Degradation of lucerne stem cell walls by five rumen bacterial species

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Abstract

The rumen bacterial strains *Butyrivibrio fibrisolvens* H17c, *Fibrobacter succinogenes* S85, *Lachnospira multiparus* 40, *Ruminococcus albus* 7 and *R. flavefaciens* FD-1 were compared individually and as a five-species mixture with a rumen inoculum for their ability to degrade lucerne (*Medicago sativa* L.) stem cell wall polysaccharides. Two maturity stages of lucerne (bud and full flower) were utilized as substrates and incubation periods of 24 and 96 h were employed. Lucerne stem substrates and fermentation residues were analysed for cell wall content and composition. Degradation of total cell wall polysaccharides and all polysaccharide fractions was greater for immature than for mature lucerne stems. The 96-h incubation resulted in significantly more degradation of all cell wall polysaccharides than did the 24-h incubation. The *R. albus* culture was able to degrade all cell wall polysaccharide fractions as well or better than any other culture, including the rumen inoculum. Co-culture of *R. albus* with the four other ruminal species did not improve degradation of any wall polysaccharides compared with *R. albus* alone. Microscopic examination indicated that *R. albus* and the five-species mixture appeared to degrade lucerne tissues in thin sections to almost the same extent as did rumen fluid.

Additional keywords: *Medicago sativa*, tissues, *in vitro*

Introduction

Lucerne (*Medicago sativa* L.) is a major forage fed to ruminant livestock around the world. While lucerne leaves are rapidly and extensively degraded in the rumen, the stem fraction is of limited degradability because of its high cell wall content (Albrecht

et al., 1987). The presence of lignin in the cell wall is generally assumed to account for the poor degradability of plant cell walls (Jung & Deetz, 1993). Stems of lucerne contain a diverse set of tissues, some of which never lignify during growth and development while other ones lignify almost immediately after cell growth has ended (Engels & Jung, 1998). In addition to differences in the presence or absence of lignin, the relative abundance of the various cell wall polysaccharides varies among tissues. Pectin is found in all primary cell walls at low contents and accumulates in collenchyma tissue in thickened primary walls. Phloem fibre is unique in that it develops a cellulose-rich secondary cell wall that does not lignify, in contrast to xylem fibre secondary wall, which is rich in cellulose, hemicellulose and lignin (Engels & Jung, 1998). The diversity in cell wall composition of lucerne stem tissues results in different rates and extents of degradation by ruminal micro-organisms with non-lignified tissues being much more degradable than lignified tissues (Jung & Engels, 2001).

The ruminal microflora is capable of utilizing diverse plant tissues and cell wall structures. While ruminal bacteria have evolved specializations that allow individual species to occupy particular ecological niches in the rumen, there is substantial overlap among the most commonly isolated bacterial species with respect to which cell wall polysaccharides they can degrade. *Fibrobacter succinogenes, Ruminococcus albus* and *R. flavefaciens* are all capable of degrading cellulose and hemicellulose, although *F. succinogenes* cannot utilize xylose (the main lucerne hemicellulosic monosaccharide) for growth (Morris, 1984). *Prevotella ruminicola* and *Butyrivibrio fibrisolvens* have been reported to be active against hemicellulose, and some *B. fibrisolvens* strains are also weakly cellulolytic (Hespell, 1988). *Lachnospira multiparus* has been reported to be unable to degrade either cellulose or hemicellulose, and while active against purified pectin, *L. multiparus* degraded orchardgrass (*Dactylis glomerata* L.) pectin to only a limited extent (Osborne & Dehority, 1989). Mixtures of bacterial species with different degradation capabilities have not altered cellulose degradation from intact forage cell walls, but hemicellulose utilization and pectin degradation were increased by combining *F. succinogenes* and *P. ruminicola* cultures (Osborne & Dehority, 1989).

While the degradation characteristics of rumen bacterial species have been well characterized using purified cell wall polysaccharide substrates, similar data for polysaccharide degradation from intact cell wall matrices are limited. And as indicated above, results for polysaccharide degradation from intact cell wall matrices by bacterial species have not always corresponded with those from purified substrates. Even less information is available on differences among bacterial species with respect to which forage tissues can be degraded. In this study we characterized both the tissue and cell wall polysaccharide degradation specificity of five common species of ruminal bacteria (*Butyrivibrio fibrisolvens, Fibrobacter succinogenes, Lachnospira multiparus, Ruminococcus albus* and *R. flavefaciens*) for lucerne stems, and compared these responses with the responses of a mixture of the five species and a mixed ruminal inoculum. Maturity of lucerne stems and length of incubation time were investigated as additional variables affecting cell wall degradation by these ruminal bacteria.

Materials and methods

Plant material

Stem samples were collected from established lucerne plants of clone 403 on the University of Minnesota – St. Paul campus. Stem growth and development of this clone have been previously described (Engels & Jung, 1998; Jung & Engels, 2002). Immature and mature stems were harvested 14 and 24 July 1997 (after 21 and 31 days of re-growth, respectively) from shoots that originated from residual nodes of stems harvested on 23 June 1997. The harvested lucerne stems had reached bud and full-flower maturity stages, respectively. The seventh internode of each sampled stem, counting from the base, was excised and stored in 50% ethanol (v/v) for microscopic analysis. The remaining stem material was lyophilized and subsequently ball-milled (Jung *et al.*, 2000).

Bacterial cultures

Cultures of *Butyrivibrio fibrisolvens* H17c, *Fibrobacter succinogenes* S85, *Lachnospira multiparus* 40, *Ruminococcus albus* 7, and *R. flavefaciens* FD-1 were grown axenically under CO_2 in 158-ml serum vials sealed with butyl stoppers and crimp-sealed aluminium closures (Miller & Wolin, 1974). Each bottle contained 100 ml of a modified Dehority medium (Weimer *et al.*, 1991) supplemented with cellobiose (4 g l^{-1}), clarified ruminal fluid (2 ml l^{-1}), and approximately 200 mg of ground lucerne stems. In addition, *B. fibrisolvens* was supplemented with glucose (3 g l^{-1}) and yeast extract (2 g l^{-1}), and *L. multiparus* was supplemented with 10 g peptone, 2 g yeast extract, and 25 mmol Na-acetate per litre. All substrates were added aseptically using hypodermic syringes, from sterile concentrated stock solutions, after autoclaving the basal medium. Cultures were started by transferring 2% (v/v) inoculum into the serum vials, and were incubated for 16 to 20 h at 39 °C. Five vials of a single pure culture were aseptically combined in a sterile Erlenmeyer flask under continuous CO_2 sparging to obtain approximately 500 ml of pure culture inoculum that was used to inoculate the sample tubes.

Incubations

Replicate 200-mg sub-samples of the immature and mature lucerne stem samples were placed in 50-ml polypropylene conical centrifuge tubes (Corning) for inoculation and degradation by the bacterial cultures. Thin sections from the ethanol-preserved seventh internode samples, mounted on slides (see description below), were also prepared for inoculation. Ground lucerne stems were briefly (about 1 minute) hydrated by addition of 3 ml of a buffered reducing agent (per litre: 8 g cysteine-HCl, 8 g Na_2CO_3, 1 ml volatile fatty acid solution [10 µl each of isobutyric, isovaleric, DL-2-methylbutyric, and n-valeric acids per ml H_2O]; pH 6.9) under CO_2 sparging. The sample tube was then inoculated with 27 ml of the above inoculum and, where appropriate, a glass slide containing the hydrated stem sections was placed into the tube. The CO_2 sparge was then discontinued, and the tube tightly sealed with a screw cap.

The inoculation was repeated for each tube for a given pure culture, and this inoculation procedure was then repeated for each of the other strains. For the mixed-culture inoculum, a single vial of each pure culture was combined into a flask and used as above. The ruminal inoculum was prepared under CO_2 using ruminal contents from a lactating, fistulated Holstein cow maintained on a mixed ration diet that included lucerne silage. The ruminal contents (pH 6.34) were squeezed through four layers of cheesecloth, the filtrate set aside. Squeezed solids (50 g) were rinsed and re-squeezed four times with about 100 ml of CO_2-gassed buffer (McDougall, 1948). The filtrate (360 ml) was combined with 100 ml of the original filtrate and used directly for the inoculum.

The initial optical density (525 nm) of the cultures added to the sample tubes, calculated from 10-fold dilutions in glass tubes of 16 mm path length, were: rumen inoculum, 12.0; *B. fibrisolvens*, 2.2; *F. succinogenes*, 2.2; *L. multiparus*, 2.5; *R. albus*, 1.57; *R. flavefaciens*, 0.95; and mixed culture, 1.75. The initial pH of inoculated sample tubes was 6.3–6.4, determined in several separate tubes not subjected to incubation.

After inoculation, tubes were incubated in an upright position at 39 °C in an anaerobic glovebag (Coy) filled with 10% H_2/40% CO_2/50% N_2 (v/v/v). Several times each day, the caps were loosened and retightened to vent fermentation gases, and the tubes gently mixed to redistribute forage particles. Samples that contained the stem sections on glass slides were incubated for 6, 24 or 96 h, after which the slides were immediately removed, rinsed by dipping several times in distilled water, and placed in tubes containing sufficient 50% (v/v) ethanol to completely cover the stem sections. Samples that contained only ground lucerne were incubated for 24 or 96 h, then chilled in ice. After rinsing the lucerne particles from the undersides of the tube caps back into the tube, the tubes were frozen at –20 °C, then lyophilized. Empty centrifuge tubes were inoculated with each culture or combination of cultures, and incubated for 24 or 96 h as blanks for calculation of cell wall polysaccharide degradation in the ground lucerne experiment. Lucerne stem cross-sections were also incubated in the buffer media without inoculation to serve as comparative controls for the microscopic evaluations of degradation.

Determination of cell wall polysaccharides

Immature and mature lucerne stem samples, before and after incubation with bacterial cultures, were analysed for cell wall content and composition by the Uppsala Dietary Fiber procedure (Theander *et al.*, 1995). The neutral sugar residues (glucose, xylose, arabinose, galactose, mannose, rhamnose and fucose) released from the cell wall polysaccharides by the two-stage sulphuric acid hydrolysis were quantified as alditol acetate derivatives by gas-liquid chromatography with flame ionization detection. Total uronic acids (galacturonic, glucuronic, and 4-*O*-methyglucuronic acids) released in the first stage acid treatment were measured colorimetrically (Ahmed & Labavitch, 1977). Klason lignin was quantified as the ash-free residue remaining after acid hydrolysis. Total cell wall content was estimated as the sum of the neutral sugar residues, total uronics, and Klason lignin. Based on the known composition of lucerne cell walls (Hatfield, 1992) cellulose was estimated as the glucose content of the samples; hemi-

cellulose as the sum of xylose, mannose, and fucose residues; and pectin as the sum of uronic acid, arabinose, galactose, and rhamnose residues. Degradation of total cell wall polysaccharides (sum of all monosaccharide residues), cellulose, hemicellulose, pectin and individual monosaccharide residues was calculated for each inoculated tube containing ground lucerne.

Microscopic evaluation

Cross-sections (100 μm) of lucerne stem internodes preserved in 50% ethanol were made, without embedding, using a Leitz-Wetzlar sliding microtome. Multiple cross-sections were made from each of two internode samples, from each lucerne maturity stage. Sections were mounted on microscopic slides using double-sided tape. Two cross-sections from each internode and both lucerne maturity stages were mounted on each slide. After mounting, the sections were allowed to air-dry. The evening before inoculation of the slide-mounted sections, the sections were re-hydrated by immersion of the slides in sterile deionized water. Inoculation and incubation of the sections was described above. After the bacterial incubations were completed, the undegraded control and the degraded lucerne stem sections were evaluated using light microscopy. The extent of cell wall development was assessed by observation of specific stem tissues, including collenchyma, phloem fibre, xylem fibre and parenchyma. Degree of cell wall degradation of tissues was scored using the following scale: 0, no observable degradation; +, some degradation visible; ++, extensive degradation; and +++, complete degradation of the tissue. To aid in the evaluation of extent of tissue degradation, control and degraded sections were stained with ruthenium red and toluidine blue to highlight non-lignified, pectin-rich tissues and lignified tissues, respectively (Jensen, 1962; Sylvester & Ruzin, 1994).

Statistical analysis

Degradations of ground lucerne stems were done in duplicate for each bacterial inoculum and both incubation time points. The data were analysed as a completely randomized design with a split-split plot arrangement of treatments (Steel & Torrie, 1960). Lucerne maturity stage was the main factor, bacterial culture was the split-factor, and incubation time (24 or 96 h) was the split-split-factor. All interactions among parameters were included in the statistical analysis. For those parameters that resulted in a significant analysis of variance F-test, means were contrasted using the least significant difference test. Treatment differences were considered statistically significant at $P < 0.05$.

Results

Effect of maturity on cell wall composition and degradation

The seventh internode of mature lucerne stems had more thick-walled, lignified xylem

tissue than did the immature stems (Figure 1). Some xylem fibre cells had deposited an additional secondary wall layer on the lumen side of the secondary cell wall. This additional secondary wall did not stain as intensely for the presence of lignin as did the rest of the secondary wall. Deposition of non-lignified, cellulose-rich secondary walls in phloem fibre cells was also evident in the mature internodes. However, cell wall content and composition of the two lucerne stages of maturity were less divergent for the ground stem samples than for the internode tissues. Total cell wall content was only 50 g kg^{-1} higher for the mature stems than for the immature stems, and cell wall compositions were similar (Table 1). Composition of the lucerne cell walls exhibited

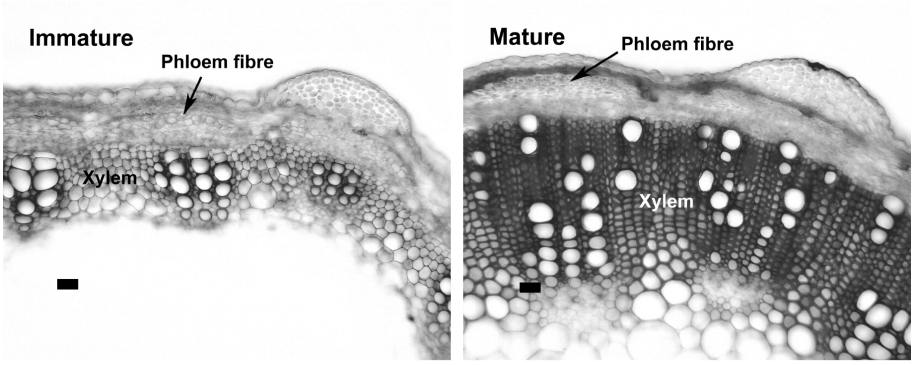

Figure 1. Cross-sections of immature and mature lucerne stems after 21 and 31 days of re-growth, respectively. Xylem tissue proliferation and development of thick secondary walls in phloem fibres were evident in the more mature internodes. Scale bar = 50 µm.

Table 1. Cell wall content and composition of lucerne stems harvested at two stages of maturity (immature and mature, i.e., after 21 and 31 days of re-growth, respectively).

Component	Stage of maturity	
	Immature	Mature
	– – – – (g per kg organic matter)	– – – –
Cell wall	655	705
	– – – – – – (g per kg cell wall)	– – – – – –
Glucose	462	478
Xylose	126	132
Arabinose	32	26
Galactose	31	26
Mannose	28	31
Rhamnose	11	11
Fucose	3	3
Uronic acids	104	88
Klason lignin	202	206

only minor shifts, with pectin content being slightly greater in the immature stems, while the other wall components were marginally higher in the mature stems.

In order to remove any potential artifacts associated with heating (weakening of the lucerne cell walls or slide-mounted lucerne sections, or production of fermentation inhibitors), the lucerne was not sterilized prior to inoculation. Lancaster & Patterson (1988) reported that autoclaving and boiling of forages resulted in reduced rate and extent of *in vitro* ruminal degradation. Instead, forages were air-dried at 50 °C to inactivate most mesophilic non-sporeforming anaerobic vegetative cells, and following addition of culture medium a large inoculum of the desired test culture was added. The fermentation products observed at the end of the 96-h fermentation were consistent with those expected from the individual pure or mixed cultures, and unexpected fermentation products were not detected by the HPLC method used. These observations suggest that the inoculated cultures were responsible for the degradative activities observed.

Figure 2 illustrates the pattern of lucerne stem tissue degradation for mature internodes by the rumen inoculum with increasing time of incubation. By 6 h after inoculation, the non-lignified tissues (collenchyma, chlorenchyma, epidermis, secondary phloem and cambium) were completely degraded. Degradation of the non-lignified phloem fibre secondary wall was extensive after 24 h of incubation and by 96 h only a thin, lignified primary wall ring-structure remained. No degradation of the primary and secondary xylem fibre cell walls could be discerned, even after 96 h, but the lightly lignified additional secondary wall located in some xylem fibre cells was partially degraded. The same pattern of tissue degradation was observed for immature internodes (data not shown).

Degradation patterns after 24 h of incubation

The degradation after 24 h of incubation of individual lucerne stem tissues from immature internodes by bacterial cultures is illustrated in Figure 3. The ruminal inoculum and the five-species pure culture mixture resulted in the degradation of all non-lignified tissues (epidermis, collenchyma, chlorenchyma, secondary phloem and cambium) including the non-lignified phloem fibre secondary wall. The *R. albus* inoculum alone was able to degrade lucerne stem tissues to the same extent as observed for the ruminal inoculum and for cultures inoculated with the mixture of pure cultures. In contrast, *B. fibrisolvens* degraded all of the non-lignified tissues completely, but not the non-lignified, cellulose-rich secondary wall of the phloem fibre. *L. multiparus* attacked all non-lignified tissues, but did not completely degrade non-lignified, thick-walled collenchyma in 24 h. Both *F. succinogenes* and *R. flavefaciens* appeared to degrade non-lignified chlorenchyma and cambium tissues. Phloem fibre cells and non-lignified collenchyma were also partially degraded by *F. succinogenes*.

Both the rate and extent of degradation for individual tissues from lucerne internodes were different among the bacterial inocula. All of the potentially degradable tissues were completely degraded after 96 h of incubation with the ruminal, *F. succinogenes*, *R. albus*, and mixture inocula (Table 2). However, after 6 and 24 h of incubation the *F. succinogenes* and mixed inocula showed less extensive degradation than did the

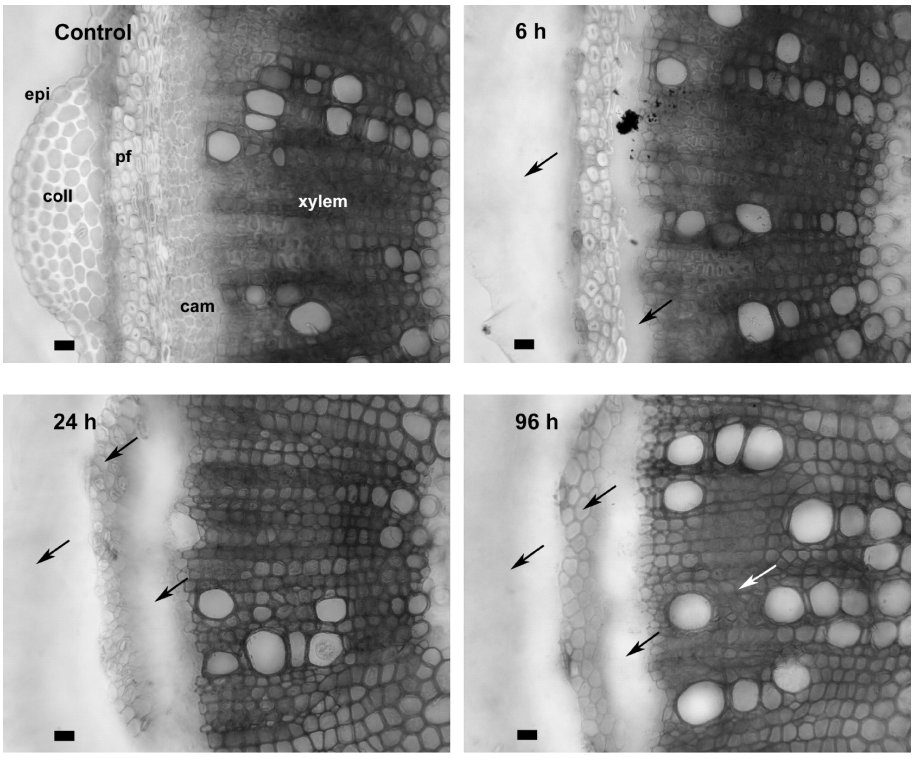

Figure 2. Impact of incubation time (6, 24 or 96 hours) on degradation of mature lucerne stem tissues by rumen inoculum. Lucerne stem tissues are identified in the Control micrograph (epi = epidermis; coll = collenchyma; pf = phloem fibres; cam = cambium and secondary phloem; xylem = xylem fibres and vessels). Arrows indicate tissues showing signs of degradation. Scale bar = 20 µm.

ruminal and *R. albus* inocula. The rate of degradation was slower for the partially lignified phloem fibres than for the non-lignified tissues. *L. multiparus* and *R. flavefaciens* were only able to partially degrade the lucerne stem tissues. Degradation of stem tissues was somewhat more extensive by *B. fibrisolvens*, but could not be assessed after 96 h because of a damaged slide. Degradation of specific tissues in the mature stem internodes by the various bacterial inocula was very similar to that observed for immature stem cross-sections, but the extent of phloem fibre degradation was less after 24 h of incubation with the ruminal, *F. succinogenes* and *R. albus* inocula (data not shown). Mature internode xylem fibre tissue contained an additional secondary wall layer on the lumen side of cells that was only lightly lignified, as viewed by phloroglucinol staining. The ruminal, *F. succinogenes*, *R. albus* and mixed inocula could degrade this wall layer completely in 96 h, whereas the other inocula showed no ability to attack this additional xylem fibre cell wall layer.

Scoring the degree of specific tissue degradation achieved by bacterial cultures was difficult in some cases. The pattern of degradation observed for the thick-walled, non-

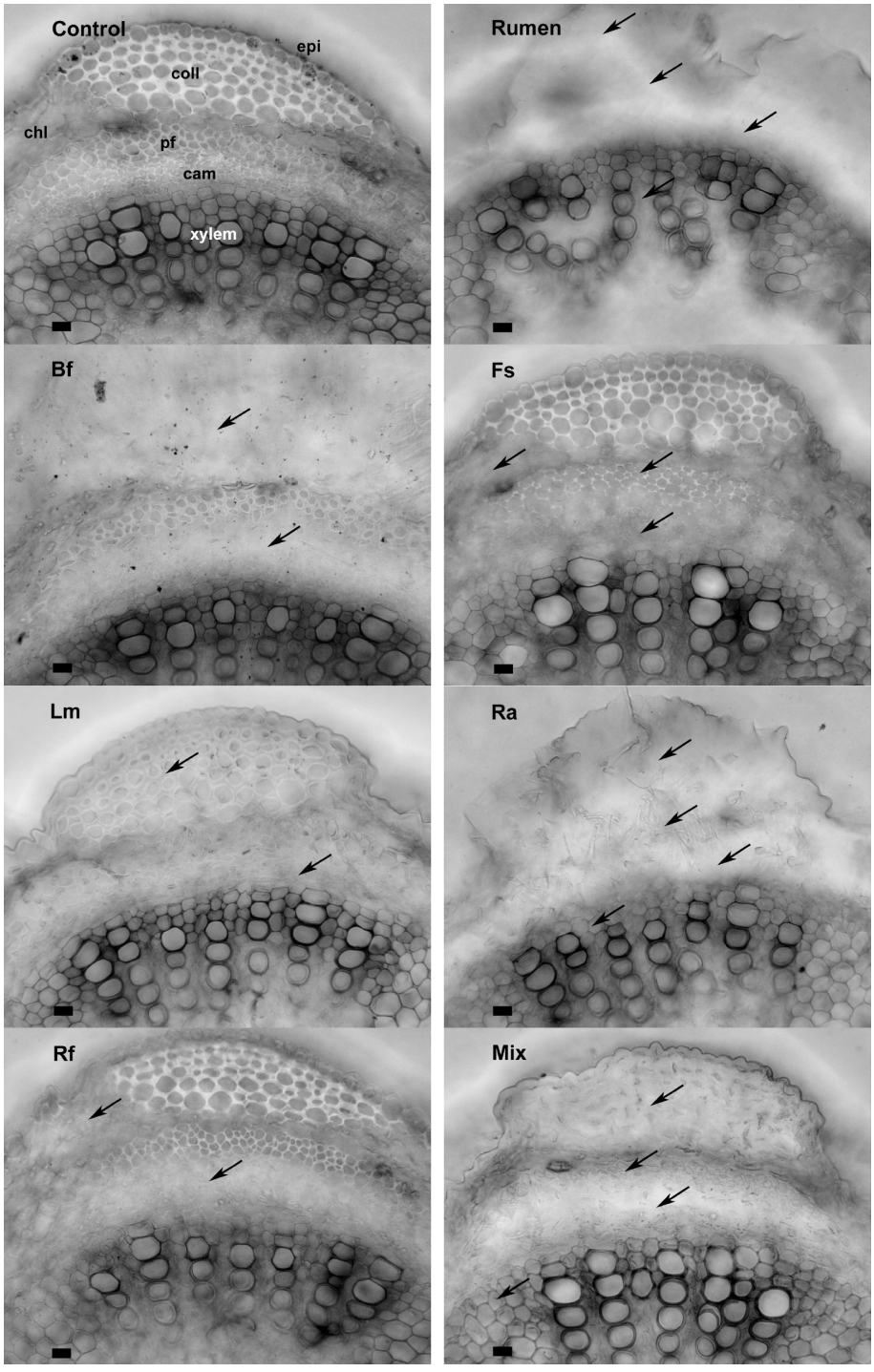

Figure 3. Degradation of immature lucerne stem tissues after 24 h of incubation with rumen inoculum (Rumen), *Butyrivibrio fibrisolvens* H17c (Bf), *Fibrobacter succinogenes* S85 (Fs), *Lachnospira multiparus* 40 (Lm), *Ruminococcus albus* 7 (Ra), *R. flavefaciens* FD-1 (Rf), or a mixture of the five pure cultures (Mix). The lucerne stem tissues are identified in the Control micrograph (epi = epidermis; coll = collenchyma; chl = chlorenchyma; pf = phloem fibres, cam = cambium and secondary phloem; and xylem = xylem fibres and vessels). Arrows indicate tissues showing signs of degradation. Scale bar = 20 µm.

Table 2. Degradation of individual lucerne stem tissues from immature internodes by various bacterial inocula[1].

Culture[2]	Incubation time (h)	Tissue[3]				
		Coll	Pf	Cam	PXP	PP
Rumen	6	+++	+	++	+++	+++
	24	+++	+++	+++	+++	+++
	96	+++	+++	+++	+++	+++
Bf	6	+	o	+	o	o
	24	++	+	+++	++	+
	96	nd[4]	nd	nd	nd	nd
Fs	6	o	o	o	o	o
	24	+	++	++	++	++
	96	+++	+++	+++	+++	+++
Lm	6	o	o	o	o	o
	24	+	o	o	o	o
	96	+	o	+	+	o
Ra	6	++	+	++	++	++
	24	+++	+++	+++	+++	+++
	96	+++	+++	+++	+++	+++
Rf	6	o	o	o	o	o
	24	o	o	o	o	o
	96	+	+	+	+	o
Mix	6	+	+	++	+	+
	24	++	++	++	++	++
	96	++	+++	+++	+++	+++

[1] Degradation was scored on the following scale based on comparison of control sections and sections exposed to bacterial inocula: o = no observable degradation; + = some degradation visible; ++ = extensive degradation; +++ = complete degradation of the tissue.

[2] Rumen = rumen inoculum; Bf = *Butyrivibrio fibrisolvens* H17c; Fs = *Fibrobacter succinogenes* S85; Lm = *Lachnospira multiparus* 40; Ra = *Ruminococcus albus* 7; Rf = *R. flavefaciens* FD-1; Mix = combination of the five pure cultures.

[3] Coll = collenchyma; Pf = phloem fibres; Cam = cambium and secondary phloem; PXP = primary xylem parenchyma; PP = pith parenchyma.

[4] nd = not determined.

lignified collenchyma tissue varied among bacterial cultures. While the ruminal, *R. albus* and mixed cultures removed all of the collenchyma tissue, only partial degradation of this tissue was observed for *F. succinogenes* and *L. multiparus*. Both *F. succinogenes* and *L. multiparus* were judged to have degraded collenchyma tissue to the same limited extent (Table 2). However, as shown in Figure 4 the residual collenchyma tissues remaining after degradation by these species were different in appearance.

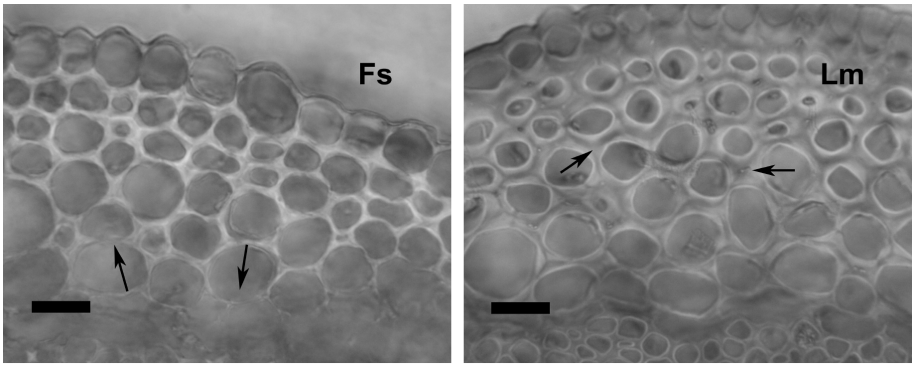

Figure 4. Collenchyma tissue residues remaining after degradation for 24 h by *Fibrobacter succinogenes* (Fs) or *Lachnospira multiparus* (Lm). Arrows indicate regions of degradation. Scale bar = 20 μm.

Degradation of collenchyma cell walls by *F. succinogenes* resulted in complete removal of some walls on the cambial side of collenchyma cell groups, whereas only thinning of walls was observed on the epidermal side of collenchyma bundles (Figure 4). Within collenchyma cell groups, some cells showed no evidence of any cell wall degradation having occurred. In contrast to the uneven pattern of collenchyma tissue degradation by *F. succinogenes*, a consistent pattern of tissue modification was observed after degradation by *L. multiparus*. The residual collenchyma tissue remaining after degradation by *L. multiparus* appeared to have thick walls that were even in thickness and all cells had a circular shape, unlike the variable pattern of cell shape and wall thickness seen in non-degraded collenchyma. The texture of the collenchyma walls appeared less dense and wall edges were less defined after degradation by *L. multiparus*. Moreover, it appeared that *L. multiparus* had degraded the middle lamella region where collenchyma cells had originally been joined, because all collenchyma cells were separated from each other.

Degradation patterns after 96 h of incubation

Degradation by the rumen inoculum of total cell wall polysaccharides from the ground immature stems was greater after 96 h than after 24 h of incubation, but a similar time effect was not observed for the mature stems (Figure 5). However, degradation averaged across all the bacterial inocula increased with incubation time from 24 to 96 h resulting in more cell wall polysaccharide degradation for both immature (37.3 versus 42.3%, $P < 0.05$) and mature (30.9 versus 38.6%, $P < 0.05$) stems. Averaged across both lucerne maturity stages and incubation times, *R. albus* degraded more total cell wall polysaccharides than did any other inoculum (Figure 6). The ruminal inoculum was slightly less capable of degrading ground lucerne stem cell walls than was *R. albus*; the five-species mixture ranked third, although it was substantially lower in total cell wall polysaccharide degradation than was either *R. albus* or the ruminal inoculum. *B. fibrisolvens* exhibited the least capacity to degrade total cell wall polysaccharides but *F. succinogenes*, *L. multiparus* and *R. flavefaciens* were only marginally more effective.

The degradation of cellulose by the bacterial inocula matched their degradation of total wall polysaccharides very closely (Table 3). However, for hemicellulose and pectin, and their component monosaccharide residues, the degree of degradation did not always match total cell wall polysaccharide degradation and different patterns were observed among the inocula. While *R. albus* was always among the most effective inocula for degradation of all lucerne cell wall polysaccharide components, the ruminal inoculum was not significantly more active than the other inocula in the removal of xylose or fucose from hemicellulose. Although able to remove almost no xylose from lucerne hemicellulose, *L. multiparus* ranked among the best inocula for degradation of fucose residues from hemicellulose. *L. multiparus* was also among the most active inoculum sources for degradation of galactose and rhamnose residues from pectin. Rhamnose was the most highly degradable cell wall polysaccharide residue from lucerne stems and uronic acids were also consistently degraded to a large extent by all inocula.

While the main effects for lucerne stem maturity, bacterial inoculum source, and length of incubation were the dominant parameters in the results of statistical analysis, some interactions were observed among these parameters for various measures of cell wall polysaccharide degradation. For example, a lucerne stem maturity × incubation time interaction was observed for cellulose degradation. Across all the inoculum sources, cellulose degradation for the 24-h incubations was greater for immature than for mature stems (30.2 versus 25.6%, $P < 0.05$), but the two stem maturities did not

Table 3. Degradation of cell wall polysaccharides and their component sugar residues[1] from lucerne stems. Data are means[2] across two lucerne maturity stages and two incubation periods (24 and 96 hours).

Culture[3]	Cellulose	Hemicellulose				Pectin				
	Glc	Xyl	Man	Fuc	Total	UA	Gal	Ara	Rha	Total
	(%)									
Rumen	55.0b	16.2b	70.8a	43.7b	26.7b	78.5a	67.5b	83.1a	94.2c	78.5a
Bf	11.6e	15.3b	14.4c	40.4b	15.6c	63.2b	22.1d	78.1a	91.0d	60.7c
Fs	19.2d	8.3c	45.1b	100.0a	16.7c	58.5b	79.2a	58.2b	99.1a	64.8bc
Lm	18.7d	5.5c	15.3c	98.3a	7.4d	77.1a	53.6c	37.1c	95.1c	67.3b
Ra	62.5a	30.8a	70.9a	100.0a	40.3a	75.0a	80.5a	75.7a	100.0a	78.1a
Rf	23.8c	8.6c	54.9b	100.0a	19.0c	64.0b	56.0c	59.8b	98.9ab	64.3bc
Mix	26.4c	17.3b	46.8b	97.0a	24.5b	77.7a	83.2a	86.5a	97.6b	81.5a
SEM[4]	1.1	1.1	5.3	1.3	1.5	2.8	3.1	3.9	0.5	1.7

[1] Glc =glucose; Xyl = xylose; Man = mannose; Fuc = fucose; UA = uronic acids; Gal = galactose; Ara = arabinose; Rha = rhamnose.

[2] Means in the same column, followed by a different letter are statistically different ($P < 0.05$).

[3] Rumen = rumen inoculum; Bf = *Butyrivibrio fibrisolvens* H17c; Fs = *Fibrobacter succinogenes* S85; Lm = *Lachnospira multiparus* 40; Ra = *Ruminococcus albus* 7; Rf = *R. flavefaciens* FD-1; Mix = combination of the five pure cultures.

[4] SEM =standard error of the means.

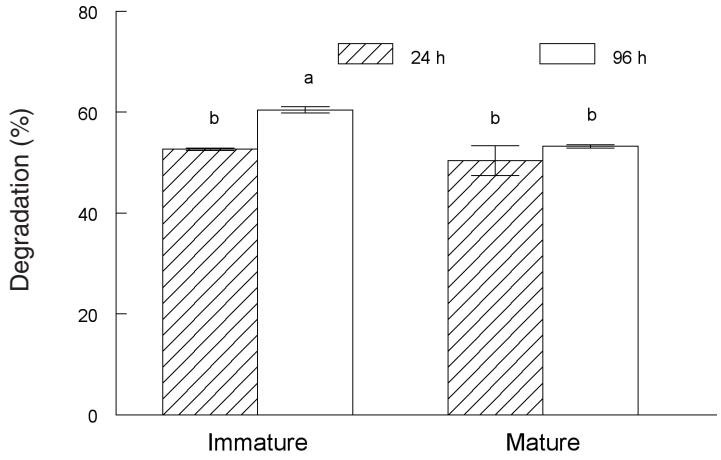

Figure 5. Degradation of total cell wall polysaccharides from immature and mature lucerne stems by rumen inoculum after 24 or 96 h of incubation. Data bars sharing the same letter are not statistically different ($P > 0.05$).

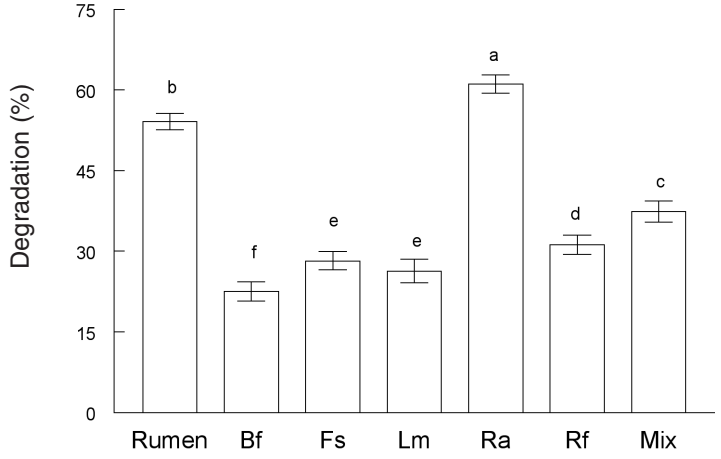

Figure 6. Degradation of total cell wall polysaccharides from lucerne stems by rumen fluid (Rumen), *Butyrivibrio fibrisolvens* H17c (Bf), *Fibrobacter succinogenes* S85 (Fs), *Lachnospira multiparus* 40 (Lm), *Ruminococcus albus* 7 (Ra), *R. flavefaciens* FD-1 (Rf), and a combination of the five pure cultures (Mix). Data are averaged across two maturity stages of lucerne, and 24 and 96 h of incubation. Data bars sharing the same letter are not statistically different ($P > 0.05$).

differ in cellulose degradation for the 96-h incubations (34.3 versus 33.9%, $P > 0.05$). Cellulose degradation also exhibited a bacterial inoculum source × time of incubation interaction. For the *F. succinogenes* and *R. albus* inocula, lengthening incubation time from 24 to 96 h did not result in increased cellulose degradation, across both immature and mature stems, whereas all the other inocula had a statistically significant increase in cellulose degradation at the longer incubation time (data not shown). In the case of pectin degradation, an inoculum × incubation time interaction indicated that the 24- and 96-h incubations did not differ for the ruminal and *R. flavefaciens* inocula while all other inocula responded to a longer incubation time with increased pectin degradation, across the two lucerne stem maturities (data not shown). Removal of xylose residues from cell wall polysaccharides was the only degradation measurement that had a three-factor interaction (lucerne stem maturity × bacterial inoculum source × length of incubation). In this case, only the five-species mixture and rumen inocula exhibited greater xylose degradation after a 24-h compared with a 96-h incubation for immature stems, but for mature stems these two inocula did not show greater xylose degradation after a 96-h incubation. In contrast, *B. fibrisolvens*, *F. succinogenes* and *R. flavefaciens* inocula did not respond to longer incubations for immature stems, but did have greater xylose degradation after 96 h for mature stems (data not shown). For both lucerne maturity stages, *R. albus* showed the greatest xylose degradation at both incubation times.

Discussion and conclusions

Degradation of lignified and non-lignified tissue

The pattern of lucerne stem tissue degradation observed for the rumen inoculum, matched those reported previously (Akin, 1989; Jung & Engels, 2002). Non-lignified tissues were rapidly and completely degraded, whereas lignified tissues were poorly degraded. The rapid and extensive degradation of pectin observed for ground lucerne stem samples corresponded to current and earlier observations that pectin-rich, non-lignified tissues are completely degraded by mixed rumen microflora (Engels & Jung, 1998). The maturing of lucerne stems is known to result in reduced cell wall degradation and reduced susceptibility of individual tissues to ruminal degradation (Jung & Engels, 2002), a result that was confirmed by current results. In general, the cultures inoculated with a single bacterial species exhibited the same trend of reduced cell wall degradation due to maturity, as was observed for the rumen inoculum, and as has been previously reported for whole lucerne herbage (Dehority & Scott, 1967).

Polysaccharide degradation

Extent of polysaccharide degradation by *R. albus* was greatest for pectin and least for hemicellulose, with cellulose being intermediate. The low degradation of hemicellulose parallelled the poor degradation of xylans. This pattern of polysaccharide degradation was similar for the ruminal, *F. succinogenes*, *L. multiparus* and *R. flavefaciens* inocu-

la. The five-species mixture deviated from this pattern in that cellulose degradation and hemicellulose degradation were similar, but less than for pectin. *B. fibrisolvens* was unique in that this species degraded hemicellulose better than it did cellulose, a result consistent with previous reports on the degradation capabilities of this species (Hespell, 1988); again, pectin was the most degradable cell wall polysaccharide. The high degradation of pectin matches previous reports for pectin degradation in general and lucerne pectin degradation in particular (Chesson & Monro, 1982; Hatfield & Weimer, 1995). The pattern of degradation of specific glycosidic residues in lucerne stems reveals both similarities and differences with respect to previous studies with whole lucerne herbage. In several studies using autoclaved whole lucerne herbage, Dehority and co-workers observed that the extent of degradation of lucerne cellulose by pure cultures generally proceeded in the order *F. succinogenes* > *R. albus* ~ *R. flavefaciens* > *B. fibrisolvens* (Dehority & Scott, 1967), while the reverse order was observed for hemicellulose degradation (Coen & Dehority, 1970). *F. succinogenes* S85 also degraded far more lucerne cell wall (sterilization and method not indicated) than did either *R. flavefaciens* FD-1 or *R. albus* 7 (46.5, 3.9 and 1.2 g per 100 g cell wall, respectively) (Miron *et al.*, 1989), and degraded more of all individual monosaccharide residues than did *B. fibrisolvens* (Miron & Ben-Ghedalia, 1993). In our study, *R. albus* was the most active of the species tested in degradation of both cellulose and hemicellulose in ground lucerne stems, and this strain clearly demonstrated the most rapid and complete degradation of individual tissue types in lucerne stem sections.

Degradation of specific tissue types

In general, the degree of cell wall polysaccharide degradation by individual bacterial species we observed for ground stems agreed with the extent of tissue degradation we observed in stem cross-sections. *R. albus* was able to degrade almost all stem tissues, with the exception of xylem, and this species degraded the cell wall polysaccharides of ground lucerne stems as well or better than any other inoculum source. The pattern of tissue degradation observed for *R. albus* matched that of the ruminal and mixed inocula fairly closely, but the mixed inoculum degraded substantially less cell wall polysaccharides from ground stems than expected based on the tissue degradation data. Data for the degradation of ground lucerne stems suggested that *F. succinogenes* and *R. flavefaciens* should have attacked specific tissues to a similar extent, but it was noted that while both species degraded thin-walled, non-lignified chlorenchyma and cambial tissues, only *F. succinogenes* was able to partially degrade the thick-walled collenchyma and phloem fibre secondary walls. While *B. fibrisolvens* and *L. multiparus* were both able to degrade cambial tissue to a great extent, *L. multiparus* showed only limited degradation of the thick pectin-rich collenchyma tissue. This result for *L. multiparus* would appear in contradiction to the general characterization of this species as a pectin-degrading organism. However, the data for degradation of the ground lucerne stems indicated a reasonably high level of activity by *L. multiparus* against pectin. *L. multiparus* appeared to exhibit a somewhat preferential degradation of the middle lamella region of collenchyma tissue compared with the thick primary walls of this tissue, a pattern not observed for any other bacterial culture. Much of the collenchyma

degradation observed for *L. multiparus* appeared to have occurred through degradation downwards from the original cut surface of the stem cross-sections, because the residues could only be viewed by focusing below the cut surface level of non-degradable xylem tissues. In contrast, degradation of collenchyma by *F. succinogenes* apparently resulted more from partial degradation from the lumen side of the collenchyma cells because residual collenchyma walls could be focused in virtually the same plane as the non-degradable xylem.

Reconciling disparities between the apparent ability of specific bacterial species to degrade lucerne stem tissues with the amount of cell wall polysaccharide degraded from ground stems is difficult. While *L. multiparus* is regarded as non-cellulolytic, we found that this bacterial species degraded as much cellulose from ground stems as *F. succinogenes*. Because all plant cell walls contain some pectin, the observed degradation of cellulose from ground lucerne stems by *L. multiparus* is a plausible result if this bacterium must degrade cellulose in order to gain access to the pectin present in primary walls. It remains unclear why *L. multiparus* did not degrade the thick, pectin-rich collenchyma tissue more completely. Perhaps this tissue contains pectin richer in arabinose residues, which *L. multiparus* degraded poorly, than found in some other tissues. The apparent preferential middle lamella degradation from collenchyma by *L. multiparus* suggests that this cell wall region was also different in its chemical structure.

As shown in previous studies (Coen & Dehority, 1970), utilization of the products of polysaccharide hydrolysis is not necessarily coupled to degradation (i.e., hydrolysis to the point of solubilization) of the source polysaccharides. In particular, *F. succinogenes* and some *Ruminococcus* strains have been shown to effectively hydrolyse hemicelluloses and pectin from intact forages, but to utilize only a very small fraction of the hydrolytic products. Degradation of a polysaccharide from intact forage by a bacterium without subsequent utilization of the hydrolytic products would seem counter-productive. But such a strategy may provide a selective advantage by (1) improving access of the organism and its degradative enzymes to other, more utilizable polysaccharides, (2) inducing synthesis of other polysaccharide-degrading enzymes, or (3) providing fermentable carbohydrates to mutualistic partners that cross-feed nutrients to the polysaccharide-degrading organisms. While the results obtained for single strains of a given species do not necessarily represent those from other strains of the same species, the more extensive degradation of cell wall tissues by *R. albus* are in agreement with observations that this species is more abundant than are the other cellulolytic species in the rumens of both dairy cattle (Weimer *et al.*, 1999) and sheep (Van Gylswyk, 1970) fed high-forage diets.

References

Ahmed, A.E.R. & J.M. Labavitch, 1977. A simplified method for accurate determination of cell wall uronide content. *Journal of Food Biochemistry* 1: 361–365.

Akin, D.E., 1989. Histological and physical factors affecting digestibility of forages. *Agronomy Journal* 81: 17–25.

Albrecht, K.A., W.F. Wedin & D.R. Buxton, 1987. Cell-wall composition and digestibility of alfalfa stems and leaves. *Crop Science* 27: 735–741.

Chesson, A. & J.A. Monro, 1982. Legume pectic substances and their degradation in the ovine rumen. *Journal of the Science of Food and Agriculture* 33: 852–859.

Coen, J.A. & B.A. Dehority, 1970. Degradation and utilization of hemicellulose from intact forages by pure cultures of rumen bacteria. *Applied Microbiology* 20: 262–268.

Dehority, B.A. & H.W. Scott, 1967. Extent of cellulose and hemicellulose digestion in various forages by pure cultures of rumen bacteria. *Journal of Dairy Science* 50: 1136–1141.

Engels, F.M. & H.G. Jung, 1998. Alfalfa stem tissues: cell-wall development and lignification. *Annals of Botany* 82: 561–568.

Hatfield, R.D., 1992. Carbohydrate composition of alfalfa cell walls isolated from stem sections differing in maturity. *Journal of Agricultural and Food Chemistry* 40: 424–430.

Hatfield, R.D. & P.J. Weimer, 1995. Degradation characteristics of isolated and *in situ* cell wall lucerne pectic polysaccharides by mixed ruminal microbes. *Journal of the Science of Food and Agriculture* 69: 185–196.

Hespell, R.B., 1988. Microbial digestion of hemicelluloses in the rumen. *Microbiological Science* 5: 362–365.

Jensen, W.A., 1962. Botanical Histochemistry; Principles and Practices. Freeman, San Francisco, California, 408 pp.

Jung, H.G. & D.A. Deetz, 1993. Cell wall lignification and degradability, In: H.G. Jung, D.R. Buxton, R.D. Hatfield & J. Ralph (Eds), Forage Cell Wall Structure and Digestibility. ASA-CSSA-SSSA, Madison, pp. 315–346.

Jung, H.G. & F.M. Engels, 2001. Alfalfa stem tissues: rate and extent of cell-wall thinning during ruminal degradation. *Netherlands Journal of Agricultural Science* 49: 3–13.

Jung, H.G. & F.M. Engels, 2002. Alfalfa stem tissues: cell-wall deposition, composition, and degradability. *Crop Science* 42: 524–534.

Jung, H.G., M.A. Jorgensen, J.G. Linn & F.M. Engels, 2000. Impact of accessibility and chemical composition on cell-wall polysaccharide degradability of maize and lucerne stems. *Journal of the Science of Food and Agriculture* 80: 419–427.

Lancaster, C.M. & J.A. Patterson, 1988. Effect of forage sterilization method on rate and extent of fiber degradation by ruminal bacteria. *Journal of Animal Science* 66: 1800–1806.

McDougall, I.M., 1948. Studies on ruminant saliva: I. the composition and output of sheep's saliva. *Biochemical Journal* 42: 99–109.

Miller, T.L. & M.J. Wolin, 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. *Applied Microbiology* 27: 985–987.

Miron, J. & D. Ben-Ghedalia, 1993. Digestion of cell-wall monosaccharides of ryegrass and alfalfa hays by the ruminal bacteria Fibrobacter succinogenes and Butyrivibrio fibrisolvens. *Canadian Journal of Microbiology* 39: 780–786.

Miron, J., M.T. Yokoyama & R. Lamed, 1989. Bacterial cell surface structures involved in lucerne cell wall degradation by pure cultures of cellulolytic rumen bacteria. *Applied Microbiology and Biotechnology* 32: 218–222.

Morris, E.J., 1984. Degradation of the intact plant cell wall of subtropical and tropical herbage by rumen bacteria. In: F.M.C. Gilchrist & R.I. Mackie (Eds), Herbivore Nutrition in the Subtropics and Tropics. Science Press, Craighall, pp. 378–396.

Osborne, J.M. & B.A. Dehority, 1989. Synergism in degradation and utilization of intact forage

cellulose, hemicellulose, and pectin by three pure cultures of ruminal bacteria. *Applied and Environmental Microbiology* 55: 2247–2250.

Steel, R.G.D. & J.H. Torrie, 1960. Principles and Procedures of Statistics: with Special Reference to the Biological Sciences. McGraw-Hill, New York, 481 pp.

Sylvester, A.W. & S.E. Ruzin, 1994. Light microscopy I: Dissection and microtechnique. In: M. Freeling & V. Walbot (Eds), The Maize Handbook. Springer, New York, pp. 83–95.

Theander, O., P. Aman, E. Westerlund, R. Andersson, & D. Pettersson, 1995. Total dietary fiber determined as neutral sugar residues, uronic acid residues, and Klason lignin (The Uppsala Method): collaborative study. *Journal of the AOAC International* 78: 1030–1044.

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. *Journal of Agricultural Science, Cambridge* 74: 169–180.

Weimer, P.J., Y. Shi & C.L. Odt, 1991. A segmented gas/liquid delivery system for continuous culture of microorganisms on solid substrates, and its use for growth of *Ruminococcus flavefaciens* on cellulose. *Applied Microbiology and Biotechnology* 36: 178–183.

Weimer, P.J., C.L. Odt, G.C. Waghorn & D.R. Mertens, 1999. Effect of diet on populations of three species of ruminal cellulolytic bacteria in lactating dairy cows. *Journal of Dairy Science* 82: 122–134.