B

[a] Based on seven runs in triplicate (21 values). Values are means ± standard errors. Means in the column without a common letter differ at $P < 0.02$.
[b] Nonspecific protease from *S. griseus*.

Stewart et al. (52) observed an inhibition in cellulose digestion by *N. frontalis* RE1 when cell culture supernatants from *R. albus* or *R. flavefaciens* fermentations were added; however, growth of the fungus on glucose was not inhibited by addition of the supernatant. The inhibitory activity was destroyed by autoclaving at 121°C for 15 min, and based on gel permeation and anion-exchange chromatography, appeared to consist of several polypeptides. The authors postulated that the inhibitory factor(s) interfered with attachment of the fungi to the substrate. In a later study, Stewart (51) reported that the inhibitor was resistant to protease enzymes, but sensitive to periodate. Since periodate is known to split the bond between two hydroxylated carbon atoms, the author speculated that it may be affecting a lipoteichoic acid associated with the proteins.

Bernalier et al. (12) also detected an extracellular factor(s) in *R. flavefaciens* culture supernatants which inhibited the cellulolytic activity of *N. frontalis*. The factor(s) was destroyed at temperatures above 60°C and could be precipitated with ammonium sulfate at 40% saturation. Using anion-exchange chromatography, sequential precipitation, dialysis, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, two proteins were identified as being responsible for the inhibition. The inhibitory factor(s) did not appear to affect fungal growth, but instead affected the activity of the fungal cellulases.

In summary, antibiosis between rumen bacteria and fungi appears to be caused by a water-soluble, protease-resistant, heat-stable factor(s) produced by the bacteria in vitro or normally occurring in RF. The present results differ somewhat from those of several previous reports on the inhibitory activity produced by cultures of *R. flavefaciens*, which was destroyed by heat and appeared to be protein in nature (12, 51). However, in a later study, the inhibitory activity described by Stewart et

al. (52) was shown to be resistant to protease treatment. It is of interest that in our study as well as those with the pure cultures, the inhibition does not seem to affect fungal growth, but rather digestion of the insoluble cellulose substrate. Interference with either the encystment of zoospores or initial thallus development and inhibition of fungal cellulases are all possible modes of action. The present results do pose a very puzzling question: if RF contains an inhibitory factor for the fungi, why are they always present in vivo? Their numbers may vary, but they occur in almost all animals and across all types of diets. Are they maintained by growth on soluble carbohydrates and contribute very little to cellulose digestion in the rumen? It is also of interest that RF appears to contain a factor which increases fungal cellulose digestion in vitro when antibiotics are included in the medium. Although further studies are obviously needed, the present information might suggest that in vivo, dilution, fluid turnover rate, or absorption decreases the concentration of the inhibitor(s), allowing limited growth of the fungi.

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