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Gas-Liquid Chromatography for Evaluating Polysaccharide Degradation by *Ruminococcus flavefaciens* C94 and *Bacteroides succinogenes* S85†

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Two predominant rumen cellulolytic bacteria, *Ruminococcus flavefaciens* C94 and *Bacteroides succinogenes* S85, were incubated with ground filter paper (Whatman no. 1), cattle manure fiber, wheat straw, Kentucky bluegrass, alfalfa, and corn silage as substrates. Analyses of the initial substrate and the recovered residue after 48 h of static incubation showed that *R. flavefaciens* C94 was quantitatively more effective than *B. succinogenes* S85 in degrading total dry matter (32.2% versus 16.1%). However, *B. succinogenes* S85 demonstrated a qualitative advantage in degrading the hemicellulose and hemicellulosic sugars of particular substrates. *R. flavefaciens* degraded a mean 29.7% of the cellulose and 35.6% of the hemicellulose in the various substrates, whereas *B. succinogenes* degraded a mean 17.9 and 31.6% of these fractions, respectively. Gas-liquid chromatography was an important aid in characterizing the polysaccharide-degrading capabilities of these rumen species.

Gas-liquid chromatography has been shown to be a useful technique for studying the chemical composition and structural integrity of plant cell wall polysaccharides (1). A logical application of this technique is in the study of microbial degradation of polysaccharides in the rumen, which has been done by Morris and Bacon (18), using the alditol acetate procedure of Sloneker (19). The technique could also be highly useful in characterizing the specificity of polysaccharide degradation by bacterial species in pure culture, as well as in studying the structural hindrances of polysaccharide complexes which limit the availability of polymeric subunits for fermentation (12).

Two of the more predominant species of cellulolytic bacteria in the rumen are *Ruminococcus flavefaciens* (5, 15) and *Bacteroides succinogenes* (3, 15). The type strain of *R. flavefaciens* (C94) was originally isolated from the rumen of a cow fed a grain mixture diet (5), whereas the type strain of *B. succinogenes* (S85) was isolated from the rumen of heifers fed an alfalfa silage diet (3). Both strains have been extensively studied by a number of investigators (7, 10, 11, 13, 14, 17, 18) with regard to their polysaccharide-degrading capabilities.

The objectives of this study were to characterize and compare the degradation of several natural polysaccharides by these rumen cellulolytic strains by using a modified gas-liquid chromatographic analytical procedure developed in our laboratory (8).

(An abstract of this study was presented at the Federation of American Societies of Experimental Biology Meeting, April 1979, Dallas, Tex.)

MATERIALS AND METHODS

Organisms and culture medium. *R. flavefaciens* C94 and *B. succinogenes* S85 were provided by M. P. Bryant, Department of Dairy Science, University of Illinois, Urbana. The strains were maintained on glucose-cellobiose-starch-rumen fluid (GCS-RF) agar slants (4) held at 4°C and were transferred at 30-day intervals. The basal medium used to evaluate the substrates is described in Table 1 and is essentially similar to the RUM10 medium used by Latham et al. (16) in their studies with *R. flavefaciens* and *B. succinogenes*. The anaerobic techniques of Hungate (15) as modified by Bryant (2) were used throughout the study.

Substrates. Six substrates (filter paper [Whatman no. 1], cattle manure fiber, wheat straw, Kentucky bluegrass, alfalfa, and corn silage) were selected to provide a wide variation of different polysaccharide complexes. Alfalfa, Kentucky bluegrass, and corn silage were selected because they are common feed forages, and wheat straw is a major agricultural crop residue. Manure fiber from cattle fed an all-corn silage diet was of interest because it had undergone previous degradation by passage through the animal. Filter paper was selected because it is a highly processed

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TABLE 1. *Composition of basal medium for substrate incubation*

Component	% in medium
Fiber substrate (wt/vol)[a]	0.2
Trypticase (BBL Microbiology Systems)	0.3
Yeast extract (BBL)	0.2
Mineral solution I[b]	7.5
Mineral solution II[c]	7.5
Volatile fatty acid solution[d]	0.3
$FeSO_4 \cdot 7H_2O$ (0.01%)	1.0
$CoCl_2 \cdot 6H_2O$ (0.01%)	1.0
Resazurin solution (0.1%)	0.1
Sodium carbonate (8%)[e]	2.0
Cysteine sulfide (2.5%)[e,f]	5.0
Distilled water	To 100 ml
Gas phase	CO_2

[a] Finely ground filter paper, manure fiber, wheat straw, Kentucky bluegrass, alfalfa, or corn silage.

[b] 0.6% K_2HPO_4.

[c] 0.6% KH_2PO_4, 1.2% $(NH_4)_2SO_4$, 1.2% NaCl, 0.25% $MgSO_4 \cdot 7H_2O$, and 0.16% $CaCl_2 \cdot H_2O$.

[d] 17.0 ml of acetic acid, 6.0 ml of propionic acid, 4.0 ml of *n*-butyric acid, 1.0 ml of isobutyric acid, 1.0 ml of DL-methylbutyric acid, 1.0 ml of *n*-valeric acid, 1.0 ml of isovaleric acid, and 1.0 g of phenylacetic acid.

[e] Aseptically added to medium after autoclaving and cooling.

[f] Prepared as described by Caldwell and Bryant (6).

polysaccharide often used as a standard substrate. All of the substrates were dried at 60°C, finely ground (0.5-mm screen) with a Wiley mill, and washed three times with distilled water. Fresh cattle manure fiber was washed with copious amounts of tap water through a coarse (1,190-μm pore size) screen (Tyler Standard Screen Scale, Cleveland, Ohio) to remove unwanted components and then processed as above. The thoroughly washed substrates were dried again at 60°C for 4 days and stored in glass bottles.

Incubation and analytical procedures. The six substrates were individually added to 300 ml of basal medium at 0.2% (wt/vol) in 500-ml round-bottom flasks and thoroughly moistened. The flasks were then brought to a rapid boil under oxygen-free CO_2, stoppered and wired down, and autoclaved at 15 lb/in^2 for 20 min. After cooling, the flasks were opened under oxygen-free CO_2, and presterilized 8% sodium carbonate and 2.5% cysteine sulfide were added. Each flask was then aseptically inoculated with 10 ml of a batch culture of *R. flavefaciens* C94 or *B. succinogenes* S85, grown for 24 h in GCS-RF medium (4) and with optical densities (600 nm) of 1.0.

A stirring plate and magnetic bar were used to keep the substrate particles in suspension, with periodic swirling by hand to prevent adherence to the flask wall. Incubation was for 48 h at 39°C.

At the end of the incubation, the flasks were opened, and the contents were centrifuged at 5,000 × *g* for 20 min. The supernatant was carefully pipetted off, and the pelleted residue was washed six times with about 50 ml of distilled water, with centrifugation at 5,000 × *g* between each washing. The washed residue was then quantitatively transferred to a preweighed sintered-glass crucible with distilled water, filtered, dried at 60°C for 48 h, and weighed for determination of dry-matter loss. The dried residues were then delignified with sodium chlorite as previously described (9), filtered through a sintered-glass crucible, and dried again at 60°C for 48 h. About 50 mg of the delignified residue (i.e., holocellulose) was then hydrolyzed with trifluoroacetic acid, and the free hemicellulose sugars were converted to their alditol acetate derivatives (8). The white residue that remained after hydrolysis by trifluoroacetic acid (i.e., cellulose) was recovered, dried at 60°C, and weighed. Unincubated substrate samples were processed in a similar manner.

Gas-liquid chromatography. The alditol acetate derivatives of the hemicellulosic sugars were analyzed with a Hewlett-Packard gas chromatograph (model 5840A), equipped with a hydrogen flame ionization detector, microprocessor, and autosampler. A stainless-steel column (120 by 0.3 cm) packed with 0.2% polyethylene glycol adipate, 0.2% polyethylene glycol succinate, and 0.4% silicone XF-1150 on Gas-Chrom P (100 to 200 mesh) was used. The analytic run was temperature programmed at 1°C increase per min between 135 and 200°C, with a 10-min hold at 135°C after sample injection. A helium carrier gas flow rate of 30 ml/min was employed, with injector temperature at 210°C, detector temperature at 250°C, and attenuation of ×32. Retention times of individual sugars were identified by injecting known derivatized sugar standards and quantified by using derivatized myo-inositol as an internal standard. Under the described conditions, the elution order of the derivatized sugars was: rhamnose, arabinose, xylose, mannose, galactose, glucose, and myo-inositol.

Calculations. Apparent dry-matter loss (percent) as a result of degradation was calculated as the difference between the initial dry weight of the substrate added minus the dry weight of the recovered residue, divided by the initial dry weight × 100. Percent cellulose loss was also calculated as the difference between the initial cellulose content of the substrate minus the cellulose content of the recovered residue, divided by the initial cellulose content × 100. Percent hemicellulose was determined by the summation of the hemicellulose sugars detected by gas-liquid chromatography, and its loss was calculated by difference. Similar calculations were made for the loss of the individual hemicellulosic sugars.

RESULTS

Composition of substrates. The cellulose, hemicellulose, hemicellulosic sugars, and lignin contents of the six substrates are shown in Table 2. Filter paper contained the highest amount of cellulose, followed in descending order by wheat straw, Kentucky bluegrass, cattle manure fiber, alfalfa, and corn silage. The mean cellulose content of the six substrates was 48.4%, with a range of 91.0 to 29.7%. In contrast, wheat straw contained the highest amount of hemicellulose, followed in descending order by Kentucky blue-

TABLE 2. *Cellulose, hemicellulose, hemicellulosic sugar, and lignin contents of substrates*[a]

Substrate	Cellulose	Hemicellulose	Hemicellulosic sugars[b]					Lignin[c]
			Glu	Gal	Man	Ara	Xyl	
Filter paper	91.04	8.52	3.95	1.22	0.00	0.00	3.35	—
Manure fiber	41.71	13.21	1.66	0.00	0.39	0.88	10.28	9.61
Wheat straw	49.27	22.87	3.02	0.64	0.00	2.71	16.50	12.36
Kentucky bluegrass	43.94	18.50	1.78	1.10	0.00	3.12	12.50	5.56
Alfalfa	34.86	13.07	5.26	0.97	0.65	1.24	4.95	6.21
Corn silage	29.69	9.79	3.44	1.67	0.53	0.15	4.00	4.18

[a] Values (percent of dry matter) were calculated by procedures described in the text.
[b] Glu, Glucose; Gal, galactose; Man, mannose, Ara, arabinose; Xyl, xylose.
[c] Lignin values were determined by the permanganate method of Van Soest and Wine (20).

grass, cattle manure fiber, alfalfa, corn silage, and filter paper. The mean hemicellulose content of the six substrates was 14.2%, with a range of 22.3 to 8.5%. Xylose was the predominant hemicellulosic sugar in almost all of the substrates and was particularly high in wheat straw, Kentucky bluegrass, and cattle manure fiber. Two exceptions were filter paper and alfalfa, in which glucose was slightly higher in content than xylose. Certain detectable sugars were notably absent in the hemicellulose of some of the substrates. Mannose was not detected in filter paper, wheat straw, and Kentucky bluegrass; galactose was not detected in cattle manure fiber; and arabinose was not detected in filter paper. Rhamnose, fucose, and ribose, which were also detectable by the gas-liquid chromatography procedure, were not found in the hemicellulose of any of the substrates. A comparison of the composition of corn silage with that of manure fiber obtained from cattle that were fed corn silage showed that both the cellulose and hemicellulose contents, as well as the xylose content of the manure fiber, were higher as a percentage of total dry matter (Table 2).

Dry-matter loss. Apparent dry-matter loss (percent) after degradation of the various substrates by *R. flavefaciens* C94 and *B. succinogenes* S85 is shown in Table 3. *R. flavefaciens* C94 was twice as effective as *B. succinogenes* S85 in degrading the six substrates (mean, 32.2% versus 16.1%). There was also a difference in the ranking of the ability of the two species to degrade the individual substrates. Corn silage was degraded most effectively by *R. flavefaciens*, followed in descending order by alfalfa, wheat straw, filter paper, Kentucky bluegrass, and cattle manure fiber. Corn silage was also the most highly degraded substrate with *B. succinogenes*, followed in descending order by alfalfa, Kentucky bluegrass, cattle manure fiber, wheat straw, and filter paper. A comparison of the corn silage and the cattle manure fiber from corn silage showed that *R. flavefaciens* and *B. succinogenes* were able to degrade only 14.4 and 8.6%, respectively, of the residual polysaccharide components found in manure fiber.

Dry-matter loss and lignin content. The relationship between apparent dry-matter loss (percent) and lignin content (percent) of the six substrates is shown in Fig. 1. With the exception of alfalfa, as lignin content increased in the substrates, there was a concomitant decrease in apparent dry-matter loss for *B. succinogenes*. However, with *R. flavefaciens*, alfalfa and wheat straw—although containing more lignin than Kentucky bluegrass or cattle manure fiber—showed a higher apparent dry-matter loss.

Cellulose and hemicellulose loss. The cellulose and hemicellulose loss (percent) from the six substrates is shown in Table 4. Mean cellulose degradation by *R. flavefaciens* was 29.7% for all substrates. The cellulose of corn silage and alfalfa underwent the most degradation by this strain. *B. succinogenes* was less effective, with a mean cellulose degradation of 17.4%. The cellulose of wheat straw and filter paper was particularly resistant to attack by *B. succinogenes*. Both species degraded hemicellulose to about the same extent on the mean of the six substrates, with *R. flavefaciens* being slightly more effective with cattle manure fiber, wheat straw, Kentucky bluegrass, and corn silage. *B. succinogenes*, however, was more effective than *R. flavefaciens* in degrading the hemicellulose of filter paper and alfalfa.

Hemicellulosic sugar loss. The degradation of the individual sugars in the hemicellulose fraction by the two rumen species is shown in Table 5. *R. flavefaciens*, in agreement with its more efficient degradation of total dry matter and the hemicellulose fraction of the substrates, demonstrated more versatility than *B. succinogenes* in attacking the hemicellulosic sugars of the substrates. Both glucose and galactose in the hemicellulose of Kentucky bluegrass, and the glucose in the hemicellulose of corn silage, were degraded by *R. flavefaciens*, but not by *B. succinogenes*. Neither species, however, appeared to degrade the glucose and galactose in the hem-

TABLE 3. *Degradation of substrate dry matter by strains C94 and S85*[a]

Substrate	*R. flavefaciens* C94				*B. succinogenes* S85			
	Input (mg)	Recovered (mg)	Degraded		Input (mg)	Recovered (mg)	Degraded	
			mg	%			mg	%
Filter paper	613	474	139	22.7	614	595	19	3.1
Manure fiber	613	525	88	14.4	605	552	52	8.6
Wheat straw	611	428	183	30.0	611	589	22	3.6
Kentucky bluegrass	606	474	132	21.8	611	522	89	14.6
Alfalfa	606	353	253	41.7	636	492	144	22.6
Corn silage	612	229	383	62.6	639	357	282	44.1
Mean	610	414	196	32.2	619	518	101	16.1
Standard error[b]	1.4	43.8	43.7	7.2	5.9	35.9	40.9	6.4

[a] Substrates were finely ground (0.5-mm screen), washed 3× with distilled water, and dried at 60°C for 4 days. Substrates were added at 0.2% (wt/vol) to basal medium (300 ml) before autoclaving. A 10-ml sample from batch cultures of the strains was aseptically inoculated into each flask and incubated for 48 h at 39°C.

[b] Standard error of the mean of six substrates.

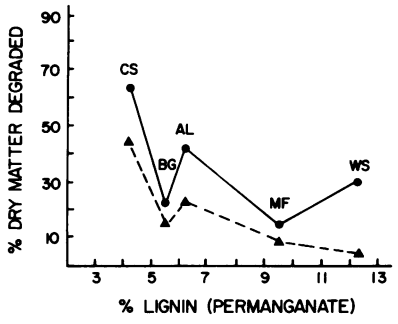

FIG. 1. *Relationship between dry matter (percent) degraded by strain C94 and strain S85 and the permanganate lignin content (percent) of substrates. Lignin values are shown in Table 2, and dry matter values are shown in Table 3. Symbols: R. flavefaciens (●); B. succinogenes (▲); corn silage (CS); Kentucky bluegrass (BG); alfalfa (AL); manure fiber (MF); wheat straw (WS).*

TABLE 4. *Degradation of cellulose and hemicellulose fractions of substrates by strains C94 and S85*[a]

Substrate	*R. flavefaciens* C94		*B. succinogenes* S85	
	Cellulose	Hemicellulose	Cellulose	Hemicellulose
Filter paper	21.5	16.5	9.9	47.0
Manure fiber	24.6	31.3	24.2	13.8
Wheat straw	29.4	48.7	5.1	20.8
Kentucky bluegrass	22.1	49.6	23.7	10.6
Alfalfa	35.3	40.9	20.2	72.5
Corn silage	45.2	26.5	21.3	25.0
Mean	29.7	35.6	17.4	31.6
Standard error[b]	3.8	5.4	3.2	9.7

[a] Incubation conditions are as described in Table 3, footnote *a*. Values are percent loss of cellulose and hemicellulose, calculated as the difference between the initial substrate and final residue content, divided by the initial substrate content × 100.

[b] Standard error of the mean of six substrates.

icellulose of wheat straw or the arabinose in the hemicellulose of corn silage. *B. succinogenes* was more effective than *R. flavefaciens* in degrading the hemicellulosic sugars of filter paper and alfalfa, particularly glucose and galactose. Of interest was the comparison between corn silage and the manure fiber from corn silage-fed cattle. Neither *R. flavefaciens* nor *B. succinogenes* could degrade the arabinose in the hemicellulose of corn silage, but *R. flavefaciens* degraded 26% of the arabinose in the hemicellulose of manure fiber, whereas *B. succinogenes* could not degrade it. Similarly, *B. succinogenes* could not degrade the glucose in the hemicellulose of corn silage, but degraded 67.5% of the glucose in the hemicellulose of cattle manure fiber. Mannose was completely degraded by both species in all of the substrates in which it was present.

DISCUSSION

The data in Table 2 indicate that there was considerable variation between the six substrates in terms of their cellulose, hemicellulose, hemicellulosic sugar, and lignin contents.

The observation that a substantial amount (8.5%) of hemicellulose was present in filter paper was of interest, since its presence is not often taken into account when this substrate is used as a standard. The distinct differences in the sugar proportions found in the hemicellulose of the substrates were clearly an indication of the heterogeneity associated with this fraction.

R. flavefaciens C94 was quantitatively more effective than *B. succinogenes* S85 in degrading all six of the substrates when compared on the basis of apparent dry-matter loss. Electron mi-

TABLE 5. *Degradation of hemicellulosic sugars by strains C94 and S85*[a]

Substrate	*R. flavefaciens* C94					B. succinogenes S85				
	Glu	Gal	Man	Ara	Xyl	Glu	Gal	Man	Ara	Xyl
Filter paper	0.0	28.0	—	—	86.2	42.3	100.0	—	—	26.5
Manure fiber	50.5	—	100.0	26.0	29.2	67.5	—	100.0	0.0	9.2
Wheat straw	0.0	0.0	—	100.0	97.5	0.0	0.0	—	66.6	48.2
Kentucky bluegrass	25.3	43.1	—	72.7	47.9	0.0	0.0	—	34.0	30.5
Alfalfa	33.1	0.0	100.0	95.3	53.5	75.4	64.9	100.0	100.0	60.3
Corn silage	28.6	46.4	100.0	0.0	14.7	0.0	79.9	100.0	0.0	13.8

[a] Incubation conditions are as described in Table 3, footnote *a*. Values are percent loss of the hemicellulosic sugars, calculated as the difference between the initial substrate and final residue content of the individual sugars, divided by the initial substrate content × 100. Abbreviations as in Table 2, footnote *b*. —, No sugar found.

croscopy studies (17) suggest that comminution of plant tissue is an important prerequisite for attachment and subsequent degradation by *R. flavefaciens*. All of the substrates in this study were finely ground, which could have influenced the results in favor of this species. However, *B. succinogenes* demonstrated a qualitative advantage in degrading the hemicellulose and certain hemicellulosic sugars of filter paper and alfalfa, which *R. flavefaciens* did not degrade well despite the comminution of the substrates. In this context, the recent observations of Latham et al. (16, 17) that *R. flavefaciens* preferentially attaches to cut edges of epidermal cell walls whereas *B. succinogenes* attaches more often to cut edges of mesophyll cell walls are of relevance.

Our finding that *B. succinogenes* S85 did not degrade ground filter paper (Whatman no. 1) efficiently was unexpected, since Halliwell and Bryant (14) previously reported that strain S85 effectively degraded filter paper and also native cotton fiber. The data shown in Table 4 indicate that the small effect we observed with *B. succinogenes* was directed to the hemicellulose fraction of filter paper. Halliwell and Bryant (14) used longer incubation periods (5 to 7 days) and ball-milled the filter paper to a fine powder, whereas in this study the incubation period was 2 days, and the filter paper was ground through a 0.5-mm screen. Halliwell and Bryant (14) also showed that *R. flavefaciens* C94 degraded 72% of ball-milled filter paper (Whatman no. 1) after 5 days of incubation, whereas in this study we observed 23% degradation after 2 days of incubation.

Studies by Dehority (10) with these two rumen species showed that *R. flavefaciens* C94 was more effective than *B. succinogenes* S85 in degrading hemicellulose isolated from flax or corn hull. In this study, *R. flavefaciens* C94 was more effective in degrading the hemicellulose of three of the six substrates examined (i.e., cattle manure fiber, wheat straw, and Kentucky bluegrass). *B. succinogenes* S85, however, was clearly more effective in degrading the hemicellulose of filter paper and alfalfa (Table 4). The hemicellulose in corn silage, on the other hand, was degraded to about the same extent by either species. Research by Coen and Dehority (7) showed that *B. succinogenes* S85 was more effective in degrading intact alfalfa hemicellulose than *R. flavefaciens* B1a and B34b. In this context, it was of interest that the hemicellulose in filter paper and alfalfa contained a much smaller xylose-to-glucose ratio than the other substrates and was more effectively degraded by *B. succinogenes* S85. This suggests that the polysaccharide complex associated with this particular sugar ratio was more amenable to degradation. *B. succinogenes* degraded the glucose in the hemicellulose of filter paper and alfalfa more effectively than *R. flavefaciens* did (Table 5). Morris and Bacon (18) previously suggested the possible importance of the xylose-to-glucose ratio in the digestibility of grasses in the rumen.

With all six of the substrates examined, there was no indication that cellulose or hemicellulose content was correlated to the total amount of dry matter degraded by either species.

The pattern of sugar losses from the hemicellulose fraction (Table 5) appeared to be a reflection of the degree of structural hindrance encountered by the two rumen species in degrading the various substrates. This was suggested by instances in which a particular hemicellulosic sugar was not degraded in a substrate by a species, but the same sugar was degraded in the other substrates. For example, *R. flavefaciens* did not degrade the glucose in wheat straw, but did degrade the glucose in alfalfa and corn silage. Similarly, *B. succinogenes* did not degrade the glucose in Kentucky bluegrass and corn silage, but did degrade the glucose in alfalfa. The pattern of sugar losses also suggested that the two rumen species differed in their manner of attack on the hemicellulose of the substrates. *R. flavefaciens* degraded the glucose in Kentucky bluegrass and corn silage, but *B. succinogenes* did not. The loss in hemicellulosic sugars, however, may not necessarily have resulted in their actual fermentation, since only a few sugars are known to be fermented by either species. *R. flavefa-*

ciens C94 does not ferment glucose, arabinose, and xylose, but will ferment cellobiose, and presumably xylobiose via xylan (5). *B. succinogenes* S85 does not ferment arabinose, galactose, or xylose, but will ferment glucose and cellobiose (3). Neither species will ferment pectin (11), which is associated with the hemicellulose fraction.

The relationship between the lignin content and the apparent dry-matter loss of the six substrates (Fig. 1) suggested that lignin content was the major but probably not the only factor influencing dry-matter degradation by these species. Both alfalfa and wheat straw contained higher lignin contents than either Kentucky bluegrass or cattle manure fiber, respectively, yet *R. flavefaciens* was more effective in degrading the total dry matter of these substrates. *B. succinogenes* showed a similar effect when comparing alfalfa and Kentucky bluegrass, but not when comparing wheat straw and manure fiber. Morris and Bacon (18) demonstrated that acetylated groups in the cell walls of grasses have an inhibitory effect on ruminal degradation.

The data show that employing the gas-liquid chromatography technique was of definitive help in evaluating the polysaccharide-degrading capabilities of the two rumen cellulolytic species. Continued studies of this nature could provide a better understanding of the complexity of polysaccharide digestion in the rumen ecosystem and its relationship to the overall fermentation process.

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