Influence of Forage Phenolics on Ruminal Fibrolytic Bacteria and In Vitro Fiber Degradation

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In vitro cultures of ruminal microorganisms were used to determine the effect of cinnamic acid and vanillin on the digestibility of cellulose and xylan. Cinnamic acid and vanillin depressed in vitro dry matter disappearance of cellulose 14 and 49%, respectively, when rumen fluid was the inoculum. The number of viable *Bacteroides succinogenes* cells, the predominant cellulolytic organism, was threefold higher for fermentations which contained vanillin than for control fermentations. When xylan replaced cellulose as the substrate, a 14% decrease in the digestibility of xylan was observed with vanillin added; however, the number of viable xylanolytic bacteria cultured from the batch fermentation was 10-fold greater than that of control fermentations. The doubling time of *B. succinogenes* was increased from 2.32 to 2.58 h when vanillin was added to cellobiose medium, and absorbance was one-half that of controls after 18 h. The growth rate of *Ruminococcus albus* and *Ruminococcus flavefaciens* was inhibited more by *p*-coumaric acid than by vanillin, although no reduction of final absorbance was observed in their growth cycles. Vanillin, and to a lesser extent cinnamic acid, appeared to prevent the attachment of *B. succinogenes* cells to cellulose particles, but did not affect dissociation of cells from the particles. *B. succinogenes*, *R. albus*, *R. flavefaciens*, and *Butyrivibrio fibrisolvens* all modified the parent monomers cinnamic acid, *p*-coumaric acid, ferulic acid, and vanillin, with *B. fibrisolvens* causing the most extensive modification. These results suggest that phenolic monomers can inhibit digestibility of cellulose and xylan, possibly by influencing attachment of the fibrolytic microorganisms to fiber particles. The reduced bacterial attachment to structural carbohydrates in the presence of vanillin may generate more free-floating fibrolytic organisms, thus giving a deceptively higher viable count.

Various studies suggest that phenolic compounds of plant cell walls inhibit digestion of forages in the rumen (10–12). Phenolic acids are toxic to rumen bacteria and protozoa (3, 6). Cellulolytic rumen bacteria are intimately associated with plant material undergoing degradation, and such organisms might encounter concentrations of phenolic acids higher than those found in rumen fluid. Nevertheless, Chesson et al. (6) showed that rumen cellulolytic strains in pure cultures are no more tolerant to added phenolic acids than rumen bacteria not normally found associated with plant particles. Hydrogenation of the more toxic phenolic acids to a less toxic form may be one mechanism of defense for organisms active in fiber degradation. Further degradation of the hydrogenated phenolic acids may then occur.

Few studies have been conducted to determine the influence of phenolic compounds on forage digestibility and the toxic effects they may exert on rumen microorganisms. Studies in our laboratories indicated that certain forage phenolic compounds routinely inhibit in vitro dry matter disappearance (IVDMD) of cellulose (10, 11). The objectives of our study were to examine the effect of two phenolic compounds, cinnamic acid and vanillin, on the population of cellulolytic and xylanolytic bacteria involved in batch fermentations. Growth rates of rumen cellulolytic bacteria in the presence of various phenolic compounds were also examined. The two phenolic compounds depressed the IVDMD of cellulose by 14 and 49%, respectively; however, the total number of *Bacteroides succinogenes* cells was threefold greater for fermentations which contained vanillin.

MATERIALS AND METHODS

Bacteria. *B. succinogenes* S85 and *Butyrivibrio fibrisolvens* 49 were a gift from the Microbiology Division, Dairy Science Department, University of Illinois, Urbana. *Ruminococcus flavefaciens* C94 and *Ruminococcus albus* 7 were gifts from I. M. Robinson, National Animal Disease Center, Ames, Iowa. The purity of the cultures was checked by examining colonies on roll tubes and by microscopic examination of Gram-stained smears.

Culture methods. The Hungate anaerobic culture method as described by Bryant (5) was used throughout the study. The composition of the cellulose and xylan agar roll tube medium was as follows (per 100 ml): clarified rumen fluid, 15 ml; mineral S2 (21), 5 ml; Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 0.2 g; yeast extract, 0.05 g; hemin, 0.000125 g; cellulose (Whatman no. 1 filter paper ball milled with flint pebbles for 18 h), 0.2 g, or xylan (larchwood; Sigma Chemical Co., St. Louis, Mo.), 0.24 g; Na$_2$CO$_3$, 0.4 g; cysteine hydrochloride and sodium sulfide, 0.0025 g each; resazurin, 0.0002 g; and purified agar (BBL), 0.5 g. The gas phase was 100% CO$_2$, and incubations were at 37°C. The composition of the IVDMD fermentation medium was as follows (per 30 ml): McDougall buffer (18), 24 ml; Solka-Floc or xylan, 0.5 g; and cinnamic acid or vanillin (Sigma), 30 mg. Rumen fluid (6 ml), collected from a fistulated steer fed an alfalfa-mixed grass diet and strained through four layers of cheesecloth, was added as a source of inoculum. Fifty-milliliter plastic centrifuge tubes with screw caps were used for the in vitro fermentations. They were inverted 10 times at 8 a.m. and 4 p.m. daily.

The in vitro fermentations were stopped by storing them at 0°C for up to 48 h or by centrifugation at 2,500 × *g* for 20

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TABLE 1. Effect of cinnamic acid and vanillin on cellulose fermentation by mixed rumen bacteria (after 48-h fermentations with phenolic compounds[a]

Fermentation medium[b]	IVDMD (%)	No. of cellulolytic bacteria (10^7/ml)[c]		VFA[d] concn (μmol/ml)			
		B. succinogenes	Others	Acet	Prop	Buty	Acet/Prop
Control	44.8*	3.03[§]	0.69	30.3[§]	13.1*	2.4*	2.33[§]
Cinnamic acid (0.1%)	38.6[†]	3.40[§]	0.68	27.1[§.‖]	10.2*	2.2[†]	2.66[§.‖]
Vanillin (0.1%)	22.8[‡]	9.48	0.56	21.7	5.0[†]	1.3[‡]	4.32[‖]
SEM	1.1	1.88	0.22	1.37	0.6	0.01	0.31

[a] Data are the means of five replicates from two experiments, with the exception of VFA data, which represent the means of two replicates from one experiment. Means in same column not showing a common superscript symbol differ. *, †, and ‡ indicate $P < 0.05$; § and ‖ indicate $P < 0.10$.
[b] Composition of IVDMD fermentation medium is given in the text. Control is without phenolic acids.
[c] Counted as zones of clearing in agar roll tube medium after 2 weeks of incubation. Others include all bacteria that formed a zone of clearing besides B. succinogenes.
[d] Acet, Acetate; Prop, propionate; Buty, butyrate.

min, and residual pellets were analyzed for cellulose by the procedure of Crampton and Maynard (7). Xylan was estimated by differences in dry weight before and after fermentation. Digestibility was calculated as the amount of xylan or cellulose that disappeared during the fermentation relative to initial concentration. These digestibility values were corrected for xylan or cellulose added with the inoculum. It is assumed that xylan is partially soluble; however, this only increases the significance of our digestibility value when differences were observed, based on a dry weight. Separate IVDMD tubes were used for determining the number of cellulolytic or xylanolytic bacteria. The contents of triplicate tubes were combined and blended under a stream of CO_2 with a Waring blender. One milliliter of this mixture was serially diluted in anaerobic buffer (4), and 10^{-5}, 10^{-6}, and 10^{-7} dilutions were used to inoculate three cellulose or xylan agar roll tubes from each dilution. Cellulose zones of clearing, with and without colonies, were counted after 10 to 14 days, and the xylan zones of clearing were counted after 24 h of incubation.

A procedure similar to that of Minato and Suto (19, 20) was used to qualitatively measure attachment of B. succinogenes to cellulose particles. B. succinogenes was cultured in cellobiose medium to mid-log phase. This culture was used to inoculate the following media: (i) cellulose broth medium alone (same as cellulose agar roll tube medium, except no agar was added and the cellulose concentration was increased to 0.8 g/100 ml) or (ii) with 0.2% vanillin, (iii) with 0.2% cinnamic acid, (iv) or without the addition of cellulose. The media were inoculated with a 50% volume from the cellulose medium, inverted 10×, incubated at 37°C for 15 min, and centrifuged (150 × g for 10 min). The culture supernatant was withdrawn, and the A_{600} was read. The change in A_{600} readings from the two controls (media i and iv) indicated that the phenolic compounds affected attachment.

Volatile fatty acid (VFA) analysis was performed on the supernatant from the in vitro cellulose fermentations. The supernatant was passed through a 0.22-μm-pore membrane filter (Millipore Corp.), acidified to pH 2.0 with H_3PO_4, and analyzed with a Hewlett-Packard model 5840A gas chromatograph as described previously (28). The roll tube medium without agar and with cellobiose (0.5%) replacing cellulose was used for the growth rate studies. The medium was inoculated with 0.05 ml of a late-log-phase culture, and the A_{600} was read every 2 h with a Bausch and Lomb Spectronic 88 spectrophotometer. Results are presented as the mean of triplicate tubes. Once the cultures reached stationary growth, they were centrifuged at 15,000 × g for 10

min, and the A_{300} of the supernatant was read to measure the amount of phenolic compound remaining. The absorbance of uninoculated fermentation broth media served as controls.

Statistics. Data were analyzed by least-squares analysis of variance and by Duncan's multiple range test (23).

RESULTS

The effect of cinnamic acid and vanillin on the cellulose fermentation by mixed rumen bacteria is shown in Table 1. After 48 h, cinnamic acid and vanillin depressed the IVDMD of cellulose by 14 and 49%, respectively. B. succinogenes, identified by zones of clearing without colony formation, Gram stain, cell morphology, and fermentation products of representative strains, was the predominant cellulolytic bacterium in all fermentation media. Although cellulose digestibility was reduced 49% with vanillin, the number of viable B. succinogenes cells was threefold greater in the fermentation containing vanillin than in the control fermentation. The number of other cellulolytic bacteria was similar between the three fermentation media. The concentration of VFA was lower in the fermentation containing vanillin than in the control. The acetate-to-propionate ratio was also greater for this fermentation, primarily because of the large decrease in the concentration of propionate.

Differences in the number of cellulolytic bacteria between the control and vanillin in vitro fermentations began at 24 h (Table 2). The differences were greatest at 36 h (1.83 × 10^7 versus 7.85 × 10^7, respectively) and then declined until none was seen at 96 h. Lysis of B. succinogenes cells may occur after 36 h in the vanillin medium (6).

When xylan replaced cellulose as the substrate in the IVDMD medium, a response similar to that of cellulose was

TABLE 2. Effect of incubation time on number of rumen cellulolytic bacteria cultured from in vitro fermentations containing phenolic compounds[a]

Incubation time (h)	No. of cellulolytic bacteria from fermentaiton media (10^7/ml)			
	Control	Cinnamic acid (0.1%)	Vanillin (0.1%)	SEM
0	0.35			
12	0.65	0.31	0.48	0.16
24	2.38*	2.35*	3.56[†]	0.11
36	1.83*	2.46*	7.85[†]	0.66
48	1.57*	1.68*	3.08[†]	0.14
96	2.83*	0.80[†]	2.22*	0.28

[a] Means of three replicates. Means in same column not showing a common superscript symbol differ at $P < 0.10$.

TABLE 3. Number of rumen xylanolytic bacteria and IVDMD of xylan after 48-h fermentations with phenolic compounds[a]

Fermentation medium[b]	IVDMD (%)	No. of xylanolytic bacteria cultured on roll tube media (10^7/ml)		
		Control	Cinnamic acid (0.2%)	Vanillin (0.2%)
Control	62.5*	4.83*	4.67	4.71[§]
Cinnamic acid (0.1%)	58.8[†]	4.42*	4.33	
Vanillin (0.1%)	53.7[‡]	46.75[†]		51.60[∥]
SEM	0.7	9.14	1.22	7.09

[a] Means of three replicates. Means in same column not showing a common superscript symbol differ: *, †, and ‡ indicate P 0.05; § and ∥ indicate P 0.10.
[b] Composition of IVDMD fermentation medium is given in the text.

observed (Table 3). For the control and fermentation containing vanillin, IVDMDS of 62.5 and 53.7%, respectively, were observed, giving a 14% decrease in xylan digestibility with vanillin present. This corresponded to a 10-fold increase of xylanolytic bacteria, 4.83×10^7 and 46.75×10^7/ml, respectively, in the control and vanillin fermentations. When the xylanolytic bacteria from the control fermentation were cultured on roll tube medium containing 0.2% vanillin, the viable count (4.71×10^7/ml) did not change compared with roll tube medium without vanillin (4.83×10^7/ml). When the xylanolytic bacteria from the fermentation containing vanillin were cultured on roll tube medium containing 0.2% vanillin, the count (51.6×10^7/ml) was much higher than the count from the control fermentation (4.71×10^7/ml). However, the count for the vanillin-cultured fermentation (51.6×10^7/ml) was not significantly greater than the count for the
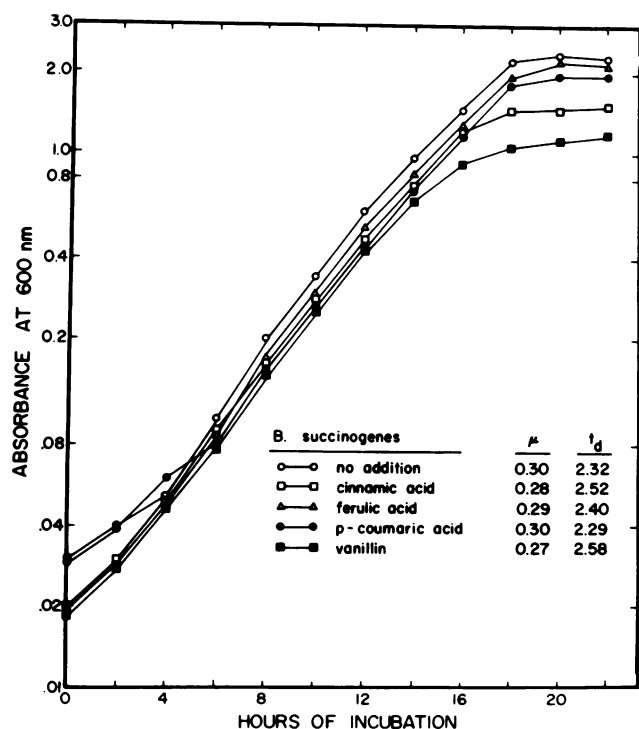

FIG. 1. Growth of *B. succinogenes* in the absence and presence of four phenolic compounds. Descending order of inhibition: vanillin > cinnamic acid > ferulic acid > *p*-coumaric acid.
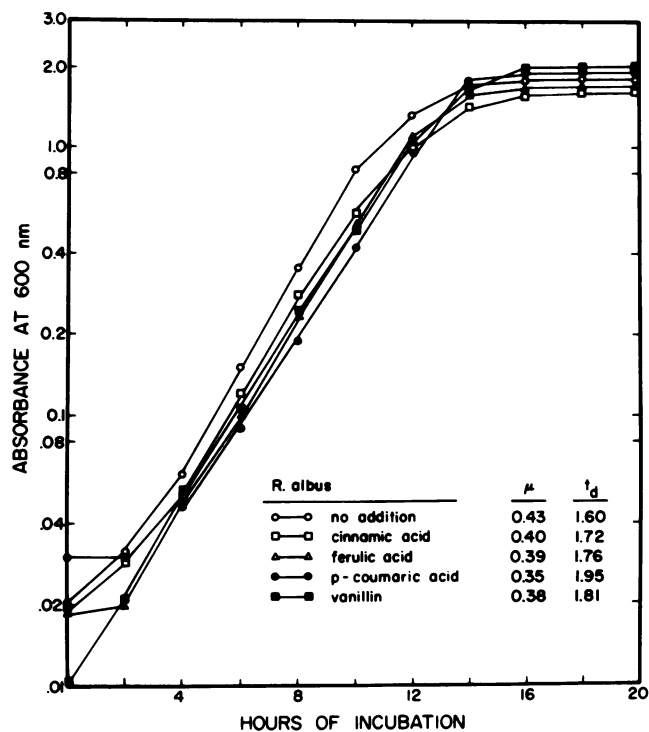

FIG. 2. Growth of *R. albus* in the absence and presence of four phenolic compounds. Descending order of inhibition: *p*-coumaric acid > vanillin > ferulic acid > cinnamic acid.

control roll tubes (46.75×10^7/ml). This suggested that vanillin in the fermentation medium exerted an effect on the xylanolytic bacteria, but vanillin did not have a subsequent effect on viability of xylanolytic organisms in roll tubes.

The effect of various phenolic compounds on the doubling time (t_d) and growth rate (μ) of *B. succinogenes* in cellobiose medium is shown in Fig. 1. In descending order of inhibition, with the t_d of the control at 2.32 h, increases in t_d were as follows: vanillin, 2.58 h; cinnamic acid, 2.52 h; ferulic acid, 2.40 h; *p*-coumaric acid had no effect on t_d. Absorbance readings at 18 h indicated that *B. succinogenes* in the presence of vanillin grew to only one-half of the cell mass of the control.

Figures 2 and 3 show the effect of these phenolic compounds on the growth of *R. albus* and *R. flavefaciens*, respectively. The descending order of inhibition by the phenolic compounds was the same for both ruminococci: *p*-coumaric acid, vanillin, ferulic acid, and cinnamic acid. In contrast to *B. succinogenes*, vanillin did not inhibit the final cell yield.

Data from three replicate experiments suggested that vanillin and cinnamic acid influence the attachment of *B. succinogenes* to filter paper (Table 4). Cultures were grown on cellobiose medium to mid-log phase, from which samples were removed and incubated with or without filter paper and in the presence of vanillin or cinnamic acid for 15 min. The cultures were centrifuged slowly to remove only the filter paper particles and the attached cells. The culture supernatants with vanillin had consistently higher absorbance readings than those with filter paper alone, indicating that vanillin inhibited attachment of *B. succinogenes* to the particles. Cinnamic acid produced a similar effect.
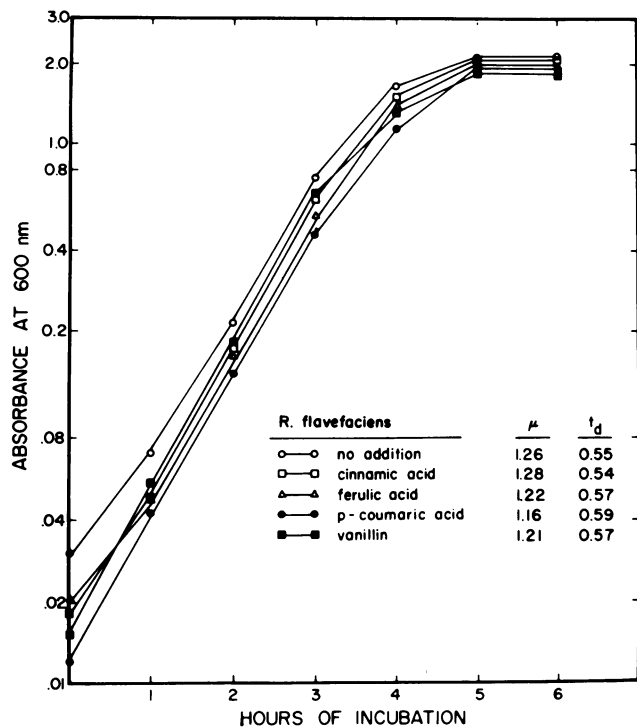
When vanillin was included in the in vitro fermentation

FIG. 3. Growth of *R. flavefaciens* in the absence and presence of four phenolic compounds. Descending order of inhibition: *p*-coumaric acid > vanillin > ferulic acid > cinnamic acid.

medium and serial dilutions were made in anaerobic buffer, the first dilution, but not the second, was immediately oxidized. It was thought that the brief oxidation may detach cellulolytic bacteria from fiber particles, thus explaining the higher count of cellulolytic bacteria in the medium containing vanillin. An experiment was done to determine if this was true. *B. succinogenes* was grown on cellulose broth medium for 48 h. The culture was diluted 1:1 without blending in anaerobic buffer containing 0.2% vanillin and in buffer free of vanillin. Each buffer was serially diluted two ways. The first dilution in one series was deliberately exposed to oxygen until the resazurin turned pink, while the following dilutions in the series were treated anaerobically. The other serial dilution was treated anaerobically throughout the dilution procedure. Four sets of cellobiose roll tubes were inoculated (vanillin buffer without O_2 exposure, vanillin

buffer with O_2 exposure, buffer without vanillin and without O_2 exposure, and buffer without vanillin but with O_2 exposure), and viable counts were determined. No differences were observed in the viable counts from the four sets of roll tubes, suggesting that brief O_2 exposure did not affect the viability or detachment of *B. succinogenes* from fiber particles. This also indicated that although vanillin may interfere with attachment of *B. succinogenes* to cellulose particles, vanillin did not enhance dissociation once the organisms were attached.

The data in Table 5 suggest that the rumen cellulolytics are able to modify phenolic monomers. *B. fibrisolvens* modified the original monomeric structures much more than did the other three cultures, with no more than 14% of any of the monomers remaining after the growth phase was completed. This may, in part, be due to the longer period of time required for *B. fibrisolvens* to reach stationary phase (40 h) compared with 6, 16, and 20 h for *R. flavefaciens*, *R. albus*, and *B. succinogenes*, respectively. Considering the means for all four monomers, *R. flavefaciens* was the second most active in modifying the monomers with roughly 50% remaining at stationary phase, followed by *B. succinogenes* with greater than 50% remaining, and finally *R. albus* with greater than 60% remaining in most cases.

DISCUSSION

It is difficult to correlate the decrease in the IVDMD of cellulose and xylan with the increase in the number of respective organisms that degrade these substrates when vanillin is present. We initially thought that vanillin-induced oxidation of anaerobic conditions might be involved during our serial dilutions and during initial set up of IVDMD fermentation medium. If vanillin initially oxidized the IVDMD fermentation medium, this may delay the initiation of cellulose or xylan hydrolyses and may explain the reduced IVDMD. However, this oxidation hypothesis does not explain the increase in the number of cellulolytic organisms which occurred when we deliberately exposed the IVDMD fermentation medium to oxygen and vanillin. After 24 h, the cellulolytic organisms in the fermentation medium containing vanillin outnumbered those in the control (Table 2). Although succinate, a product of *B. succinogenes* which is rapidly decarboxylated to propionate in the rumen (22), was not determined in our fatty acid analysis, the VFA data (Table 1), especially the lower propionate concentration in the fermentation medium containing vanillin, suggest that the metabolism of cellulose by *B. succinogenes* may be inhibited by vanillin. The lower cell densities and slower

TABLE 4. Effect of vanillin and cinnamic acid on attachment of *B. succinogenes* to ball-milled filter paper[a]

Replicate exp	A_{600} with treatment				
	Without filter paper	With filter paper	Filter paper + vanillin	Filter paper + cinnamic acid	SEM
1	0.39*	0.33[†]	0.38*	0.35*,[†]	0.01
2	0.57*	0.44[†]	0.51[‡]	0.49[‡]	0.02
3	0.69*	0.49[†]	0.55[‡]	0.53[‡]	0.02

[a] See text for details.
[b] Means in same column not showing a common superscript differ at $P < 0.05$.

TABLE 5. Phenolic compound remaining after incubation of pure cultures to stationary growth phase

Species	Phenolic compound remaining (%)[a]			
	Cinnamic acid	*p*-Coumaric acid	Ferulic acid	Vanillin
B. succinogenes	51.2*	58.8*	74.2*	46.2*
B. fibrisolvens	0.3[†]	4.3[†]	8.2[†]	13.9[†]
R. albus	66.8[‡]	63.0*	82.5*	58.4[‡]
R. flavefaciens	46.8*	54.8*	50.0[‡]	52.3*
SEM	7.1	7.2	8.4	5.2

[a] Means in same column not showing a common superscript symbol differ at $P < 0.05$.

doubling times of *B. succinogenes* when grown on a soluble substrate such as cellobiose in the presence of vanillin (Fig. 1) contradict the increased viable count when insoluble cellulose is the substrate. However, these data (Fig. 1) support the decreased IVDMD, i.e., fewer organisms, less activity, and thus less IVDMD. Chesson et al. (6) found that growth of various rumen bacteria in simple sugars medium is affected to a variable degree by different phenolic acids. Ferulic acid and *p*-coumaric acid were most toxic, but the growth of the cellulolytic organisms, *B. succinogenes*, *R. flavefaciens*, and *R. albus*, was retarded rather than suppressed at concentrations of these acids which might arise from the amounts ingested by the animal. However when vanillin concentrations were approximately 13 mM (0.2%), cell densities declined; Chesson et al. (6) also found that increasing concentrations of 1, 5, 10, and 20 mM decrease final cell densities.

Vanillin may interfere with attachment of *B. succinogenes* to cellulose (Table 4). Several studies suggest that *B. succinogenes* must attach to fiber particles to degrade the plant cell wall components (1, 16). Mandatory attachment is reasonable in that *B. succinogenes* is assumed to have a membrane-bound cellulase system (8); therefore, it must be in close proximity to its substrate for degradation to take place. Vanillin may in some way inhibit attachment and thus reduce cellulose degradation. This reduction in attachment due to vanillin may be one explanation for the increase in *B. succinogenes* counts when vanillin is present in the medium. When bacterial numbers were estimated by the roll tube method, the free-floating and attached bacteria were both counted. However, if bacteria remain attached to the cellulose particles during serial dilution, each cellulose particle will result in a clearing, which may be due to the activities of several individual cells. In contrast, each free-floating bacterium results in a separate clearing. However, the phenolic compounds may also modify the normal spatial existence, pairs, and short chains of *B. succinogenes*. Phenolic compounds could affect growth habit, septum formations, and cell divisions, resulting in increased free-floating viable counts.

Akin (3) found that 0.1% *p*-coumaric acid delays the growth of unidentified cellobiose-utilizing rumen bacteria initially; however, after 24 h, absorbance is the same. This acid also delays cellulose degradation by two to three times. Interestingly, his study showed that *p*-coumaric acid inhibits the growth of xylan-utilizing bacteria and also causes a reduction of about 50% of the colonies appearing on xylan roll tubes. This compares with a 10-fold increase that occurred with vanillin. This marked difference may be due to the different inocula used. Akin (3) used rumen fluid from cattle fed Bermuda grass, whereas we used rumen fluid from cattle fed an alfalfa-mixed grass diet.

Based on other studies (9, 24–26), it may seem somewhat unusual that we routinely found *B. succinogenes* to be the predominant cellulolytic organism in the IVDMD fermentation tubes. However, in past studies 2% agar was used in cellulose roll tube medium which appears to restrict movement of *B. succinogenes*, thus making it difficult to observe zones of clearing. Macy et al. (17) demonstrated that selective isolation of *B. succinogenes* from rat gastrointestinal tracts is not successful unless the purified agar concentration is lowered to 0.5%. We found this to hold true for isolation of *B. succinogenes* from the pig gastrointestinal tract (27).

No effort was made to identify the products formed from the phenolic monomers given in Table 5. In some cases a hydrogenation of the 2-propenoic side chain may occur (5).

Of interest was the extensive modification that *B. fibrisolvens* caused in relation to the other cellulolytic organisms. Chesson et al. (6) found that *Streptococcus bovis* and the cellulolytic bacteria, in particular the ruminococci, show at least a limited ability to modify ferulic and *p*-coumaric acids, which they found most toxic, by hydrogenation of the 2-propenoic side chain. The hydrogenated products are considerably less toxic to these organisms than the parent acid.

Akin (2) has shown that a rumen bacterial strain uses ferulic and sinapic acids under anaerobic conditions as the sole carbon and energy sources. However, growth is sparse and slow. It has not been reported that *B. succinogenes* can utilize phenolic acids for growth. Krumholz and Bryant (13) isolated a new bacterial species from the rumen that uses vanillin as an energy source for growth by converting it to butyrate, protocatechuic aldehyde, and protocatechuate. It can also catabolize ferulate and syringate. Other organisms that they recently isolated from the rumen include *Eubacterium oxidoreducens*, which is capable of anaerobic degradation of gallate, pyrogallol, phloroglucinol, and quercetin (14), and *Syntrophococcus sucromutans*, which demethoxylates various phenolic compounds (15).

It would be of interest to combine some of the strains that Krumholz and Bryant (13–15) have recently isolated with some of the cellulolytic organisms to determine if increased digestibility of forage cell walls occurs. Even though some of these new isolates are found in the rumen in low numbers ($<10^6$/ml of rumen fluid), they may be required only in catalytic numbers to degrade the phenolic compounds, thus making the cellulose and hemicellulose more accessible to the respective organisms that degrade these substrates.

LITERATURE CITED

1. **Akin, D. E.** 1980. Evaluation by electron microscopy and anaerobic culture of types of rumen bacteria associated with digestion of forage cell walls. Appl. Environ. Microbiol. **39:**242–252.
2. **Akin, D. E.** 1980. Attack on lignified grass cell walls by a facultatively anaerobic bacterium. Appl. Environ. Microbiol. **40:**809–820.
3. **Akin, D. E.** 1982. Forage cell wall degradation and *p*-coumaric, ferulic and sinapic acids. Agron. J. **74:**424–482.
4. **Betian, H. G., B. A. Linehan, M. P. Bryant, and L. V. Holdeman.** 1977. Isolation of cellulolytic *Bacteroides* sp. from human feces. Appl. Environ. Microbiol. **33:**1009–1010.
5. **Bryant, M. P.** 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. **25:**1324–1328.
6. **Chesson, A., C. S. Stewart, and R. J. Wallace.** 1982. Influence of plant phenolic acids on growth and cellulolytic activity of rumen bacteria. Appl. Environ. Microbiol. **44:**597–603.
7. **Crampton, E. W., and L. A. Maynard.** 1938. The relation of cellulose and lignin content to the nutritive value of feeds. J. Nutr. **15:**383–388.
8. **Forsberg, C. W., T. J. Beveridge, and A. Hellstrom.** 1981. Cellulase and xylanase release from *Bacteroides succinogenes* and its importance in the rumen environment. Appl. Environ. Microbiol. **42:**886–896.
9. **Hungate, R. E.** 1957. Microorganisms in the rumen of cattle fed a constant ration. Can. J. Microbiol. **3:**289–311.
10. **Jung, H. G.** 1985. Inhibition of structural carbohydrate fermentation by forage phenolics. J. Sci. Food Agric. **36:**74–80.
11. **Jung, H. G., and G. C. Fahey, Jr.** 1983. Interactions among

phenolic monomers and in vitro fermentations. J. Dairy Sci. **66:**1255–1263.

12. **Jung, H. G., and G. C. Fahey, Jr.** 1983. Nutritional implications of phenolic monomers and lignin: a review. J. Anim. Sci. **57:**206–219.

13. **Krumholz, L. R., and M. P. Bryant.** 1985. *Clostridium pfennigii* sp. nov. uses methoxyl groups of monobenzenoids and produces butyrate. Int. J. Syst. Bacteriol. **35:**454–456.

14. **Krumholz, L. R., and M. P. Bryant.** 1986. *Eubacterium* sp. nov. requiring H₂ or formate to degrade gallate, pyrogallol, phloroglucinol and quercetin. Arch. Microbiol. **144:**8–14.

15. **Krumholz, L. R., and M. P. Bryant.** 1986. *Syntrophococcus sucromutans* sp. nov. gen. uses carbohydrates as electron donors and formate, methoxymonobenzenoids or *Methanobrevibacter* as electron acceptor systems. Arch. Microbiol. **143:**313–318.

16. **Latham, M. J., B. E. Brooker, G. L. Pettipher, and P. J. Harris.** 1978. Adhesion of *Bacteroides succinogenes* in pure culture and in the presence of *Ruminococcus flavefaciens* to cell walls in leaves of perennial ryegrass (*Lolium perenne*). Appl. Environ. Microbiol. **35:**1166–1173.

17. **Macy, J. M., J. R. Farrand, and L. Montgomery.** 1982. Cellulolytic and non-cellulolytic bacteria in rat gastrointestinal tracts. Appl. Environ. Microbiol. **44:**1428–1434.

18. **McDougall, E. I.** 1948. Studies on ruminant saliva. I. The composition and output of sheep's saliva. Biochem. J. **42:**99–104.

19. **Minato, H., and T. Suto.** 1976. Techniques for fractionation of bacteria in rumen microbial ecosystem. I. Attachment of rumen bacteria to starch granules and elution of bacteria attached to them. J. Gen. Appl. Microbiol. **22:**259–276.

20. **Minato, H., and T. Suto.** 1981. Techniques for fractionation of

bacteria in rumen microbial ecosystem. IV. Attachment of rumen bacteria to cellulose powder and elution of bacteria to it. J. Gen. Appl. Microbiol. **27:**21–31.

21. **Salanitro, J. P., I. G. Fairchilds, and Y. D. Zgornicki.** 1974. Isolation, culture characteristics, and identification of anaerobic bacteria from the chicken cecum. Appl. Microbiol. **27:**678–687.

22. **Scheifinger, C. C., and M. J. Wolin.** 1973. Propionate formation from cellulose and soluble sugars by combined cultures of *Bacteroides succinogenes* and *Selenomonas ruminantium*. Appl. Microbiol. **26:**789–795.

23. **Steel, R. G. D., and J. H. Torrie.** 1960. Principles and procedures of statistics, p. 107–109. McGraw-Hill Book Co., New York.

24. **Stewart, C. S., C. Paniagua, D. Dinsdale, K.-J. Cheng, and S. H. Garrow.** 1981. Selective isolation and characteristics of *Bacteroides succinogenes* from the rumen of a cow. Appl. Environ. Microbiol. **41:**504–510.

25. **van Gylswyk, N. O.** 1970. The effect of supplementing a low protein hay on the cellulolytic bacteria in the rumen of sheep and on the digestibility of cellulose and hemicellulose. J. Agric. Sci. **74:**169–180.

26. **van Gylswyk, N. O., and C. E. G. Roche.** 1970. Characteristics of *Ruminococcus* and cellulolytic *Butyrivibrio* species from the rumens of sheep fed differently supplemented teff (*Eragrostis tef*) hay diets. J. Gen. Microbiol. **64:**11–17.

27. **Varel, V. H., S. J. Fryda, and I. M. Robinson.** 1984. Cellulolytic bacteria from pig large intestine. Appl. Environ. Microbiol. **47:**219–221.

28. **Varel, V. H., A. G. Hashimoto, and Y. R. Chen.** 1980. Effect of temperature and retention time on methane production from beef cattle waste. Appl. Environ. Microbiol. **40:**217–222.