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Influence of Plant Phenolic Acids on Growth and Cellulolytic Activity of Rumen Bacteria

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Isolated rumen bacteria were examined for growth and, where appropriate, for their ability to degrade cellulose in the presence of the hydroxycinnamic acids *trans-p*-coumaric acid and *trans*-ferulic acid and the hydroxybenzoic acids vanillic acid and 4-hydroxybenzoic acid. Ferulic and *p*-coumaric acids proved to be the most toxic of the acids examined and suppressed the growth of the cellulolytic strains *Ruminococcus albus, Ruminococcus flavefaciens*, and *Bacteroides succinogenes* when included in a simple sugars medium at concentrations of >5 mM. The extent of cellulose digestion by *R. flavefaciens* and *B. succinogenes* but not *R. albus* was also substantially reduced. Examination of rumen fluid from sheep maintained on dried grass containing 0.51% phenolic acids showed the presence of phloretic acid (0.1 mM) and 3-methoxyphloretic acid (trace) produced by hydrogenation of the 2-propenoic side chain of *p*-coumaric and ferulic acids, respectively. The parent acids were found in trace amounts only, although they represented the major phenolic acids ingested. Phloretic and 3-methoxyphloretic acids proved to be considerably less toxic than their parent acids. All of the cellulolytic strains (and *Streptococcus bovis*) showed at least a limited ability to hydrogenate hydroxycinnamic acids, with *Ruminococcus* spp. proving the most effective. No further modification of hydroxycinnamic acids was produced by the single strains of bacteria examined. However, a considerable shortfall in the recovery of added phenolic acids was noted in media inoculated with rumen fluid. It is suggested that hydrogenation may serve to protect cellulolytic strains from hydroxycinnamic acids.

Phenolic acids are common constituents of forage fed to ruminants, where they occur most frequently as hydroxycinnamic acids ester-linked to polysaccharide. Ferulic and *p*-coumaric acids, the major phenolic acids found in this form, may represent up to 2.5% by weight of the cell walls of temperate grasses (7, 11). Hydroxybenzoic acids are also detected in lesser amounts. Occasionally, phenolic acids may also be consumed in a soluble form if alkali-treated cereal straws are included in an animal's diet (14).

In view of the toxicity of phenolic acids toward many of the soil and other microorganisms which come into contact with decaying plant material (13, 21), we examined the effect of such acids on specific rumen bacteria. Since it is known that plant phenolic acids repress the production of glycan hydrolases by certain fungi (18, 19), special consideration was given to the effect of these acids on cellulose digestion. The cellulolytic rumen bacteria are intimately associated with plant material undergoing degradation in the rumen (2) and thus seem likely to encounter localized high concentrations of released phenolic acids.

Although the effect of phenolic acids on the rumen microflora has not been previously reported, the effect of rumen microorganisms on phenolic acids has received attention (12). Microorganisms present in sheep rumen liquor will extensively decarboxylate 4-hydroxybenzoic acid and will additionally demethoxylate 3-methoxy-4-hydroxybenzoic (vanillic) acid. Decarboxylation of 4-hydroxycinnamic acids, however, is slight, as side chains are rapidly hydrogenated to give 3-[4-hydroxyphenyl]propionic (phloretic) acid and its derivatives. These, Martin (12) suggests, are further modified by the rumen microflora and give rise to the 3-phenylpropionic acid found in rumen liquor.

MATERIALS AND METHODS

Bacteria. All bacteria were from the culture collection of the Rowett Research Institute, Bucksburn, Aberdeen, United Kingdom, except *Bacteroides succinogenes* S85, which was a gift from M. P. Bryant, University of Illinois, Urbana. The purity of cultures

was checked by microscopic examination of Gram-stained smears.

Culture methods. Anaerobic culture techniques (10) and a growth temperature of 39°C were used throughout. Stock cultures of bacteria intended for growth experiments in the simple sugars medium described below were maintained in Hobson medium 2 (9), except for *Megasphaera elsdenii* and *Veillonella alcalescens*, which were kept on a similar medium lacking glucose, cellobiose, and maltose. Two drops of 48-h cultures grown on the liquid form of medium 2 were used to inoculate 10 ml of the simple sugars medium. Cellulolytic cultures were maintained on MOD SD medium (16) containing 0.3% (wt/vol) cellobiose. It was found necessary to supplement this medium with 30% (vol/vol) clarified rumen fluid in the case of *Ruminococcus albus* SY3 to retain cellulolytic activity. An overnight culture (1 ml) was used to inoculate 10 ml of cellulose medium.

The simple sugars medium contained, per liter, 150 ml each of Hobson mineral solutions a and b (9); 5 ml of trace metals solution (4); 10 g of Casitone (Difco); 2.5 g of yeast extract; 2 g each of glucose, cellobiose, and maltose; 10 ml of 70% (wt/vol) sodium lactate; vitamins to the final concentrations used by Scott and Dehority (15); 1 mM each isobutyric, isovaleric, valeric, and DL-2-methylbutyric acids; 1 mg of hemin; 1 mg of resazurin; and, after boiling and flushing with CO_2, 4 g of $NaHCO_3$ and 0.5 g of cysteine hydrochloride. Agar (6 g liter^{-1}) was added to the *B. succinogenes* S85 culture medium. For the determination of cellulolytic activity, the basal medium was MOD SD (16), with the substitution of soluble sugars by a preweighed amount of cellulose (approximately 25 mg in 10 ml of medium). Cotton fibers, dewaxed by a modification of the method of Corbett (5), were used for *B. succinogenes* BL2 and *Ruminococcus flavefaciens* 007. *R. albus* SY3 was grown on HCl-swollen ground filter paper, since this strain does not produce appreciable losses of weight of cotton. The amount of cellulose remaining after incubation at 39°C for 7 to 10 days was estimated by the method of Updegraff (17).

Phenolic acids were added to both media to the required final concentrations, and the pH was adjusted to between 6.7 and 6.9 before the media were dispensed under CO_2 and sterilized by autoclaving in tubes sealed with butyl rubber septum stoppers at 121°C.

Extraction and identification of phenolic acids. Phenolic acids released from plant samples by the overnight action of 2 M NaOH were freed from plant debris by filtration. The aqueous extracts obtained were adjusted to pH 2.5 with concentrated HCl and extracted three times with 20 ml of diethyl ether each time. Suitable volumes of bacterial culture filtrates (1 to 5 ml) and rumen fluid (approximately 25 ml) from sheep maintained solely on dried grass (1 kg day^{-1}) or, for short periods, on alkali-treated barley straw (fed ad libitum) were similarly acidified and extracted. Ether was removed by evaporation, and the phenolic acids were dissolved in methanol containing 2 mg of malic acid per ml as an internal standard. Trimethylsilyl derivatives of phenolic acids were prepared (6) and were separated by gas-liquid chromatography as previously described (3). Saponification of rumen fluid did not influence the recovery of phenolic acids.

Individual phenolic acids (Table 1) were identified by comparison of their retention times and their mass spectra with those of reference compounds. 3-[3-Methoxyphenyl]propionic acid, 3-[4-hydroxy-3-methoxyphenyl]propionic acid, and 3-[4-hydroxy-3,5-dimethoxyphenyl]propionic acid were prepared by the catalytic (5% palladium on charcoal) reduction of 1 g each of their corresponding unsaturated acids (Table 1) in 200 ml of methanol with H_2 at room temperature overnight. Hydrogenation products were recovered in virtually theoretical yields. The remaining phenolic acids were purchased from Fluka AG.

RESULTS

Phenolic acid content of feeds and rumen liquor. An indication of the identities and amounts of phenolic acids likely to be encountered by rumen bacteria was obtained by the analysis of dried grass and barley straw feeds and of rumen liquor obtained from sheep maintained on these diets (Fig. 1). Phenolic acids comprised 0.51% of the dry weight of the dried grass feed; *trans-p*-coumaric acid (0.23%), *trans*-ferulic acid (0.25%), and vanillic acid (0.03%) were the only acids detected in greater than trace amounts. These acids were also found in untreated barley straw at 0.63, 0.56, and 0.03% of the dry weight of the straw, respectively. Less than 5% of the total phenolic acids initially present in straw were water soluble. After treatment with NaOH (10 g of NaOH per 100 g of straw applied as a spray), virtually all could be extracted with water alone. About 45% of bound phenolic acids were solubilized after the treatment of the straw with gaseous NH_3 (4.5 g of NH_3 per 100 g of straw).

Phenylpropionic acid was the major phenolic acid identified in rumen liquor from grass-fed sheep (Fig. 1) and the only phenolic acid detected in rumen liquor from sheep maintained on treated straw. In both grass- and straw-fed animals, it was detected at concentrations of 0.4 to 0.8 mM. As Fig. 1 shows, a number of other acids were present in the rumen liquor of grass-fed sheep, including phloretic acid (0.1 mM), 3-methoxyphloretic acid (trace amounts only), vanillic acid (0.03 mM), and hydroxyphenylacetic acid (0.05 mM). Ferulic and *p*-coumaric acids, the major phenolic acids ingested, were detected only in trace amounts in rumen liquor from grass-fed animals and were absent from rumen liquor from animals fed treated barley straw.

Tolerance of rumen bacteria to phenolic acids. The growth of rumen bacteria in the simple sugars medium was affected to a variable degree by the different phenolic acids added to the medium (Table 2). Ferulic and *p*-coumaric acids were most toxic, but only the growth of the cellulolytic organisms *B. succinogenes*, *R. albus*, and *R. flavefaciens* was influenced at the concentrations of these acids which might arise

TABLE 1. Cinnamic acids and hydroxycinnamic acids and their hydrogenation products

R^1	R^2	R^3	
H	H	H	cinnamic acid
H	OH	H	p-coumaric acid
OCH_3	H	H	3-methoxycinnamic acid
OCH_3	OH	H	ferulic acid
OCH_3	OH	OCH_3	sinapic acid
+2(H)			
H	H	H	3-phenylpropionic acid
H	OH	H	3-[4-hydroxyphenyl] propionic acid (Phloretic acid)
OCH_3	H	H	3-[3 methoxyphenyl] propionic acid
OCH_3	OH	H	3-[4-hydroxy-3-methoxyphenyl] propionic acid (3-methoxyphloretic acid)
OCH_3	OH	OCH_3	3-[4-hydroxy-3,5-dimethoxyphenyl] propionic acid (3,5-dimethoxyphloretic acid)

from the amounts ingested. Even then, growth was retarded rather than suppressed. Hydrogenated products of hydroxycinnamic acids were less toxic than their parent acids. All species examined were able to grow in the presence of 20 mM phloretic acid or 10 mM 3-methoxyphloretic acid (the highest concentration tested), and six of the nine could grow in a 50 mM solution of phloretic acid. The most tolerant of the species tested was *Streptococcus bovis*, which grew vigorously in all acids except 50 mM *p*-coumaric acid. This did not mean that the acids had no effect, however. In most cases, the cell density could be seen to decline at the higher concentrations of the more toxic acids. For example, the turbidity at 650 nm of the 5-day cultures of *S. bovis* containing *p*-coumaric acid was 1.87, 1.66, 1.49, 1.38, and 0.81 at concentrations of 0, 1, 5, 10, and 20 mM respectively. Microscopic examination showed that at the higher concentrations, the appearance of the cells changed as well. The bacteria were more gram variable, and some lysis and escape of cell contents were visible. Evidence of similar structural damage was also seen with *V. alcalescens* and *R. albus* at higher concentrations of phenolic acids, even with the relatively nontoxic vanillic acid.

Effect of phenolic acids on cellulolysis. Strains representing the three major species of bacteria responsible for cellulose degradation in sheep rumens were examined for activity in the presence of phenolic acids (Table 3). *B. succinogenes* BL2 was used in preference to strain S85 because, under the conditions tested, it displayed greater cellulolytic activity. Cellulolytic activity was unaffected when 1 mM phenolic acids were incorporated in the culture medium, but the activity of two strains, *R. flavefaciens* and *B. succinogenes*, was substantially reduced when 5 mM hydroxycinnamic acids or 4-hydroxybenzoic acid were included. *R. albus*, however, remained unaffected by any except the highest concentration of acid examined. Phloretic acid proved not to reduce cellulolysis except at the highest concentration tested, at which the activity of *B. succinogenes* alone was markedly reduced.

Effect of rumen bacteria on phenolic acids in

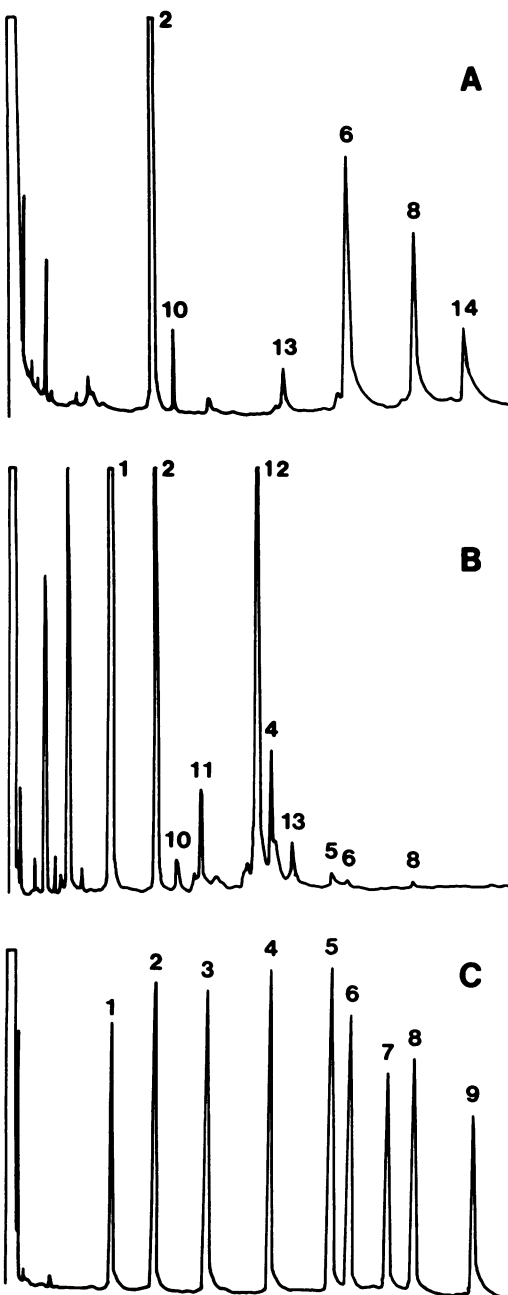

FIG. 1. Separation of trimethylsilyl derivatives of phenolic acids by gas-liquid chromatography. (A) Phenolic acids released from dried grass by NaOH. (B) Phenolic acids extracted from rumen liquor of grass-fed sheep. (C) Standard mixture. 1, 3-Phenylpropionic acid; 2, malic acid standard; 3, 3-[3-methoxyphenyl]-propionic acid; 4, phloretic acid; 5, 3-methoxyphloretic acid; 6, *trans-p*-coumaric acid; 7, 3,5-dimethoxyphloretic acid; 8, *trans*-ferulic acid; 9, *trans*-sinapic acid; 10, unknown; 11, hydroxyphenylacetic acid; 12, unknown; 13, vanillic acid; 14, unknown.

vitro. The benzoic acids 4-hydroxybenzoic acid and vanillic acid were unaffected by the bacterial strains examined. In every case, they were recovered at their initial concentrations from culture broths after incubation, and no evidence for decarboxylation or, in the case of vanillic acid, demethoxylation was found. The effect of a rumen fluid inoculum was not examined.

The effect of individual strains of bacteria on hydroxycinnamic acids was varied (Table 4). In simple sugars medium, *p*-coumaric and ferulic acids at an initial concentration of 1 mM were rapidly hydrogenated by the two strains of *Ruminococcus* and by *S. bovis* to form phloretic acid and 3-methoxyphloretic acid, respectively. *R. albus* and *R. flavefaciens* also displayed a similar but reduced response in cellulose medium. One of the two remaining cellulolytic strains, *Butyrivibrio fibrisolvens*, also showed a limited ability to bring about this conversion. With the exception of *S. bovis*, the non-cellulolytic bacteria did not modify the acids. *S. bovis* and the ruminococci were also able to convert sinapic acid to 3,5-dimethoxyphloretic acid. The ability to hydrogenate hydroxycinnamic acids was also related to the initial concentration of the acids incorporated into media. At 5 mM, only *S. bovis* and *R. flavefaciens* produced appreciable amounts of hydrogenated product. With the remaining strains, added acids were essentially recovered in full from culture broths. As was the case with the benzoic acids, no evidence of decarboxylation was apparent.

Inoculation of the simple sugars medium with rumen fluid also resulted in the rapid and complete hydrogenation of ferulic and *p*-coumaric acids when these were incorporated at a concentration of 1 mM. At 5 mM, however, although there was evidence of hydrogenation, there was a considerable shortfall in the recovery of phenolic acids. In cellulose medium, no phenolic acids could be recovered from medium inoculated with rumen fluid, although this was possible from uninoculated controls.

Phenylpropionic acid was detected in culture filtrates from both media after inoculation with isolated bacteria or rumen fluid in the absence of added phenolic acids. Inclusion of hydroxycinnamic acids in either medium did not increase the level at which 3-phenylpropionic acid was detected. Any conversion of hydroxycinnamic acid to 3-phenylpropionic acid would require, in addition to the hydrogenation of the C_3 side chain, removal of the 4-OH group and, in the case of ferulic and sinapic acids, demethoxylation. Possible intermediates in this conversion, cinnamic and 3-methoxycinnamic acids arising through dehydroxylation without prior hydrogenation and 3-[3-methoxyphenyl]propionic acid arising by dehydroxylation after hydrogenation,

were not detected in any culture broth examined or in extracts of rumen liquor from sheep fed grass or straw diets. Phloretic and 3-methoxyphloretic acids were also recovered from culture broths after inoculation and incubation at their initial concentrations. The only exception was cellulose medium inoculated with rumen fluid. In this case, although there was a shortfall in recovery, no corresponding increase in 3-phenylpropionic acid was detected.

TABLE 2. Tolerance of some rumen bacteria to phenolic acids in simple sugars medium[a]

Phenolic acid	Maximum acid concn at which growth was observed[b] (mM)																	
	Streptococcus bovis 26		*Megasphaera elsdenii* J1		*Selenomonas ruminantium* WPL 151/1		*Bacteroides ruminicola* 223/M2/7		*Veillonella alcalescens* 692		*Butyrivibrio fibrisolvens* B834		*Bacteroides succinogenes* S85		*Ruminococcus flavefaciens* 007		*Ruminococcus albus* SY3	
	1 day	5 days	1 day	5 days	1 day	5 days	1 day	5 days	1 day	5 days	1 day	5 days	1 day	5 days	1 day	5 days	1 day	5 days
p-Coumaric acid	20	20	10	10	10	20	5	10	5	5	10	10	5	5	1	5	5	5
Ferulic acid[c]	10	20	5	10	10	20	5	10	5	10	5	5	1	5	1	5	5	5
Phloretic acid	50	50	10	20	50	50	5	20	10	20	50	50	50	50	20	50	50	50
3-Methoxyphloretic acid[d]	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
3-Phenylpropionic acid	50	50	20	20	50	50	10	10	10	10	50	50	50	50	50	50	50	50
4-Hydroxybenzoic acid	50	50	50	50	50	50	10	50	20	50	50	50	20	20	50	50	50	50
Vanillic acid	50	50	20	50	20	50	10	20	10	50	10	10	5	20	20	20	20	20

[a] Cultures were examined for growth after 1 and 5 days.
[b] Concentrations tested were 1, 5, 10, 20, and 50 mM, except for ferulic acid, which did not dissolve completely at 50 mM.
[c] The highest concentration tested was 20 mM.
[d] The highest concentration tested was 10 mM.

DISCUSSION

The spatial organization of the rumen flora involves an adherent or "associated" flora found in close proximity to fragments of plant material. This associated population is generally thought to be rich in strains of the cellulolytic bacteria, *R. albus*, *R. flavefaciens*, *B. succinogenes*, and *B. fibrisolvens*, largely responsible for fiber degradation in the rumen (2). In this microenvironment, such organisms would be expected to encounter phenolic acids released during cell wall digestion at concentrations far higher than those suggested by the analysis of rumen liquor. Yet the cellulolytic strains, when examined in pure culture, were found to be no more tolerant of added phenolic acids than rumen bacteria not normally found associated with plant particles (Table 2). Either such organisms do not regularly encounter toxic levels of phenolic acids in the rumen, or there exists some mechanism for their protection.

The cellulolytic bacteria (and *S. bovis*) showed at least a limited ability to modify the more toxic ferulic and *p*-coumaric acids by hydrogenation of the 2-propenoic side chain, the products proving considerably less toxic to these organisms than the parent acid. The two *Ruminococcus* spp. were the most effective at bringing about the conversion, although their ability to do so was dependent on the initial concentration of acid introduced into the medium (Table 4). In the rumen, where the release of wall-bound total phenolic parallels the relatively slow rate of wall degradation (3), it seems unlikely that localized concentrations of phenolic acids would develop to a level at which hydrogenation reactions would be inhibited. The limited activity shown toward hydroxycinnamic acids by *B. succinogenes* and the weakly cellulolytic *B. fibrisolvens* seems unlikely to be a significant factor in the ecology of these organisms. However, both might benefit from the presence of *Ruminococcus*.

Although hydrogenation may provide an immediate defense for organisms active in fiber degradation, it does not account for the ultimate disappearance of hydroxycinnamic and phloretic acids from rumen liquor. Sheep fed dried grass at 1 kg day $^{-1}$ ingest sufficient phenolic

TABLE 3. Effect of phenolic acids on the cellulolytic activity of rumen bacteria[a]

Phenolic acid	Concn (mM)	% of cellulolytic activity retained		
		B. succinogenes BL2	*R. flavefaciens* 007	*R. albus* SY3
p-Coumaric acid	1	89	96	100
	5	87	71	83
	10	37	39	55
Ferulic acid	1	93	96	97
	5	58	47	84
	10	29	27	81
Phloretic acid	1	100	100	100
	5	100	100	100
	10	31	92	89
4-Hydroxybenzoic acid	1	96	100	100
	5	63	64	89
	10	62	53	83
Vanillic acid	1	100	100	100
	5	90	100	100
	10	89	100	100

[a] The amount of cellulose digested after 7 to 10 days of incubation at 39°C is expressed as a percent of that digested by control cultures without added acid under the same conditions. Values given are the averages of three replicated determinations made on two occasions.

acids to provide concentrations in rumen liquor of approximately 10 mM in the absence of any mechanism for their removal. Clearly, such a mechanism does exist, since neither hydroxycinnamic acids nor their hydrogenation products could be detected in greater than trace amounts. However, no evidence could be found to support the suggestion (12) that hydroxycinnamic acids are precursors of the 3-phenylpropionic acid found in rumen liquor. Inoculation of media containing *p*-coumaric or ferulic acids with single strains of bacteria or with rumen fluid did not

TABLE 4. Effect of rumen bacteria and a rumen fluid inoculum on the recovery of *p*-coumaric and ferulic acids from simple sugars medium and cellulose growth medium[a]

Medium and bacteria	Acids (mM) recovered							
	p-Coumaric acid				Ferulic acid			
	1 mM		5 mM		1 mM		5 mM	
	PCA	PA	PCA	PA	FA	MPA	FA	MPA
Simple sugars medium								
None	1.0	0	5.0	0	1.0	0	5.0	0
Streptococcus bovis 26	0	1.0	3.7	1.4	0	1.0	4.2	0.8
Megasphaera elsdenii J1	0.9	0.1	4.7	0.1	0.7	0	5.0	0
Selenomonas ruminantium WPL 151/1	0.9	0.1	4.9	0	0.8	0.2	4.7	0.2
Bacteroides ruminicola 223/M2/7	1.0	0	4.9	0	0.6	0.1	4.6	0.4
Veillonella alcalescens 692	1.0	0			0.9	0	5.0	0
Butyrivibrio fibrisolvens B834	0.8	0.2	4.3	0.3	0.8	0.2	4.8	0.2
Bacteroides succinogenes S85	0.9	0.1	4.8	0.2	0.5	0.5		
Ruminococcus flavefaciens 007	0	1.0	3.1	1.9	0	1.0	4.1	0.7
Ruminococcus albus SY3	0	1.0	4.8	0.2	0	0.9	4.0	0.9
Rumen fluid	0	1.0	0	0.4	0	1.0	0	3.8
Cellulose medium								
Bacteroides succinogenes BL2	0.9	0	1.73	0	0.8	0	3.2	0
Ruminococcus flavefaciens 007	0.2	0.8	4.6	0.3	0.8	0.1	3.0	1.9
Ruminococcus albus SY3	0.7	0.3	4.7	0.3	0.8	0.1	3.0	1.9
Rumen fluid	0.1	0	0	0	0	0	0	0

[a] Recovered: PCA, *p*-coumaric acid; PA, phloretic acid; FA, ferulic acid; MPA, 3-methoxyphloretic acid.

lead to elevated levels of 3-phenylpropionic acid, and, other than hydrogenation products, no other possible intermediate in this conversion was detected.

Phenolic acids may be lost from rumen fluid by nonspecific absorption to microbial surfaces or by specific uptake and utilization by some rumen microorganisms. Routes for the anaerobic breakdown of syringyl and guaiacyl structures are now well established (8, 20). A rumen bacterial strain able to utilize ferulic and sinapic (but not *p*-coumaric) acids under anaerobic conditions as the sole carbon and energy sources has also been described (1). However, growth on these substrates was sparse and slow and may have arisen solely at the expense of methoxyl side chains, leaving the aromatic ring intact. Because of thermodynamic considerations, fermentation of aromatic compounds in which the ring is cleaved is generally performed only by mixed cultures. In this investigation, shortfalls in the recovery of phenolic acids from culture filtrates were associated only with rumen fluid (mixed cultures) inocula and not with media inoculated with single strains of bacteria.

The experiments described here provide information about the concentrations of phenolic acids toxic to rumen bacteria (Table 2), but only in the case of cellulolysis (Table 3) were the effects of sublethal concentrations examined in detail. The delay in the detection of growth in some cultures (Table 2) may, for example, be indicative of a decrease in the specific growth rate of these bacteria. Similarly, detrimental effects on the final cell density and on the microscopic appearance of some species were observed. Further experimental work is required to define the precise nature of these effects.

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