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Degradation of Polysaccharides and Lignin by Ruminal Bacteria and Fungi

D. E. AKIN[1]* AND RONALD BENNER[2]†

Richard B. Russell Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture,[1] and Environmental Research Laboratory, U.S. Environmental Protection Agency,[2] Athens, Georgia 30613

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Bermudagrass (*Cynodon dactylon*) leaf blades and whole cordgrass (*Spartina alterniflora*) fiber were evaluated for degradation of cell walls by microbial groups in ruminal fluid. The groups were selected by the addition of antibiotics to the inoculum as follows: (i) whole ruminal fluid (WRF), no antibiotics; (ii) cycloheximide (C) to inhibit fungi, thus showing potential bacterial activity; (iii) streptomycin and penicillin (S,P) to inhibit fiber-degrading bacteria, showing potential fungal activity; (iv) streptomycin, penicillin, and chloramphenicol (S,P,CAM) to inhibit all bacteria including methanogens; (v) streptomycin, penicillin, and cycloheximide (S,P,C) to inhibit all microbial activity as a control; and (vi) autoclaved ruminal fluid (ARF) to inhibit all biological activity as a second control. Scanning electron microscopy of tissue degradation indicated that tissues not giving a positive histological reaction for lignin were more readily degraded. Cordgrass was more highly lignified, with more tissues resisting degradation than in bermudagrass. Patterns of degradation due to treatment resulted in three distinct groups of data based on the extent of fiber or component losses: WRF and C > S,P and S,P, CAM > S,P,C and ARF. Therefore, bacterial activity was responsible for most of the fiber loss. Fiber degradation by anaerobic fungi was significantly less ($P = 0.05$). Cupric oxide oxidation of undigested and digested bermudagrass fiber indicated that phenolic constitutents differed in their order of resistance to removal or solubilization. Vanillyl and syringyl components of lignin were the most resistant to decomposition, whereas ferulic acid was readily solubilized from fiber in the absence of microbial activity. Studies utilizing [^{14}C]lignocellulose from cordgrass indicated that little lignin was converted to CO_2 or CH_4 and that most losses represented solubilization from fiber. Ruminal fungi were less effective in converting lignin to gases and were less able to solubilize the lignin component compared with ruminal bacteria.

The use of antibiotics to select for the activity of single groups of microorganisms from mixed populations has provided useful information on the physiological ecology of complex systems (17, 49). Although it is clear that microorganisms synergistically interact, such as the relationship between the anaerobic fungus *Neocallimastix frontalis* and methanogenic bacteria (11) and that between *Treponema bryantii* and cellulolytic bacteria (37), the selection by antibiotics for a particular microbial group does allow for evaluation of the potential activities for species within the group. The addition of streptomycin and penicillin to ruminal fluid in vitro has been shown to increase fungal development on plant tissue and to prevent bacterial fiber degradation; the addition of cycloheximide eliminated fungal growth, allowing bacterial activities to be assessed (4, 49).

In the rumen, anaerobic bacteria, protozoa, and fungi have all been shown to contribute to fiber degradation (3). To date, we have not found protozoa that physically associate with or ingest fiber of warm-season leaf blades (7; unpublished data). In most systems, the bacteria appear to be the most dominant fiber-digesting group; *Ruminococcus albus*, *R. flavefaciens*, and *Bacteroides succinogens* are especially active in physically adhering to and degrading the more slowly digestible tissues in plants (18, 34). These bacteria possess endo- and exoglucanases and appear to have a full complement of enzymes for fiber degradation (22, 28, 43).

The contribution of the recently discovered ruminal fungi (41) to fiber digestion requires further attention. Microscopy studies (5, 9) indicated that the fungi are better able than the bacteria to colonize the lignocellulosic components of plants and that they could effectively degrade and weaken lignified tissues (6). However, their role in the degradation of lignin is not clear. Orpin (42) reported that an anaerobic fungus could solubilize up to 16% of the lignin from fiber. Studies (49) with antibiotics to select for the activity of fungal populations demonstrated no substantial loss of lignin (72% H_2SO_4 method [27]). Despite their colonization and attack on lignified tissue, mixed populations of ruminal fungi were not significantly stimulated by free phenolic compounds at the 1 mM level and were inhibited by phenolic compounds at the 10 mM level in in vitro studies (6).

In the present study, the roles of bacteria and fungi in the degradation of lignocellulosic plant tissues in the rumen are investigated. Antibiotics are used to select for bacterial or fungal activities, and a variety of methods are used to determine morphological and chemical changes in plant tissues due to specific microbial groups. Scanning electron microscopy (SEM) was used to determine morphological changes in plant tissues and to characterize the sites of attachment of specific microbial groups. Specifically radiolabeled [^{14}C]lignocellulose preparations were used to monitor both the solubilization and the mineralization to gaseous end products of the polysaccharide and lignin components of plant tissues. Chemical oxidations of plant tissues with CuO and capillary gas chromatography were used to characterize changes in the phenolic composition of lignin.

MATERIALS AND METHODS

Preparation of bermudagrass and cordgrass substrates for in vitro degradation. Coastal bermudagrass (*Cynodon dacty-*

* Corresponding author.
† Present address: The University of Texas at Austin, Marine Science Institute, Port Aransas, TX 78373.

lon (L.) Pers) was grown in well-managed plots near Athens, Ga. Plant regrowth was harvested at 6 or 9 weeks of age and maintained at −10°C until studies were done. Leaf blade sections 1 cm long were cut and used as substrate for dry weight loss studies. For SEM, leaf blade sections were cut 3 mm long. Prereduced, anaerobically sterile media were prepared in Hungate tubes containing 15 1-cm-long or 10 3-mm-long leaf sections per tube as previously described (4) with the basal medium of Caldwell and Bryant (20).

Fresh samples (20 cm high) of smooth cordgrass (*Spartina alterniflora*) were collected from Sapelo Island, Ga., and stored at −10°C until used. For microscopic evaluation of tissue degradation by microbes, 3-mm sections from halfway up the stem (including stem plus rolled leaf sheaths) in each of three plants and the associated leaf blades were excised and placed into 50-ml centrifuge tubes (six sections per tube per treatment) for inoculation with ruminal fluid. For in vitro digestibility of fiber, whole plants from the sample used to supply sections for microscopy studies were freeze-dried and extracted with neutral detergent solution (27) to remove soluble plant components. The fiber was ground to pass a 20-mesh screen and then subjected (400 mg) to in vitro dry matter determination (see below).

Preparation of radiolabeled lignocellulose. The procedure used for radiolabeling the lignin and polysaccharide components of cordgrass has been described previously (14). Living plant cuttings were incubated with sterile aqueous solutions containing either *trans*-[U-^{14}C]cinnamic acid to label lignin or D-[U-^{14}C]glucose to label polysaccharides. The [U-^{14}C]cinnamic acid was enzymatically produced from L-[U-^{14}C]phenylalanine by using a commercially available preparation (Sigma Chemical Co.) of potato-derived phenylalanine ammonia lyase (44). Unincorporated precursor and soluble components of the plant material were removed during Soxhlet extractions with 95% ethanol, benzene-ethanol (2:1), and water (14), leaving lignocellulosic fiber that was specifically radiolabeled either in the lignin or polysaccharide component. Specific activities of the radiolabeled lignocellulose preparations were determined by combusting weighed portions to carbon dioxide and water (OX 200 Biological Oxidizer; R. J. Harvey Instrument Co., Hillsdale, N.J.) and radioassaying the trapped $^{14}CO_2$. The specific activities of polysaccharide- and lignin-labeled lignocelluloses were 703 and 3,176 dpm/mg, respectively. Radioactivity was quantified in a Tracor BetaTrac 6895 liquid scintillation counter.

In vitro incubations with ruminal fluid. The ruminal digesta was removed through a cannula from a cow fed a diet of 40% sorghum (*Sorghum bicolor* (L.) Moench) silage plus 51% corn (*Zea mays* L.) grain and 9% soybean (*Glycine max* (L.) Merr) meal. The digesta was strained at the animal pen through cheesecloth into a 39°C vacuum bottle and transported to the laboratory, where it was again strained through cheesecloth. The following antibiotic treatments were prepared and added to tubes and bottles before inoculation with ruminal fluid to evaluate the potential activity of specific microbial groups: (i) WRF, whole ruminal fluid as a positive control to measure activity of all microbial groups; (ii) S,P, ruminal fluid plus 130 U of streptomycin plus 2,000 U of penicillin per ml of broth to inhibit fiber-digesting bacteria; (iii) S,P,CAM, S,P treatment plus 30 μg of chloramphenicol per ml of broth to inhibit methanogenic bacteria; (iv) C, ruminal fluid plus 0.5 mg of cycloheximide per ml of broth to inhibit fungi for evaluating potential bacterial degradation; (v) S,P,C, ruminal fluid plus streptomycin, penicillin, and cycloheximide as above to inhibit fiber-degrading bacteria and fungi as a negtive control; and (vi) autoclaved ruminal fluid (ARF) (121°C for 30 min), also as a negative control. Antibiotics were prepared such that 0.1 ml of solution was added per ml of broth to give the desired concentrations. In controls or treatments with only one antibiotic, water was added to keep the amount of additional liquids equivalent. Antibiotics were added to the incubation vessels before inoculating with ruminal fluid. For inoculation of Hungate tubes with intact bermudagrass leaf sections in 5 ml of medium, 0.4 ml of ruminal fluid was inoculated by syringe (25-guage needle) through the septa. Seven tubes per inoculum treatment were utilized for determination of dry matter digestibility. For microscopy, one tube containing 10 3-mm-long sections was inoculated per treatment. Tubes were incubated for 7 days at 39°C.

For inoculation of cordgrass, ruminal fluid was diluted 1:2 with McDougall carbonate buffer (39). This inoculum was then automatically dispensed (20 ml per bottle) under CO_2 into serum bottles with radiolabeled lignocellulose containing the antibiotic treatments listed above. Bottles were inoculated in triplicate for each treatment, flushed with CO_2, stoppered with butyl rubber stoppers and aluminum crimp seals, and incubated at 39°C for 7 days. For the ARF control, bottles which were inoculated with viable fluid were immediately autoclaved. Triplicate 50-ml centrifuge tubes for in vitro dry matter determination of ground cordgrass fiber and single tubes of intact 3-mm sections for microscopic study were inoculated with 30 ml of the ruminal fluid-carbonate buffer inoculum for WRF and S,P treatments only, and the tubes were incubated at 39°C for 48 h.

Evaluation of dry weight loss. For determination of ash-free dry weight loss, 1-cm leaf blades of bermudagrass were washed twice with distilled water, freeze-dried overnight, and weighed after stabilization in a desiccator for 30 min. Portions of the plant material were combusted at 550°C for 2 h for determination of ash content. The residues from each treatment were then combined, and the fiber was evaluated for lignin-derived phenolic components after CuO combustion and analysis by gas chromatography (see below). Gravimetric fiber loss of neutral detergent fiber of cordgrass was calculated by the method of Tilley and Terry (48), and 3-mm sections of intact leaves and stems were examined by SEM.

Preparation for micoscopy. For SEM, sections 3 mm long were retrieved from the incubation vessels and fixed in 4% glutaraldehyde buffered at pH 7.4 with 0.1 M cacodylate buffer, postfixed for 4 h in 1.5% buffered OsO_4, dehydrated in an ethanol series, and critical point dried in liquid CO_2. Sections were then coated with Au-Pd alloy and observed in a scanning electron microscope.

For evaluation of sites and types of lignification within tissues, free-hand sections of cordgrass parts from sections similar to those used for in vitro digestion of intact tissues were stained for lignin with acid phloroglucinol or 2% chlorine-sulfite (35). The area within sections occupied by specific tissues was determined from free-hand sections evaluated with a digitizer associated with a light microscope.

Evaluation of [^{14}C]lignocellulose degradation. At the end of the incubation period the contents of the bottles were acidified (pH <2), and the headspace was flushed (120 ml/min for 4 min) through traps to remove carbon dioxide and volatile fatty acids and then into a combustion furnace to convert methane to carbon dioxide, which was subsequently trapped in a liquid scintillation medium (13, 15). Radioactivity in gaseous end products was quantified by liquid scintillation spectrometry. The acidified contents of the bottles were filtered (Whatman GF/C filters), and the radioactivity

in 1-ml portions was quantified by liquid scintillation spectrometry (15).

Lignin oxidation products determined in bermudagrass residue. Samples (25 mg) of fresh bermudagrass and residues from incubations with ruminal fluid were oxidized with alkaline cupric oxide in stainless steel bombs at 170°C for 3 h on a platform shaker to produce simple phenols from the lignin polymer (13, 31). After oxidation, the bombs were quickly cooled in an ice bath, and ethylvanillin was added to the contents of the bombs as an internal standard. The overall recovery of all lignin-derived phenols was estimated from the recovery of the internal standard. Samples were centrifuged and washed, and the supernatant was acidified to pH 1 with 6 N HCl. The acidified supernatant was thoroughly extracted with freshly distilled ethyl ether. The combined extracts were passed through an anhydrous Na_2SO_4 column and dried under a stream of nitrogen. Samples were dissolved in pyridine, and 100-μl portions of the samples were mixed with equal volumes of Sylon BFT derivitizing reagent (Supelco, Inc.) and heated to 60°C for 10 min. After cooling, the Me_3Si derivatives of the lignin-derived phenols were analyzed with a Hewlett-Packard 5890 gas chromatograph fitted with a 30-m by 0.25-mm (inner diameter) fused silica capillary column (SE-30; Supelco) and flame ionization detector (31).

Total lignin concentrations were estimated by using the yields of lignin-derived phenols recovered after CuO oxidation and several factors to correct for the production efficiency of individual phenols from the lignin polymer. Vanillyl phenols are produced from the lignin polymer with approximately 30% efficiency (38, 45), and the recovered yield of vanillyl phenols was therefore divided by 0.3. Syringyl phenols are produced from the lignin polymer with approximately 90% efficiency (45), and the recovered yield of syringyl phenols was therefore divided by 0.9. There is little information on the production efficiency of *p*-hydroxyl phenols from the lignin polymer, and we therefore assume 100% recovery. Likewise, we assume 100% recovery for *p*-coumaric and ferulic acids. We include the *p*-hydroxyl phenols in the estimation of total lignin content even though they have been detected among the CuO oxidation products of a variety of lignin-free organic materials (J. I. Hedges, Ph.D. thesis, University of Texas, Austin, 1975). Of the three *p*-hydroxyl phenols analyzed in the CuO reaction products, *p*-hydroxyacetophenone appears to be derived largely from lignin, whereas *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid can have nonlignin sources (J. I. Hedges, W. A. Clark, and G. L. Cowie, Limnol. Oceanogr., in press). The relative proportion of *p*-hydroxyacetophenone to total yield of *p*-hydroxyl phenols has been used to indicate a lignin source versus a nonlignin source of the *p*-hydroxyl phenols (24). In the bermudagrass samples we analyzed, all of the degraded grass tissues yielded a lower ratio of *p*-hydroxyacetophenone to total yield of *p*-hydroxyl phenols (0.15) than did fresh material (0.29). Inclusion of *p*-coumaric and ferulic acids in the calculation of lignin content is somewhat arbitrary, since an unknown fraction of these cinnamic acids may not be associated with core lignin (29, 30). The combined corrected yields of the lignin-derived phenols, excluding *p*-coumaric and ferulic acids, are multiplied by a factor of 1.25 to account for the carbon atoms (average loss of 2 out of 10 C atoms per phenol) that are cleaved from the side chain of lignin-derived phenols during CuO oxidation. The formula used for calculating total lignin concentrations from concentrations of lignin-derived phenols (milligrams of phenol per gram of ash-free dry weight) produced during CuO oxidation of plant material is % lignin = [1.25(SUM *P* + SUM *V*/0.3 + SUM *S*/0.9) + PCA + FA]/10, where SUM *P* is the sum of *p*-hydroxyl phenols (aldehyde plus ketone plus acid), SUM *V* is the sum of vanillyl phenols (aldehyde plus ketone plus acid), SUM *S* is the sum of syringyl phenols (aldehyde plus ketone plus acid), PCA is *p*-coumaric acid, and FA is ferulic acid. The value (6.4%) obtained for the concentration of lignin in bermudagrass with lignin oxidation products (Table 1) and the above formula is similar to values (5 to 7%) obtained for comparable samples of bermudagrass with the acid-detergent-lignin analysis of Goering and Van Soest (27).

Statistics. Means were evaluated in a two-way (treatment versus replication) analysis of variance by using SAS procedures (46). Means were tested by using the Scheffe multiple comparison procedure described by Kleinbaum and Kupper (36).

RESULTS

Microscopic evaluation of tissue degradation in bermudagrass. WRF caused extensive degradation and loss of leaf morphology (Fig. 1). The mesophyll and phloem were totally degraded, whereas the epidermis and parenchyma bundle sheath were partially removed. The sclerenchyma was partially present at the abaxial site of large vascular bundles, and xylem cells and the cuticle appeared totally intact. Little evidence of fungal colonization was seen with WRF. Similar patterns of tissue degradation were observed with the C treatment (not shown). Treatment with S,P resulted in a

TABLE 1. Lignin oxidation products from CuO oxidation of bermudagrass leaf tissue after incubation for 7 days with bovine ruminal fluid treated with various antibiotics

Treatment	Concn (mg of phenol/g of tissue)[a]										
	p-Hydroxyl phenols			Vanillyl phenols			Syringyl phenols			Cinnamyl phenols	
	Ph	Po	Pa	Vh	Vo	Va	Sh	So	Sa	PCA	FA
Untreated	2.5	1.4	0.8	7.1	2.0	1.9	2.7	1.3	0.9	3.8	1.4
WRF	4.2	1.0	1.3	14.1	3.3	3.8	5.5	2.1	1.7	5.6	0.8
C	4.2	1.0	1.3	14.6	3.4	3.8	5.2	1.8	1.5	5.6	0.6
S,P	3.8	0.9	1.2	10.3	2.6	2.7	3.9	1.7	1.3	4.6	0.7
S,P,CAM	3.8	0.8	1.2	10.3	2.6	2.7	4.1	1.9	1.4	4.8	0.7
S,P,C	2.6	0.6	0.7	7.2	1.8	1.7	1.6	0.4	0.4	4.1	0.3
ARF	2.4	0.6	0.8	6.5	1.5	1.4	1.1	0.2	0.3	4.1	0.2

[a] Values from samples composited from seven tubes per treatment and evaluated on an ash-free dry weight basis. Abbreviations: Ph, *p*-hydroxybenzaldehyde; Po, *p*-hydroxyacetophenone; Pa, *p*-hydroxybenzoic acid; Vh, vanillin; Vo, acetovanillone; Va, vanillic acid; Sh, syringaldehyde; So, acetosyringone; Sa, syringic acid; PCA, *p*-coumaric acid; FA, ferulic acid.

mostly intact leaf blade with colonization by fungi in some regions only (Fig. 2). Where fungal colonization occurred, tissue loss was marked, but in uncolonized regions even the most fragile tissues (e.g., mesophyll) remained intact. These observations confirmed that the S,P treatment was effective in limiting bacterial degradation. Fungal colonization was prevalent at times on the lignified tissues, especially the sclerenchyma cells, but degradation was not extensive (Fig. 2, inset). The S,P,CAM treatment resulted in more fungal growth (sporangia and rhizomycelium) and slightly more tissue loss than did S,P treatment (Fig. 3); however, tissue loss was less than that with WRF or C. No bacteria were observed on plant cell walls with S,P or S,P,CAM. S,P,C treatment resulted in the presence of bacteria and a slight weakening of phloem (not shown). This treatment greatly reduced the degradation of plant fiber even though all bacterial growth was not prevented. Incubation with ARF resulted in no tissue loss (not shown).

Microbial degradation of bermudagrass lignin. The oxidation of lignin with alkaline cupric oxide produces as major reaction products *p*-hydroxyl, vanillyl, and syringyl phenols in the forms of aldehydes, ketones, and carboxylic acids (31, 32). The cinnamyl phenols *p*-coumaric acid and ferulic acid are also major products from the oxidation of lignin in herbaceous plant tissues, such as grasses (31, 32). The major oxidation products from bermudagrass lignin were vanillin, *p*-coumaric acid, syringaldehyde, and *p*-hydroxybenzaldehyde (Table 1). The various treatments used to evaluate the potential roles of bacteria and fungi in the degradation of plant tissues in the rumen resulted in three distinct groups of data corresponding to (i) WRF and C treatments, (ii) S,P and S,P,CAM treatments, and (iii) S,P,C and ARF treatments. Relative concentrations of individual phenols were highest in incubations with active microbial populations, since lignin is more resistant to degradation than other plant components such as polysaccharides (Table 1). Differences between the relative concentrations of lignin-derived phenols in untreated bermudagrass samples and samples exposed to ruminal fluid in the absence of microbial activity (S,P,C and ARF) indicated a marked loss, apparently due to solubilization, of *p*-hydroxyacetophenone, syringyl phenols, and ferulic acid.

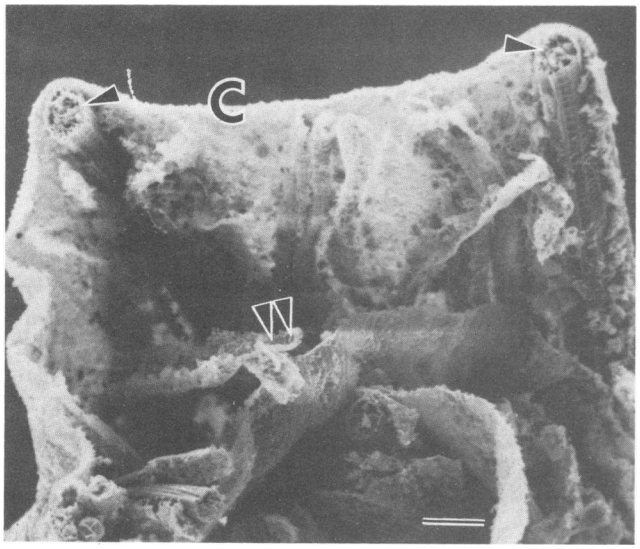

FIG. 1. SEM of bermudagrass leaf blade incubated for 7 days with WRF, showing a residue of sclerenchyma and vascular tissue (arrow), parenchyma bundle sheath (double arrows), and cuticle (C) with epidermal remnants. Bar, 100 μm.

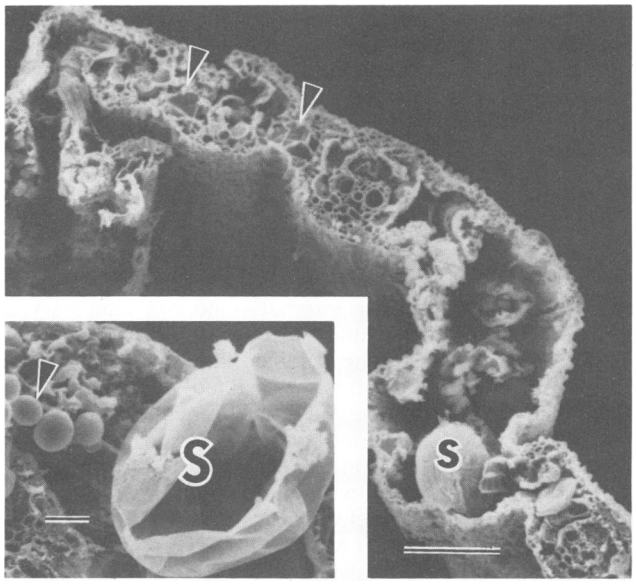

FIG. 2. SEM of bermudagrass leaf blade incubated for 7 days with S,P treatment. Fungal colonization results in loss of the more susceptible tissue such as mesophyll, whereas mesophyll (arrow) remains in other sites. A large sporangium (S) is located in the mesophyll area. Inset shows fungal colonization of lignified sclerenchyma, with a large, empty sporangium (S) and younger sporangia (arrow). Bar, 100 μm; inset bar, 10 μm.

Total weight losses from bermudagrass and weight losses from the individual families (aldehydes, ketones, and acids with similar patterns of methoxylation) of lignin-derived phenols are presented in Table 2. The greatest overall weight losses (about 80% on an ash-free dry weight basis) from bermudagrass occurred in the WRF and C treatments, indicating that bacterial activity was potentially sufficient to account for all of the observed degradation. Fungal activity alone, as indicated in the S,P and S,P,CAM treatments, resulted in significantly ($P = 0.05$) less weight loss (about 70% of ash-free dry weight). Weight losses (about 30% of ash-free dry weight) occurring in the absence of microbial activity (S,P,C and ARF treatments) were attributed to the loss of soluble components.

Total weight losses of lignin were high in all treatments and ranged from 36 to 66% (Table 2). Weight losses due to

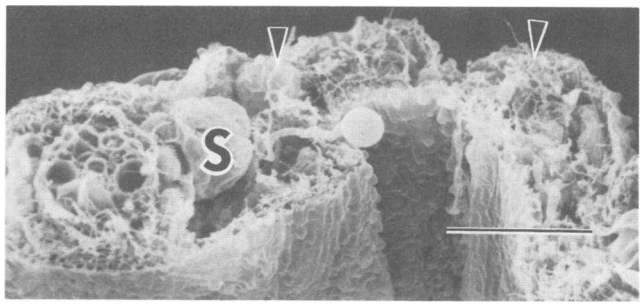

FIG. 3. SEM of bermudagrass leaf blade incubated for 7 days with S,P,CAM treatment. Fungal colonization as shown by sporangia (S) and rhizoids (arrows) is greater than that in Fig. 2, and tissue loss is slightly greater. Bar, 100 μm.

TABLE 2. Total weight loss and loss of individual components of lignin from bermudagrass leaf blades after incubation for 7 days with bovine ruminal fluid treated with various antibiotics

Treatment	% Ash-free dry weight loss[a]							% Lignin concn[b]
	Total	SUM *P*	SUM *V*	SUM *S*	PCA	FA	Lignin[b]	
Untreated								6.4
WRF	78.8	70.1	59.2	60.0	68.5	90.0	61.6	11.7
C	81.0	74.6	62.4	67.1	72.2	90.0	65.6	11.7
S,P	68.8	61.9	55.5	55.4	62.7	84.2	57.5	8.8
S,P,CAM	71.3	64.5	59.6	56.9	64.0	84.2	60.5	8.9
S,P,C	28.9	40.6	31.1	67.2	23.1	84.2	36.1	5.8
ARF	34.1	48.5	43.2	78.3	28.3	90.0	47.6	5.1

[a] Values from samples composited from seven tubes per treatment and evaluated on an ash-free dry weight basis. Abbreviations: SUM *P*, sum of *p*-hydroxyl phenols; SUM *V*, sum of vanillyl phenols; SUM *S*, sum of syringyl phenols; PCA, *p*-coumaric acid, FA, ferulic acid.

[b] Lignin percentages were calculated by using the yield of lignin-derived phenols recovered after CuO oxidation and the formula [1.25 (SUM *P* + SUM *V*/0.3 + SUM *S*/0.9) + PCA + FA]/10.

solubilization and the production of gaseous end products were not differentiated, but presumably (see results with cordgrass) most of the weight loss was from solubilization of lignin. There were significant weight losses of lignin in sterile controls (S,P,C and ARF), but weight losses of lignin in treatments with active microbial populations were much higher, indicating that microbial degradative processes were responsible for mediating approximately half of the total weight loss of lignin. The relative concentration of lignin increased in all incubations with active microbial populations, indicating that the lignin component was generally more resistant to degradation than other components of the plant material (Table 2). There was considerable variability in the relative susceptibilities of the various submolecular components of lignin to degradation. The phenolic constitutents were ranked in the following order of decreasing resistance to degradation-solubilization in treatments with active microbial populations: vanillyl = syringyl > *p*-hydroxyl = *p*-coumaric acid ⩾ ferulic acid. The order of decreasing resistance to solubilization in the sterile controls was markedly different: *p*-coumaric acid > vanillyl > *p*-hydroxyl ⩾ syringyl ⩾ ferulic acid.

Microscopic evaluation of tissue degradation in cordgrass. SEM examination of leaf blade digestion by WRF (Fig. 4) indicated that bacteria and fungi colonized the plant cell walls. Only phloem and mesophyll were substantially degraded by this inoculum. These tissues plus the vacuoles occupied about 48% of the leaf cross section, with the remaining tissues (i.e., epidermis, parenchyma bundle sheath, sclerenchyma, and xylem) resisting degradation for the most part. Mesophyll and phloem were not lignified, whereas the sclerenchyma and xylem showed definite reactions for lignin with both acid phloroglucinol and chlorine sulfite. The epidermis gave a weak reaction for lignin, whereas a reaction did not occur for the parenchyma bundle sheath even though this tissue was not degraded.

WRF degraded the mesophyll tissue of the leaf sheath but did not substantially degrade the epidermis, sclerenchyma, or xylem (Fig. 5). Sheath mesophyll did not react to lignin strains, whereas the other tissues (i.e., epidermis, sclerenchyma, and xylem) gave intense positive histological reactions to both lignin tests.

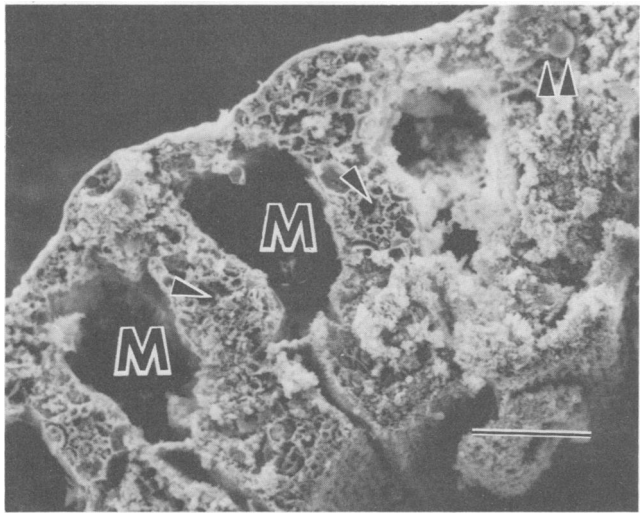

FIG. 4. SEM of cordgrass leaf blade incubated for 2 days with WRF showing loss only in mesophyll (M) and phloem (arrow) regions. Bacteria and fungi (double arrows) are present. Bar, 100 μm.

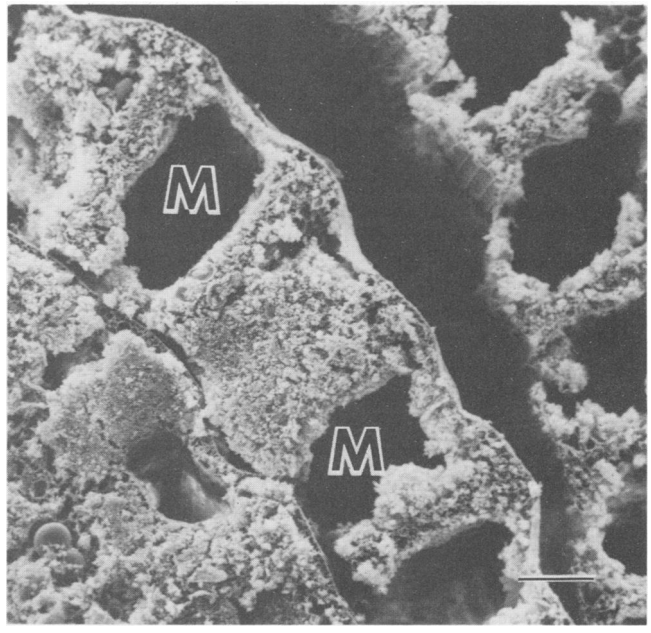

FIG. 5. SEM of cordgrass leaf stem and sheath surrounding the stem incubated for 2 days with WRF. The mesophyll (M) of the sheath is degraded whereas other tissues are intact in the sheath and the stem. Bar, 100 μm.

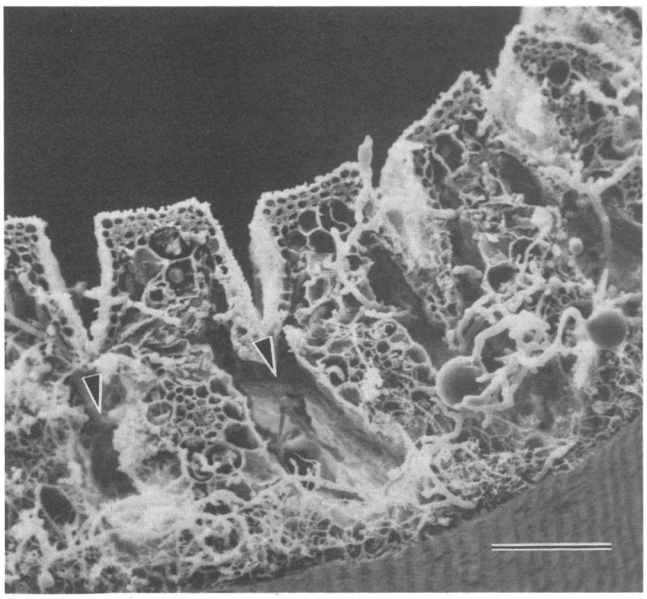

FIG. 6. SEM of cordgrass leaf blade incubated for 2 days with S,P treatment. Colonization occurred by fungi of a single morphotype. Little loss of tissue occurred, with mesophyll regions (arrows) showing the most degradation. Bar, 100 μm.

Incubation of leaf blades with S,P treatment resulted in a fungal population attached to leaf tissues (Fig. 6). The fungi, which were markedly more prevalent than in WRF-treated leaves, appeared to be primarily of a single morphotype, consisting of globose sporangia 50 to 100 μm in diameter and a substantial rhizomycelium. The fungi colonized the cut edge of the plants and not the leaf surface. The fungi removed only a small amount of tissue from the leaf blade, primarily in the mesophyll region (Fig. 6). Sites away from fungal localization within a leaf blade showed an intact mesophyll tissue, indicating that degradation occurred only in the vicinity of fungal colonization and that bacterial degradation did not occur with the S,P treatment. The fungi predominated on the lignocellulosic tissues, being especially prevalent on the abaxial epidermis (Fig. 6). These lignified sites, although heavily colonized by the rhizomycelium, were not removed and still supported the fungal mass. Ruminal fungi, although enhanced in the S,P treatment, did not colonize leaf sheaths extensively, and tissue loss in sheaths was minimal (Fig. 7).

All tissues, i.e., parenchyma and xylem, in cordgrass stems were strongly positive for lignin with both histological tests. Although a microbial mat of bacteria plus a few globose sporangia developed on the stem sections incubated with WRF, no substantial loss of tissue occurred (Fig. 5). The fungi showed marked growth and colonization of stem sections with S,P treatment (Fig. 7). The fungal morphotype was identical to that colonizing the blades, but more globose sporangia were present on stems. Stem tissues, even though overgrown by the rhizomycelium, showed little degradation by the fungi.

Degradation of cordgrass lignin and polysaccharides. Cordgrass was 68.3% neutral detergent fiber. Studies of the degradation of cordgrass differed from those with bermudagrass in that only the more refractory lignocellulosic fiber component of cordgrass was used, whereas whole plant tissues were used in bermudagrass studies. The in vitro dry matter digestibility of the cordgrass fiber with WRF was 35.8 ± 1.1% compared with values of 50 to 66% typically observed for bermudagrass leaf material (8). Specifically ^{14}C-labeled lignocellulosic fiber from cordgrass was used to investigate the microbial degradation of the polysaccharide and lignin components. Dissolved organic matter, rather than gaseous end products, was the major degradation product recovered from both the polysaccharide and lignin components (Table 3).

The total decomposition of polysaccharides was similar in WRF and C treatments (23.0 and 21.1%), as was the production of gaseous end products (3.1 and 3.1%), indicating that bacteria were potentially capable of all of the degradation observed in untreated ruminal fluid (Table 3). Treatments to inhibit bacteria (S,P and S,P,CAM) resulted in slightly less dissolved organic matter and about half as much gaseous end products. The S,P,C control had significantly higher amounts of dissolved organic matter and gaseous end products than did the ARF control, indicating that not all microbial activity was inhibited with these antibiotics. Low densities of bacteria were observed by SEM on parallel samples, also suggesting that bacterial activity was not completely inhibited by these antibiotics. Ratios of gaseous end products (volatile fatty acids plus carbon dioxide/methane) from polysaccharide degradation ranged from 6.1 to 9.5 (data not shown).

The total decomposition of lignin was also similar with WRF and C treatments (20.0 and 18.5%), as was the production of gaseous end products (0.3 and 0.3%) (Table 3). The production of dissolved organic matter and gaseous end products in treatments to inhibit bacteria (S,P and S,P,CAM) were not significantly different from those in the controls but were much lower than the degradation observed in the WRF and C treatments, indicating that fungi did not contribute to the degradation of cordgrass lignin. The total percentage of lignin that was degraded in the WRF and C treatments was similar to the total percentage of polysaccharides that was degraded in the same treatments. The percentage of gaseous end products from lignin, however, was 10-fold lower than the percentage of gaseous end products from polysaccha-

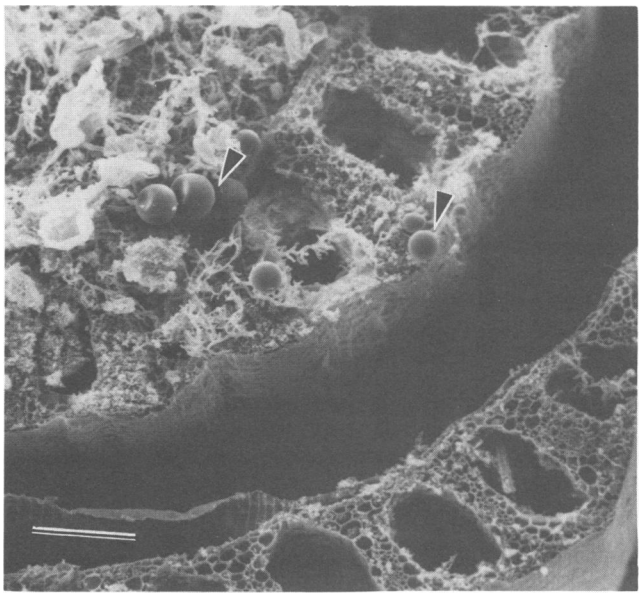

FIG. 7. SEM of cordgrass leaf sheath and stem incubated for 2 days with S,P treatment. Sheath and stem show negligible digestion, although the fungi (arrows) heavily colonized the stem. Bar, 200 μm.

TABLE 3. Production of gaseous (volatile fatty acids plus CO_2 plus CH_4) and dissolved organic matter from radiolabeled cordgrass incubated for 7 days with bovine ruminal fluid treated with antibiotics

Treatment	Degradation of specifically labeled lignocellulose[a]					
	% Gaseous		% Dissolved organic matter		% Total decomposition	
	^{14}C-PS	^{14}C-Lig	^{14}C-PS	^{14}C-Lig	^{14}C-PS	^{14}C-Lig
WRF	3.1a[b]	0.3a	19.9a	19.7a	23.0a	20.0a
C	3.1a	0.3a	18.0ab	18.2a	21.1ab	18.5a
S,P	1.4cd	0.1c	16.7ab	6.6c	18.1ab	6.7c
S,P,CAM	1.6c	0.1c	16.0ab	9.0c	17.6ab	9.1c
S,P,C	1.8c	0.1c	13.5b	5.1c	15.4b	5.2c
ARF	0.3d	0.1c	7.7c	5.7c	8.1c	5.9c

[a] ^{14}C labeled in polysaccharide (^{14}C-PS) or lignin (^{14}C-Lig) component. Values are means ± 1 standard deviation. Values followed by unlike letters within columns are significantly different ($P = 0.05$).
[b] Only one value.

rides, indicating that the dissolved lignin components were much less fermentable than the dissolved polysaccharide components. Ratios of gaseous end products (volatile fatty acids plus carbon dioxide/methane) from lignin degradation ranged from 2.3 to 4.3 (data not shown).

Large differences between the decomposition of bermudagrass and cordgrass tissues were observed in the present study. In part these differences resulted from the use of whole tissues from bermudagrass and only the cell wall (lignocellulose) portion of cordgrass tissues. The total percentage of cordgrass lignocellulose that was degraded (LC_d, mineralized and solubilized) during the incubation period is calculated as $LC_d = [L_d(L)+P_d(P)]/100$, where L_d is the percentage of lignin degraded, P_d is the percentage of polysaccharide degraded, L is the percentage of lignin in lignocellulose (7.8%), and P is the percentage of polysaccharide in the lignocellulose (92.2%). Using this formula and values from Table 3, we calculated that the total decomposition of cordgrass lignocellulose ranged from 8% in ARF to 23% in WRF. In incubations with bermudagrass tissues total weight losses ranged from 29 to 79% (Table 2). Lignin losses from bermudagrass tissues, as estimated from analyses of lignin oxidation products, ranged from 36 to 66% (Table 2). Lignin losses from cordgrass tissues, as estimated with radiolabeled lignin, ranged from 5 to 20% (Table 3). There was a strong linear correlation ($r = 0.95$; $P < 0.001$) between the percentage of organic matter and lignin lost from plant tissues (Fig. 8).

DISCUSSION

The plant samples used in this study (i.e., bermudagrass leaf blades and cordgrass fiber) represented substrates of different availabilities for microbial utilization. The bermudagrass leaves, which came from a plant of high fiber content (8), contained soluble sugars and showed complete or partial degradation of most of the tissues, which was typical for bermudagrass (1). Cordgrass plant parts, on the other hand, were highly lignified as shown histologically, and only a small proportion of tissues in leaves or stems was degraded. Values of the digestibilities and SEM of specific tissue loss substantiated the differences between the substrates. Despite differences in the structural complexity of the substrates, bacteria were more effective fiber degraders than the fungi for all chemical components investigated in both substrates.

A combination of chemical and radiotracer methods was used to investigate the microbial transformations of bermudagrass and cordgrass lignins in ruminal fluid. Each method provided unique information on the fate of lignin and an estimate of total lignin loss. It is important to point out that total lignin losses estimated from the analysis of lignin oxidation products are comparable to lignin losses determined with specifically radiolabeled lignin. For instance, Benner et al. (12) estimated from analyses of lignin oxidation products that 18% of cordgrass lignin was lost during 29-day incubations of cordgrass tissues in anaerobic sediments at 15°C. During that same period 20% of the total tissue mass was lost, resulting in a mass/lignin loss ratio of 1.11. In the present study with radiolabeled cordgrass lignin we estimated a lignin loss of 20% and a mass/lignin loss ratio of 1.14 during 7-day incubations in ruminal fluid at 39°C. The higher absolute rates of lignin and mass loss in ruminal fluid incubations relative to sediment incubations are most likely due to the relatively low incubation temperature in sediments (16). The important point is that the rate of lignin loss relative to the rate of total mass loss is almost identical in the two studies, indicating that the chemical and radiotracer methods yield similar results. The mass/lignin loss ratio of bermudagrass tissues in unamended ruminal fluid (1.28) was similar to the ratio for cordgrass tissues, indicating similar patterns of decomposition even though bermudagrass was much more susceptible to degradation. Thus it appears that lignin losses are tightly coupled to overall weight losses during decomposition.

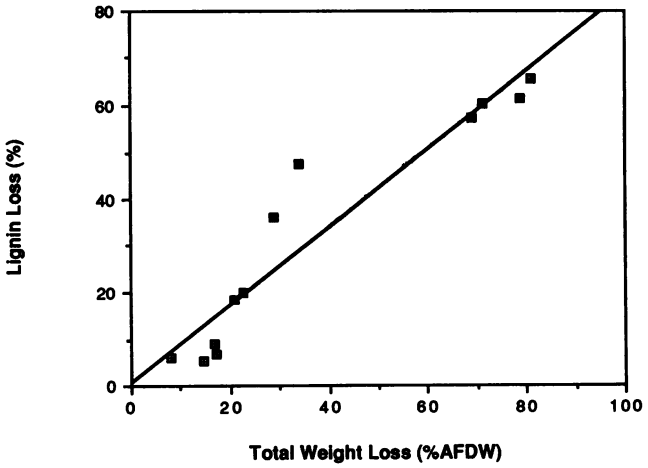

FIG. 8. Relationship between the loss of lignin and total weight loss from bermudagrass and cordgrass tissues during 7 day incubations in ruminal fluid. A linear regression of the data is shown.

The analysis of lignin oxidation products by capillary gas chromatography yielded molecular-level information on the relative reactivities of the various structural units of lignin. Guaiacyl structural units, which yield vanillyl phenols upon CuO oxidation, were the most resistant to decomposition, whereas ferulic acid was the least resistant. These results are not surprising since guaiacyl units are often highly cross-linked by ether and C-C bonds (45), whereas ferulic acid is bound by ester linkages to core lignin (33) and to carbohydrates (30). Analysis of lignin oxidation products in degraded plant tissues, however, yields no information on the fate or pathway of lignin loss from the plant tissues. This information is available from the experiments with specifically radiolabeled lignin preparations. Radiotracer experiments indicated that practically all of the losses of lignin from plant tissues resulted from solubilization rather than direct fermentation. It is likely that the relatively high concentrations of dissolved lignins produced in the rumen are condensed and precipitated during passage from the rumen to the acidic abomasum of the gastrointestinal tract (26).

The fungi elicited by the diet (40% sorghum silage, 51% corn grain 9% soybean meal) of the donor animal used in this study appeared to be of one predominate type. This type of fungus, demonstrating polycentric growth characteristics as shown by multiple sporangia on a single rhizoid of indeterminant growth (W. S. Borneman and D. E. Akin, unpublished data), has recently been reported to inhabit the rumen (6) but differs from those reported earlier (10, 41). Currently, the taxonomic status of this fungal type is not known.

Fungi selected in our in vitro system colonized the lignocellulosic tissues like those described previously (5, 9). Fungi did not preferentially degrade the lignin component, and it is likely that the lignin loss we observed in this study and the 12 to 17% loss of lignin reported in other studies (42) are due to solubilization from the plant cell wall. Indeed, the small amount of gaseous end products recovered from the lignin component with respect to the large amount of dissolved organic matter produced suggests that little lignin utilization occurred. Other studies with gravimetric analysis of various fiber components (49) and synthetic radiolabeled lignin (D. O. Mountfort, Abstr. 4th Int. Symp. Microb. Ecol., p. 13, 1986; G. L. R. Gordon, Abstr. 2nd Int. Symp. Nutr. Herbivores, p. 135–136, 1987) also indicate that the ruminal fungi studied to date appear to have little ability to mineralize lignin. While the attack of ruminal fungi on plant lignocellulose involves fermentation of the carbohydrate moiety, the weakening and physical breakdown of lignified tissues could be of particular benefit in feed utilization.

Bauchop and Mountfort (11) reported that cultures of *N. frontalis* initially isolated with streptomycin and penicillin were cocultured with methanogenic bacteria. Incorporation of chloramphenicol with subsequent transfers successively inhibited the methanogenic bacteria, allowing the study of the fungus alone. They reported that the methanogenic bacteria stimulated cellulose degradation over that by the fungus alone. In the present study, similar antibiotic treatments that were used to inhibit methanogens (Borneman, unpublished data) did not affect the digestibility of fiber components over that produced with additions of streptomycin and penicillin alone. Radiolabeled methane was recovered from all incubations containing chloramphenicol, indicating that methane production was not inhibited in the present study by the addition of chloramphenicol.

Plant tissues showing a chlorine-sulfite reaction are reported to contain substantial amounts of syringyl units, whereas acid phloroglucinol-positive tissues are characterized by vanillyl units (45, 47). Further, the syringyl-type tissues have been reported to be more susceptible to microbial degradation than the vanillyl-type tissues (1, 25). In the present study, the syringyl subunits of bermudagrass were much more susceptible to abiotic solubilization than the vanillyl subunits, but vanillyl and syringyl subunits were degraded to approximately the same extent in live incubations.

The position and function of phenolic acids such as *p*-coumaric and ferulic acid in the plant cell wall are not clear (23, 40). In the present study, *p*-coumaric acid was much more resistant than ferulic acid to microbial degradation. *p*-Coumaric acid has been reported to be more closely associated to the less-digestible fiber than ferulic acid and to exist in a different structural environment in the cell wall (29). *p*-Coumaric acid has been statistically correlated with the least-digestible component in several forages (19, 21), and its in vitro toxicity is greater than that of ferulic acid in many cases (2).

In the present study, lignin was solubilized to a great extent under the anaerobic conditions of the rumen. Gaillard and Richards (26) reported that substantial amounts of phenolic compounds were released from forage fiber at the pH of the rumen (about 6.7). The total percentage of decomposition (solubilization plus production of gaseous end products) of cordgrass lignin during 7-day incubations with ruminal fluid was similar to the percentage of decomposition observed during incubations with anoxic sediments lasting 10 months (15). In sediment incubations, however, most of the degraded lignin was converted to gaseous end products. Under our in vitro conditions simulating the ruminal environment, it appears that the solubilized lignin is not further degraded to gaseous end products. At the present time, we do not know the molecular weight range of the dissolved lignin or the potential toxicity of the lignin-derived phenols to ruminal microflora. Characterization of the dissolved degradation products of lignin may yield information on the chemical factors that affect the digestibility of plant substrates, particularly of warm-season grasses that appear to possess higher concentrations of the more toxic phenolic components (3).

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