Protein degradation and fermentation characteristics of unwilted red clover and alfalfa silage harvested at various times during the day

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Abstract

Extensive proteolysis during fermentation of high-protein legumes reduces dietary N-use efficiency in ruminants. Research has demonstrated that enhancing the level of fermentable carbohydrates in crops entering the silo may reduce protein degradation by increasing the rate of decline in pH. The objective was to evaluate whether delaying cutting time during the day, to allow accumulation of total non-structural carbohydrates (TNC), would inhibit proteolysis in the silo. Red clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.) were harvested at 06.00, 10.00, 14.00 and 18.00 hours in 1993, 1994 and 1995, and ensiled without wilting. TNC accumulated in fresh forage during the day, with starch accounting for more than 0·50 of the daily change in TNC in fresh herbage of both species, except in red clover in 1995. The level of TNC in fresh forage did not consistently affect the extent of protein degradation in either species and, in all instances, alfalfa underwent more extensive proteolysis than red clover. Silage pH typically decreased and starch concentration increased as cutting time was delayed from 06.00 to 18.00 hours. Although the extent of proteolysis was largely unaffected by inherent increases in TNC, harvesting in the afternoon did provide several benefits including increased dry-matter content, lower silage pH and higher starch concentrations. Effluent production is a concern in any unwilted silage system; there was therefore an added advantage of lower moisture content from cutting in the afternoon.

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Introduction

Fresh forage is occasionally ensiled directly to avoid inclement weather, accelerate the harvesting process and improve uniformity of regrowth by eliminating windrows on the ground. Extensive protein degradation during fermentation has been documented in many legumes (McKersie, 1985; Charmley and Thomas, 1987; Albrecht and Muck, 1991). This may reduce the efficiency with which N is utilized by ruminants (Waldo, 1985) and increases the risk of environmental contamination, as excess N is excreted in urine (Tamminga, 1992).

The extent of protein degradation in the silo is influenced by forage species (Papadopoulos and McKersie, 1983; Albrecht and Muck, 1991), pH (Brady, 1961; Finley *et al*., 1980; Scalet *et al*., 1984; McKersie, 1985), dry-matter (DM) content of the crop at ensiling (Carpintero *et al*., 1979; Muck, 1987) and temperature (Brady, 1961; Muck and Dickerson, 1988). Using alfalfa, Muck (1987) found that proteolysis was negatively correlated with DM content, particularly when the DM content exceeded 500 g kg^{-1}. Tannin-containing species, such as birdsfoot trefoil (*Lotus corniculatus* L.), undergo less protein degradation in the silo than species, such as alfalfa, that do not contain tannins (Albrecht and Muck, 1991). Red clover and cicer milkvetch (*Astragalus cicer* L.) were consistently found to undergo less protein degradation than alfalfa during fermentation, although neither species contains measurable levels of tannin (Albrecht and Muck, 1991). Others have also noted more extensive proteolysis during fermentation of alfalfa compared with red clover (Papadopoulos and McKersie, 1983; McKersie, 1985; Jones *et al*., 1995c; Owens *et al*., 1999a;b).

A rapid decline in pH helps limit the extent of protein degradation in the silo by reducing the activity of plant proteases. Although a pH of 6·0 is optimum for most plant proteolytic enzymes, most will maintain 0·15–0·35 of their activity at a pH of 4·0 (Brady, 1961; Finley *et al.*, 1980; McKersie, 1985). Physically, silos must be filled rapidly and packed and sealed thoroughly to promote a rapid drop in pH. Biochemically, anaerobic conditions should be attained as quickly as possible and sufficient substrate must be available for fermentation by lactic acid bacteria. The amount of substrate required depends primarily on buffering capacity (defined as the amount of acid per unit DM needed to reduce forage pH from 6·0 to 4·0) and DM content of the crop at ensiling (Muck, 1988). Legumes typically have a higher buffering capacity than grasses (Pitt, 1990) and, among legumes, alfalfa is usually more highly buffered (McDonald *et al.*, 1991; p.31) and has less fermentable substrate (Raguse and Smith, 1966) than red clover. Consequently, pH values tend to be lower in red clover than alfalfa silage.

The amount of fermentable substrate in forage crops varies seasonally and diurnally. Starch tends to accumulate more in second- than first-growth alfalfa, probably as a result of higher light intensities and temperatures during midsummer (Lechtenberg *et al.*, 1971). Holt and Hilst (1969) and Lechtenberg *et al.* (1971) determined that total non-structural carbohydrates (TNC), comprising sugar and starch in legumes, were lowest early in the morning and increased through the mid to late afternoon. Our objective was to determine whether protein degradation during fermentation was lower in unwilted red clover and alfalfa when forage was harvested later in the day, thus allowing TNC to accumulate and promote a rapid pH decline in the silo.

Materials and methods

Plant material used in this research was harvested in 1993, 1994 and 1995 at the University of Wisconsin, Arlington Agricultural Research Station, Arlington, WI (43°18′N, 89°21′W). 'Marathon' red clover (*Trifolium pratense* L.) and 'Dart' alfalfa (*Medicago sativa* L.) were established in the spring of 1993 on a well drained to moderately-well-drained Plano silt-loam soil (fine-silty, mixed, mesic, Typic Argiudoll). Soil nutrients and pH were maintained at recommended levels for alfalfa throughout the experiment (Kelling *et al.*, 1999). Eptam (S-ethyl dipropylcarbamothioate; Syngenta Crop Protection, Greensboro, NC, USA) was applied to the seedbed before sowing to control weeds during establishment, and malathion (O,O-dimethyldithiophosphate of diethyl mercaptosuccinate; United Agri-Products, Platte Chemical, Greeley, CO, USA) was used as needed to control alfalfa weevil (*Hypera postica* Gyll.) and potato leafhopper (*Empoasca fabae* Harris).

Harvesting and sample preparation

Forage for unwilted silage was harvested with hand clippers on 11 August 1993 (growth cycle succeeding new seeding growth), 30 June 1994 (second growth cycle) and 26 June 1995 (second growth cycle). Plants were cut to a 5-cm stubble height at 06.00, 10.00, 14.00 and 18.00 hours in 1993 and 1994. In 1995, forage was harvested at 06.00, 10.00, and 14.00 hours on 26 June, and the 18.00 hours cut was harvested the next day (27 June) because of inclement weather from approximately 16.00–20.00 hours on 26 June. Environmental conditions on 27 June were similar to the preceding harvest day; therefore, it was decided that samples from the 18.00 hours harvest should be used for analysis. With this exception, harvest days were sunny. Fresh forage from each species and cutting-time was bulked, conditioned by crimping the stem at approximately 2·5-cm intervals, and thoroughly mixed for sampling. Four samples (500 g wet weight) in 1993 and 1994 and three samples (500 g wet weight) in 1995 (each representing a replicate for statistical analyses) were randomly taken from the bulk forage of each species at each cutting time.

The 500 g fresh plant portions were immediately placed on ice and taken into the laboratory where they were chopped into 1-cm pieces with a paper cutter and thoroughly mixed. Approximately 100 g of fresh forage from each sample was placed in a forced air oven for 48 h at 60 °C for DM determination. Two 50-g subsamples were placed in separate 530-ml sterile sampling bags and immediately placed on dry ice. One of these subsamples was later used for determination of total nitrogen (TN) and non-protein nitrogen (NPN) and the other was used for sugar (primarily glucose, fructose and sucrose) (Lechtenberg *et al.*, 1971) and starch analysis. The subsample used to determine N fractions was kept at −70 °C, and the subsample used for determination of sugar and starch was lyophilized and stored at −20 °C until analyses could be completed.

Ensiling

Duplicate 50-g subsamples from the 500-g fresh forage were ensiled using methods described previously (Owens *et al.*, 1999a). Briefly, forage was ensiled in 100-ml polypropylene centrifuge tubes after inoculating with $3{\cdot}3 \times 10^7$ lactic acid bacteria (*Lactobacillus*

plantarum and *Pediococcus cerevisiae*; Chr. Hansen Biosystems, Milwaukee, WI, USA). Silos, with gas traps attached, were placed in a 30 °C water bath for 35 days, after which they were placed in storage at −20 °C until further chemical analyses were performed.

Characterization of fresh forage and unwilted silage

First, 10 g of the frozen fresh forage and unwilted silage were diluted to 100 g with distilled water and macerated for 30 s at high speed in a Waring™ blender. The pH of the homogenate was measured immediately after blending and then prepared for analysis of NPN and fermentation products (Owens *et al*., 1999a). Two 20-ml aliquots were dispensed into separate 50-ml polypropylene centrifuge tubes. Five ml of 25% (w/v) trichloroacetic acid (TCA) was added to one of the tubes to precipitate protein from the solution. Tubes with and without TCA were centrifuged and the supernatant decanted into 20-ml scintillation vials and stored at −20 °C. The solution to which TCA had been added (TCA extract) was evaluated for NPN concentration using the micro-Kjeldahl procedure of Bremner and Breitenbeck (1983). The solution to which TCA had not been added (water extract) was used for determination of fermentation products using high pressure liquid chromatography according to the method of Muck and Dickerson (1988).

The remaining unmacerated material was weighed and dried in a forced-air oven at 60 °C for DM determination in 1993. In 1994 and 1995, unmacerated fresh forage and unwilted silage were lyophilized for DM determination owing to concerns about potential loss or transformation of carbohydrate or N components during drying at 60 °C (Raguse and Smith, 1965). It is suspected that some NH_3 was lost from several of the silage samples during drying at 60 °C in 1993. To correct the data from these samples, values for TN used in statistical analyses were calculated by combining the measurements for TN (measured from oven-dried silage) and NH_3-N (determined from the aqueous extract). In 1994 and 1995, this problem was avoided by lyophilizing the remainder of the frozen material. Oven-dried samples from 1993 and lyophilized samples from 1994 and 1995 were ground to pass a 0·85-mm (20-mesh) screen. Total N was measured on these samples using the micro-Kjeldahl method of Bremner and Breitenbeck (1983). The oven-dried or lyophilized silage samples were also used for sugar and starch analysis. Sugar and starch concentrations in fresh forage were determined using the subsamples that had been lyophilized and stored at −20 °C.

TNC analysis

Sugar extraction and measurement

Sugar and starch were determined using the methods described by Rong *et al*. (1996). Approximately 45-mg lyophilized and ground tissue was rinsed four times with 80% (v/v) ethanol (1·0 ml ethanol per rinse) and centrifuged at 16 000 *g* for 10 min. The supernatants from each rinse were combined and diluted with 80% (v/v) ethanol to a final volume of 10 ml. Anthrone reagent was added to an aliquot containing up to 200-μg free sugar, boiled for 8 min, and then cooled to room temperature in a cold water bath. Absorbance at 625 nm was determined using a Shimadzu UV-1201 spectrophotometer attached to a ASC-5 auto sample changer (Shimadzu Corporation, Kyoto, Japan). Total sugars were expressed as glucose equivalents based on standards containing 0, 5, 10, 50, 100, 150 and 200 μg glucose ml^{-1} 80% ethanol.

Starch hydrolysis and measurement

The tube containing the ethanol-insoluble pellet was placed in a 55 °C oven for 24 h to evaporate any residual ethanol before starch hydrolysis. Distilled deionized water (0·5 ml) was added to each tube and starch gelatinized by boiling samples for 10 min. After cooling to room temperature, 0·4 ml of 0·2 mol l^{-1} Na acetate buffer was added to each tube and starch hydrolysed by adding 1 U amyloglucosidase (Sigma Chemical product A3514) and 40 U α-amylase (Sigma Chemical product A2643) in 0·1 ml of 0·2 mol l^{-1} Na acetate buffer. Tubes were incubated at 55 °C for 24 h. At the conclusion of the incubation period, the samples were centrifuged at 16 000 *g* for 10 min. Glucose released from starch hydrolysis was determined using glucose Trinder reagent (Sigma Chemical Diagnostic kit no. 315). Absorbance was read at 505 nm and the results compared with glucose standards containing 0, 5, 10, 20, 40, 60 and 80 μg glucose ml^{-1} of distilled deionized water. Starch was calculated by multiplying the glucose concentration by 0·9.

Statistical analysis

When duplicate samples were evaluated, the average of the two values was used for statistical analysis. The experiment was conducted as a 2 × 4 factorial, with two levels of species and four levels of cutting time, in a completely randomized design with four replicates in 1993 and 1994, and three replicates in 1995. Numerous year × treatment interactions and other higher order interactions were detected when data were analysed

across years. Some of these interactions resulted in changes in order of treatments; therefore, data were analysed by individual years. Analysis of variance was used to test statistical significance of species, cutting time of day (CT), and species × CT interactions using the general linear model (GLM) of SAS (SAS Institute, 1985). Species and cutting time were treated as fixed effects in the analysis of variance model. Means were separated using Fisher's protected least significant difference (LSD $P = 0{\cdot}05$) when *F*-tests were determined to be significant. The CORR procedure of SAS (SAS Institute, 1985) was used to generate Pearson correlation coefficients to evaluate the relationship of DM, sugar and TNC concentrations of fresh forage with silage NPN levels and silage pH values, and silage pH values with silage NPN concentrations.

Results and discussion

General characteristics of fresh forage and unwilted silage

Forage was cut when the stage of maturity was typical of what would be harvested and preserved as silage on most farms. Red clover was harvested at first flower in 1993 and 1994, and about 0·33 bloom in 1995. Alfalfa was harvested at late bud in 1993 and first flower in 1994 and 1995. The concentration of TN in fresh red clover averaged 35·4–37·0 g kg^{-1} DM and alfalfa 37·8–41·2 g kg^{-1} DM in 1993 (Table 1). Total N concentration was lower in 1994 (Table 2) and 1995 (Table 3) because both species were harvested at more advanced stages of maturity. The concentration of TN in both species tended to decrease slightly throughout each harvest day, in agreement with findings by Youngberg *et al*. (1972) with alfalfa. The decrease in TN concentration throughout the day is likely to be due to the dilution of N compounds by daily increases in non-structural carbohydrates. Total N concentration was slightly higher in silage compared with fresh forage and is the result of dry-matter losses associated with respiration and fermentation. The observed changes are comparable to those reported by Albrecht and Muck (1991) for unwilted red clover and alfalfa silage and by Owens *et al*. (1999b) for wilted red clover and alfalfa silage.

The DM content of fresh forage of both species tended to increase to a maximum by early afternoon, except in 1995, when DM contents were similar throughout the day (Table 3). High temperatures and minimal precipitation for several weeks preceding the 1995 harvest were the most likely reasons for the lack of change in DM content. Clostridial activity, and the associated production of butyrate, is a concern with unwilted silages. Harvesting in the afternoon, to maximize daily DM levels, may help alleviate this problem when fresh forage is ensiled.

The pH of fresh red clover and alfalfa was similar from year to year and across cutting times, ranging from 6·01 to 6·42 (Tables 1–3). The pH dropped by 1·62–2·03 units in red clover and 1·05–2·00 units in alfalfa during ensiling. All silages were well preserved with the exception of three silos in 1993. The presence of mould and accompanying high pH in silage from these silos were indicators of a poor seal and data from these silos were discarded.

Non-protein N concentration in fresh forage and unwilted silage

Non-protein N concentration in fresh forage, on a TN basis, varied little within a species from year to year, ranging from 100 to 130 g kg^{-1} TN in red clover and 112–150 g kg^{-1} TN in alfalfa. Albrecht and Muck (1991) and Owens *et al*. (1999b) reported similar values for NPN concentration in fresh herbage from these species. Averaged across cutting times, fresh alfalfa contained higher ($P < 0{\cdot}05$) concentrations of NPN than fresh red clover in 1994 (Table 2) and 1995 (Table 3), and lower ($P > 0{\cdot}05$) concentrations in 1993 (Table 1). Cutting time did not consistently affect concentration of NPN in fresh red clover or alfalfa, although NPN concentration usually increased slightly as cutting time was delayed from 06.00 to 18.00 hours, particularly in alfalfa in 1994 and 1995.

Protein hydrolysis in the silo resulted in large increases in NPN concentration in the silage of both species. The extent of protein degradation was species-dependent, however, with red clover silage containing 0·31, 0·30 and 0·37 less NPN than alfalfa silage in the three respective harvest years. Although it is generally accepted that plant enzymes are responsible for the hydrolysis of protein to peptides and amino acids before and during the initial stages of ensiling (Oshima and McDonald, 1978), the reasons for differences between species are not fully understood. Jones *et al*. (1995b) concluded that, as inherent proteolytic activity of red clover and alfalfa was not different, other factors were involved in protein protection in red clover. K. A. Albrecht (unpublished observations) observed some protein precipitation associated with red clover extracts and speculated that this would contribute to the mechanism of protein protection during ensiling. Jones *et al*. (1995a) provided evidence that polyphenol oxidase may be involved in the mechanism of protein protection in red clover during ensiling. Polyphenol oxidase oxidizes plant phenols which may then bind to proteins, thus protecting them from degradation by proteolytic enzymes.

Table 1 Mean dry-matter (DM) content, total N (TN), non-protein N (NPN), sugar (primarily glucose, fructose, and sucrose), starch and total non-structural carbohydrate (TNC) concentrations, and pH in fresh forage and unwilted silage harvested on 11 August 1993.

Cutting time (CT) (h)	Fresh forage							Unwilted silage						
	DM (g kg^{-1})	TN (g kg^{-1} DM)	NPN (g kg^{-1} TN)	Sugar† (g kg^{-1} DM)	Starch (g kg^{-1} DM)	TNC (g kg^{-1} DM)	pH	DM (g kg^{-1})	TN (g kg^{-1} DM)	NPN (g kg^{-1} TN)	Sugar (g kg^{-1} DM)	Starch (g kg^{-1} DM)	TNC (g kg^{-1} DM)	pH
Red clover														
06.00	121	36·5	102	84·6	9·6	94·2	6·25	118	37·7	532	5·41	7·1	12·5	4·58
10.00	132	37·0	113	87·6	10·1	97·7	6·26	124	37·7	526	5·54	8·9	14·5	4·34
14.00	139	36·2	112	96·8	25·1	121·9	6·26	132	36·5	520	5·48	27·6	33·1	4·27
18.00	143	35·4	114	104·9	35·6	140·5	6·29	134	36·2	541	5·25	37·8	43·1	4·30
Mean	134	36·3	110	93·5	20·1	113·6	6·26	127	37·0	535	5·42	20·4	25·8	4·40
Alfalfa														
06.00	160	41·2	115	50·3	12·2	62·6	6·37	145	41·4	801	8·36	4·5	12·9	5·32
10.00	186	40·5	113	53·9	19·4	73·3	6·34	168	40·6	778	8·39	9·8	18·2	5·23
14.00	196	37·8	127	61·7	37·3	99·0	6·39	184	38·7	737	8·84	26·0	34·8	4·74
18.00	204	37·9	112	62·8	62·0	124·8	6·42	189	38·8	786	9·68	43·7	53·3	5·08
Mean	186	39·4	117	57·2	32·7	89·9	6·38	172	39·9	776	8·82	21·0	29·8	5·10
Cutting time means														
06.00	141	38·9	109	67·5	10·9	78·4	6·31	131	39·5	677	6·89	5·8	12·7	4·95
10.00	159	38·7	113	70·8	14·8	85·5	6·30	146	39·1	652	7·00	9·4	16·3	4·78
14.00	168	37·0	119	79·2	31·2	110·4	6·32	158	37·6	629	7·16	26·8	33·9	4·51
18.00	173	36·7	113	83·9	48·8	132·7	6·35	161	37·5	663	7·46	40·7	48·2	4·69
LSD‡														
Species (S)		1·19	NS	3·15		4·60	0·031		1·26	19·9		NS	3·18	
Cutting time		1·68	NS	4·46		6·51	NS		NS	28·2		4·29	4·50	
S × CT	10·8	NS	NS	NS	6·83	NS	NS	10·2	NS	NS	0·573	NS	NS	0·187

†Ethanol-soluble sugars expressed on a glucose-equivalent basis.

‡Fisher's protected LSD (0·05) for comparing species and cutting time means. Interaction LSD (0·05), when significant, is used to compare data from any two cutting times within and between species.

NS = not significant.

Table 2 Mean dry-matter (DM) content, total N (TN), non-protein N (NPN), sugar (primarily glucose, fructose and sucrose), starch and total non-structural carbohydrate (TNC) concentrations, and pH in fresh forage and unwilted silage harvested on 30 June 1994.

Cutting time (CT) (h)	Fresh forage							Unwilted silage						
	DM (g kg^{-1})	TN (g kg^{-1} DM)	NPN (g kg^{-1} TN)	Sugar† (g kg^{-1} DM)	Starch (g kg^{-1} DM)	TNC (g kg^{-1} DM)	pH	DM (g kg^{-1})	TN (g kg^{-1} DM)	NPN (g kg^{-1} TN)	Sugar (g kg^{-1} DM)	Starch (g kg^{-1} DM)	TNC (g kg^{-1} DM)	pH
Red clover														
06.00	133	34·8	112	71·4	7·7	79·2	6·14	128	37·4	524	7·6	3·9	11·5	4·38
10.00	158	33·3	100	77·8	13·4	91·2	6·14	151	35·8	514	8·0	10·3	18·3	4·29
14.00	167	32·6	120	85·6	32·2	117·8	6·17	165	35·7	482	9·0	27·7	36·7	4·17
18.00	177	31·6	113	89·0	41·9	130·9	6·06	175	34·6	478	9·4	40·9	50·3	4·12
Mean	159	33·1	111	81·0	23·8	104·8	6·13	155	35·9	500	8·5	20·7	29·2	4·24
Alfalfa														
06.00	177	33·9	119	55·6	14·3	69·8	6·36	170	35·9	735	10·5	5·9	16·4	4·69
10.00	210	33·0	124	58·3	26·4	84·8	6·29	204	35·9	703	11·0	15·0	25·9	4·51
14.00	218	32·8	128	62·5	47·6	110·2	6·32	212	34·9	709	12·1	34·0	46·1	4·44
18.00	221	33·3	136	60·6	60·6	121·2	6·37	218	35·3	711	12·4	45·0	57·4	4·37
Mean	207	33·2	127	59·3	37·2	96·5	6·33	201	35·5	714	11·5	25·0	36·4	4·50
Cutting time means														
06.00	155	34·3	115	63·5	11·0	74·5	6·25	149	36·7	630	9·1	4·9	13·9	4·54
10.00	184	33·1	112	68·1	19·9	88·0	6·22	178	35·9	608	9·5	12·6	22·1	4·40
14.00	192	32·7	124	74·1	39·9	114·0	6·24	189	35·3	596	10·5	30·9	41·4	4·30
18.00	199	32·5	124	74·8	51·3	126·1	6·22	196	35·0	595	10·9	43·0	53·9	4·24
LSD‡														
Species (S)	5·2	NS	7·0	3·21	3·69	5·50		4·8	NS	18·2	0·51	3·14	3·37	0·068
Cutting time	7·4	1·05	10·0	4·54	5·22	7·78		6·8	1·22	25·7	0·72	4·45	4·76	0·096
S × CT	NS	NS	NS	NS	NS	NS	0·062	NS	NS	NS	NS	NS	NS	NS

†Ethanol-soluble sugars expressed on a glucose-equivalent basis.

‡Fisher's protected LSD (0·05) for comparing species and cutting time means. Interaction LSD (0·05), when significant, is used to compare data from any two cutting times within and between species.

NS = not significant.

Table 3 Mean dry-matter (DM) content, total N (TN), non-protein N (NPN), sugar (primarily glucose, fructose, and sucrose), starch and total non-structural carbohydrate (TNC) concentrations, and pH in fresh forage and unwilted silage harvested on 26 and 27 June 1995.

Cutting time (CT) (h)	Fresh forage							Unwilted silage						
	DM (g kg^{-1})	TN (g kg^{-1} DM)	NPN (g kg^{-1} TN)	Sugar† (g kg^{-1} DM)	Starch (g kg^{-1} DM)	TNC (g kg^{-1} DM)	pH	DM (g kg^{-1})	TN (g kg^{-1} DM)	NPN (g kg^{-1} TN)	Sugar (g kg^{-1} DM)	Starch (g kg^{-1} DM)	TNC (g kg^{-1} DM)	pH
Red clover														
06.00	201	29·1	110	98·6	6·7	105·2	6·02	198	29·3	425	13·7	6·2	19·9	4·01
10.00	215	28·5	115	102·7	7·1	109·8	6·03	213	29·4	419	18·0	8·8	26·8	4·01
14.00	211	27·3	116	121·6	13·8	135·4	6·01	205	28·6	449	18·7	16·8	35·5	3·98
18.00	197	26·7	130	99·3	21·7	121·0	6·03	192	28·0	503	11·7	26·8	38·5	4·05
Mean	206	27·9	118	105·5	12·3	117·8	6·02	202	28·8	449	15·5	14·7	30·2	4·01
Alfalfa														
06.00	220	31·6	132	65·9	14·6	80·5	6·02	212	33·8	738	13·6	12·8	26·4	4·72
10.00	239	30·6	130	68·2	25·4	93·6	6·06	238	31·8	690	15·7	26·1	41·8	4·37
14.00	235	29·5	136	71·4	37·9	109·3	6·05	234	31·5	677	14·7	39·9	54·6	4·33
18.00	236	29·1	150	66·2	48·3	114·5	6·07	232	31·2	725	13·4	43·1	56·6	4·45
Mean	233	30·2	137	67·9	31·5	99·5	6·05	229	32·1	708	14·3	30·5	44·8	4·46
Cutting time means														
06.00	210	30·4	121	82·3	10·6	92·9	6·02	205	31·6	581	13·6	9·5	23·2	4·37
10.00	227	29·5	122	85·5	16·2	101·7	6·05	225	30·6	555	16·8	17·4	34·3	4·19
14.00	223	28·4	126	96·5	25·9	122·4	6·03	220	30·0	563	16·7	28·3	45·1	4·15
18.00	216	27·9	140	82·7	35·0	117·7	6·05	212	29·6	614	12·6	35·0	47·6	4·25
LSD‡														
Species (S)	6·8	1·46	9·1	7·07		7·40	NS	6·5	1·22		NS		3·87	
Cutting time	9·6	NS	12·9	10·00		10·46	NS	9·3	NS		NS		5·47	
S × CT	NS	NS	NS	NS	3·64	NS	NS	NS	NS	31·1	NS	4·35	NS	0·093

†Ethanol-soluble sugars expressed on a glucose-equivalent basis.

‡Fisher's protected LSD (0·05) for comparing species and cutting time means. Interaction LSD (0·05), when significant, is used to compare data from any two cutting times within and between species.

NS = not significant.

There was a significant ($P < 0{\cdot}05$) cutting time effect on NPN concentrations in unwilted silage in 1993 (Table 1) and 1994 (Table 2) and a species × cutting time interaction in 1995 (Table 3), but the magnitude and nature of these effects were variable. In 1993, silage NPN concentrations decreased as cutting time was delayed from 06.00 to 14.00 hours, then increased in silage from the 18.00 hours cutting to levels similar to the early morning (Table 1). Although the NPN concentration of silage from both species tended to decrease at each successive cutting time in 1994, the concentration of NPN in silage from the 10.00, 14.00 and 18.00 hours harvests was not significantly different (Table 2). In 1995, red clover silage harvested at 18.00 hours contained a greater concentration of NPN than other cutting times, while alfalfa silage from the 06.00 and 18.00 hours cutting times had similar levels of NPN resulting in a species × cutting time interaction (Table 3). It is speculated that a more rapid decline in pH would have occurred in forage harvested later in the day by increasing the level of TNC, especially sugars, going into the silo, thus inhibiting the extent of protein degradation during fermentation. Even though delaying cutting time did not result in large increases in sugar concentration in either species, the possibility is not excluded that addition of a rapidly fermentable substrate could increase the rate of decline in pH and reduce proteolysis in the silo. Similar to work by Owens *et al.* (1999b) with wilted red clover and alfalfa silage, the data here indicate that the effect of harvesting later in the day, to achieve higher concentrations of TNC, on silage NPN concentrations is not consistent or great enough by itself to warrant its use as a management tool for decreasing proteolysis during fermentation of unwilted red clover and alfalfa.

Generally non-significant correlations ($P > 0{\cdot}05$) between silage NPN concentration and sugar or TNC concentrations of fresh forage provide further evidence that inherent increases in TNC concentration during the day will not consistently inhibit protein degradation in the silo (Table 4). In 1994, when the sugar concentration of fresh forage was at the lowest level of the three harvest years, a significant ($P < 0{\cdot}05$) negative correlation was detected. This indicates that management practices to maintain or increase sugar concentrations of fresh forage, or addition of readily fermentable substrate at the silo, may affect the decline in pH and subsequently reduce proteolysis when the sugar concentration of fresh forage is low. The relationship between silage NPN concentration and sugar or TNC concentration of fresh forage tended to be stronger for alfalfa than red clover because of higher sugar concentrations (Tables 1–3) and lower buffering capacity of red clover (McDonald *et al.*, 1991). Therefore, addition of fermentable substrate may have a greater beneficial impact on proteolysis in fresh ensiled alfalfa by effecting a more rapid and extensive decline in pH, particularly at low DM contents associated with unwilted alfalfa.

Although the DM content at initiation of ensiling is known to affect proteolysis in the silo (Muck, 1987), the relationship between silage NPN concentration and DM content at ensiling was not consistent (Table 4). This relationship was significant ($P < 0{\cdot}05$) in red clover in 1993 (DM content of 121–143 g kg^{-1}) and 1994 (DM content of 133–177 g kg^{-1}) when DM levels were very low, but not ($P > 0{\cdot}05$) in 1995 when the DM content

Table 4 Simple correlation (*r*) of dry-matter (DM) content, concentrations of sugars and total non-structural carbohydrates (TNC) of fresh forage with non-protein N (NPN) concentration and pH of unwilted silage, and silage pH with silage NPN concentration.

		r					
		Red clover			Alfalfa		
Independent variable	Dependent variable	1993	1994	1995	1993	1994	1995
DM†	NPN‡	−0·65**	−0·68**	−0·48	−0·40	−0·45	−0·61*
DM	pH	−0·84***	−0·88***	−0·80**	−0·59*	−0·71**	−0·88***
Sugar	NPN	−0·12	−0·55*	−0·01	−0.49	−0·73**	−0·49
Sugar	pH	−0·47	−0·86***	−0·48	−0.66**	−0·54*	−0·47
TNC	NPN	−0·10	−0·60*	−0·38	−0·18	−0·46	−0·30
TNC	pH	−0·53*	−0·89***	−0·35	−0·48	−0·73**	−0·65*
pH	NPN	0·64**	0·81***	0·26	0·66**	0·61*	0·83***

*Significant at 0·05 level of probability; **significant at 0·01; ***significant at 0·001. Values followed by no indication of significance are not significant.

†Data for DM, sugar and TNC are taken from fresh forage.

‡Data for NPN and pH are taken from unwilted silage.

did not fall below 197 g kg^{-1}. Silage pH was positively correlated with NPN concentration, with the exception of red clover in 1995 (Table 4). Consequently, NPN and pH levels tended to be lower in silage from forage harvested later in the day when the sugar and TNC concentrations were greatest, despite the fact that the opposite trend occurred for NPN concentration in fresh forage. The effect of pH on protein degradation agrees with other research on alfalfa and red clover silage (Brady, 1961; McKersie, 1985; Jones *et al*., 1995b), and demonstrates the complexity and interactive effects of DM and fermentable substrate on pH decline and protein hydrolysis during the fermentation process.

Total non-structural carbohydrates, sugar and starch in fresh forage and unwilted silage

The concentration of TNC in freshly cut forage increased throughout the day, particularly during peak solar radiation of midday (Tables 1–3). Lechtenberg *et al*. (1971) observed a linear increase in TNC from 06.00 to 18.00 hours in first and second growth alfalfa. The TNC concentration of fresh red clover was consistently greater than alfalfa as a result of higher sugar concentrations. Higher sugar concentrations in red clover compared with alfalfa were observed by Raguse and Smith (1966), especially when forage was harvested at the early bud to green pod stages of maturity. Our results concur with those of Raguse and Smith (1966) as forage was harvested within the range of maturity stages they evaluated.

In contrast with sugar concentrations, quantitative increases in starch concentrations were greater in alfalfa than red clover from 06.00 to 18.00 hours. Close examination of the data in Tables 1–3 reveals that starch accounted for 0·79–0·92 of the total increase in TNC concentration in alfalfa and 0·39–0·57 in red clover. In other research with alfalfa, Lechtenberg *et al*. (1971) reported that starch accounted for 0·65 of the daily increase in TNC concentration. As starch is the primary storage carbohydrate of both species, it is reasonable that at peak periods of photosynthesis (mid-morning to late afternoon) excess photosynthate production would result in increased starch synthesis and storage in leaves.

Total non-structural carbohydrates decreased by 0·62–0·86 in red clover and 0·50–0·79 in alfalfa during fermentation, and in silage from both species the concentration of TNC increased as cutting time was delayed from 06.00 to 18.00 hours (Tables 1–3). A reduction in sugar concentration was primarily responsible for the decline in TNC concentration in both species.

The majority of starch is degraded when forages are wilted in the field before ensiling (Owens *et al*., 1999b). Starch concentrations are less likely to decline during fermentation because most lactic acid bacteria are not able to utilize it directly (McDonald *et al*., 1991; p. 251). Some starch (Melvin, 1965) or other complex carbohydrates (Dewar *et al*., 1963), however, may be hydrolysed for use by lactic acid bacteria. In several cases (red clover harvested at 14.00 and 18.00 hours in 1993 and 10.00, 14.00 and 18.00 hours in 1995 and alfalfa harvested at 10.00 and 14.00 hours in 1995) starch concentration increased slightly during ensiling, suggesting that little or no hydrolysis occurred. Muck (1990) noted that the rate of starch hydrolysis in the silo was proportional to the starch concentration at the beginning of ensiling and decreased linearly with time. Consequently, the amount of starch used for fermentation was probably greater in alfalfa harvested in the afternoon, except in 1995 when little change in starch concentration was observed in alfalfa silage from all cuttings (Table 3). Muck (1990) also found that adding glucose to alfalfa reduced starch hydrolysis in the silo. Similarly, owing to lower sugar concentrations in fresh forage, a greater quantity of starch was utilized in alfalfa compared with red clover during the fermentation process. In two instances (alfalfa harvested at 06.00 hours in 1993 and 1994) starch concentration decreased by more than 0·50 during ensiling. Declines of this magnitude can be attributed primarily to two factors: (i) the low concentrations of sugar and starch in fresh alfalfa harvested at 06.00 hours; and (ii) the low DM content of fresh forage harvested early in the morning (160 g kg^{-1} in 1993 and 177 g kg^{-1} in 1994 compared with 220 g kg^{-1} in 1995).

Higher concentrations of starch in fresh-ensiled alfalfa and red clover harvested in the afternoon could prove useful in balancing the ration, if a significant portion of the diet comes from forages. In the USA, rations for high producing dairy cows typically contain approximately 0·50 forage with the remainder as cereals, such as corn grain (≈0·72 starch). Averaged over the 3 years of the current experiment, starch concentrations in unwilted silage from the 18.00 hours harvest were 29·5 g kg^{-1} DM higher in red clover and 36·2 g kg^{-1} DM higher in alfalfa than the respective silage made from herbage harvested early in the morning. From these results, we can calculate that by ensiling fresh red clover and alfalfa later in the afternoon, a producer could replace approximately 0·04–0·05 of the starch in corn with starch from unwilted red clover and alfalfa silage. High moisture contents and subsequent effluent production may be a problem with direct-cut silage, but in dry regions where standing alfalfa often contains greater than 25 g kg^{-1} DM, direct-cut silage could be a viable option.

pH and fermentation products of fresh forage and unwilted silage

Silage pH ranged from 3·98 to 4·58 in red clover and 4·33–5·32 in alfalfa and was usually highest in forage

Table 5 Concentration of fermentation products in unwilted red clover and alfalfa silage (g kg^{-1} DM).

Cutting time (CT) (h)	1993							1994							1995						
	Lac†	Ace	Suc	Pro	For	But	Eth	Lac	Ace	Suc	Pro	For	But	Eth	Lac	Ace	Suc	Pro	For	But	Eth
Red clover																					
06.00	90·2	44·7	12·8	0·47	ND‡	ND	ND	89·5	31·2	5·39	0·58	ND	ND	1·86	103·6	11·5	1·97	0·14	4·19	ND	1·64
10.00	114·7	28·2	8·6	ND	ND	ND	ND	85·7	25·1	5·21	1·84	ND	ND	1·77	102·4	7·8	1·21	0·30	3·42	ND	1·29
14.00	113·7	21·9	6·7	0·54	ND	ND	ND	87·8	17·1	4·01	ND	0·39	ND	2·13	104·2	9·2	2·12	0·27	3·02	ND	1·77
18.00	120·8	27·0	11·3	0·67	1·54	ND	0·39	84·8	16·2	3·58	0·30	ND	ND	1·57	112·9	16·4	3·74	ND	3·44	ND	1·83
Mean	109·9	30·4	9·8	0·42	0·39	ND	0·10	86·9	22·4	4·55	0·68	0·10	ND	1·83	105·8	11·2	2·26	0·18	3·52	ND	1·63
Alfalfa																					
06.00	36·6	67·0	20·9	0·31	3·49	1·49	4·87	58·3	32·9	4·32	0·50	2·31	ND	4·32	63·0	36·2	4·53	0·14	0·56	ND	2·24
10.00	45·5	58·1	24·0	1·16	1·08	ND	5·08	70·8	23·8	3·64	0·29	ND	ND	4·27	71·0	13·6	1·76	0·36	1·11	ND	1·42
14.00	81·2	32·1	7·0	ND	3·49	ND	2·47	68·9	31·3	4·11	ND	ND	ND	4·71	71·9	12·5	1·74	0·15	0·89	ND	1·61
18.00	61·6	40·2	21·9	1·51	0·76	0·61	5·28	73·9	15·7	3·24	0·26	ND	ND	3·74	78·5	19·4	4·27	0·31	ND	ND	3·10
Mean	56·2	49·4	18·4	0·75	2·20	0·52	4·42	68·0	25·9	3·83	0·26	0·58	ND	4·26	71·1	20·4	3·07	0·24	0·64	ND	2·09
Cutting time means																					
06.00	63·4	55·9	16·8	0·39	1·74	0·75	2·44	73·9	32·0	4·86	0·54	1·16	ND	3·09	83·3	23·8	3·25	0·14	2·37	ND	1·94
10.00	80·1	43·1	16·3	0·58	0·54	ND	2·54	78·3	24·5	4·42	1·06	ND	ND	3·02	86·7	10·7	1·49	0·33	2·27	ND	1·36
14.00	97·4	27·0	6·8	0·27	1·74	ND	1·23	78·3	24·2	4·06	ND	0·20	ND	3·42	88·1	10·9	1·93	0·21	1·96	ND	1·69
18.00	91·2	33·6	16·6	1·09	1·15	0·30	2·84	79·4	16·0	3·41	0·28	ND	ND	2·65	95·7	17·9	4·01	0·16	1·72	ND	2·47
LSD§																					
Species (S)		6·16		NS		NS			NS	NS	NS			1·531	5·19		NS	NS	0·689		NS
Cutting time		8·71		NS		NS			NS	NS	NS			NS	7·34		1·375	NS	NS		NS
S × CT	14·97	NS	5·06	NS	2·411	NS	1·072	8·00	NS	NS	NS	0·449		NS	NS	9·23	NS	NS	NS		NS

†Lac = lactate, Ace = acetate, Suc = succinate, Pro = propionate, For = formate, But = butyrate, and Eth = ethanol.

‡ND = none detected.

§Fisher's protected LSD (0·05) for comparing species and cutting time means. Interaction LSD (0·05) is used to compare data from any two cutting times within and between species.

NS = not significant.

harvested at 06.00 hours and lowest in forage harvested in the afternoon (Tables 1–3). A species × cutting time interaction for silage pH was detected in 1993 (Table 1) and 1995 (Table 3). In both instances, the pH of alfalfa silage decreased as cutting time went from 06.00 to 14.00 hours and then increased between 14.00 and 18.00 hours, whereas the pH of red clover silage remained low at the 18.00 hours harvest.

Higher sugar concentrations in fresh forage were associated with larger pH reductions in red clover than alfalfa during ensiling in all 3 years, regardless of time of day at harvest (Tables 1–3). For this reason, cutting time appeared to have a greater impact on the decline in pH in alfalfa than red clover. The relationship between fresh herbage TNC concentration and silage pH resulted in similar correlation values for both species, particularly in forage with low DM contents harvested in 1993 and 1994 (Table 4). Although a greater decline in pH of silage harvested in the afternoon did not consistently reduce proteolysis in the silo, a lower pH will help maintain forage quality and extend the life of unwilted silage in the silo by inhibiting the activity of other detrimental micro-organisms.

A significant ($P < 0{\cdot}05$) negative relationship was detected between silage pH and DM content of fresh forage (Table 4). Silage pH usually increases with increasing DM content because lactic acid bacterial activity ceases at a higher pH (Muck, 1988). However, in this research the level of fermentable substrate increased concurrently with DM content throughout the day, resulting in a greater decline in pH in the drier forage harvested in the afternoon.

Lactate and acetate are the predominant end-products of a successful fermentation, and the concentrations of these two acids were greater than other fermentation products in all silages. As lactate is a stronger acid than acetate, a desirable fermentation is one in which the lactate:acetate ratio (LAR) is 2·0 or greater (Pitt, 1990). A LAR less than 2·0 can be an indicator of insufficient sugars for fermentation, although this is not the only evidence of improperly preserved silage.

Lactate generally increased and acetate decreased as cutting time in the day was delayed (Table 5). Therefore, the LAR of silage from both species also tended to increase at later cutting times (Table 6). Red clover silage consistently attained an LAR greater than 2 and was consistently higher than that of alfalfa silage, regardless of cutting time. The LAR of alfalfa silage from the 06.00 hours harvest was always 2·0 or less as a result of low sugar concentration, high buffering capacity (McDonald *et al.*, 1991) and low DM content. In 1993, alfalfa silage from both morning harvests was particularly low in lactate and high in acetate resulting in LAR values < 1·0 and pH values > 5·0. Silage from these samples was extremely pungent due to the high acetate content. These results, in conjunction with silage data from other years, demonstrate that fresh alfalfa harvested early in the morning in humid environments does not contain sufficient DM and sugars for adequate fermentation.

Cutting time had a greater and more consistent enhancing effect on lactate production in alfalfa than red clover silage. This is not surprising as alfalfa is more likely than red clover to be deficient in fermentable substrate. At the start of ensiling, bacteria produce a substantial quantity of acetate, but as the pH decreases there are changes in the population of silage microorganisms, and a shift to the predominant production of lactate occurs (Muck, 1990). Under conditions of low sugar availability, such as in alfalfa harvested early in the morning, a smaller fraction of lactate would be produced, as much of the sugar would be used to generate acetate. In 1993 and 1994, alfalfa silage from the afternoon harvests contained a greater concentration of lactate and lower level of acetate as a direct result of higher TNC concentration and DM content in fresh forage.

Table 6 Lactate:acetate ratio in unwilted red clover and alfalfa silage. Forage was harvested at 06.00, 10.00, 14.00 and 18.00 hours and ensiled without wilting.

Cutting time (CT)	1993	1994	1995
Red clover			
06.00	2·18	2·89	9·08
10.00	4·26	3·44	13·21
14.00	5·21	5·16	12·07
18.00	4·46	5·27	7·00
Mean	4·03	4·19	10·34
Alfalfa			
06.00	0·55	1·78	2·00
10.00	0·79	3·02	5·22
14·00	2·54	3·40	5·85
18.00	1·59	5·00	4·05
Mean	1·36	3·30	4·28
Cutting time means			
06.00	1·37	2·33	5·54
10.00	2·52	3·23	9·22
14.00	3·87	4·28	8·96
18.00	3·03	5·14	5·53
LSD†			
Species (S)	0·46	0·84	1·40
Cutting time	0·65	1·18	1·98
S × CT	NS	NS	NS

†Fisher's protected LSD (0·05) for comparing species and cutting time means. Interaction LSD (0·05), when significant, is used to compare data from any two cutting times within and between species.
NS = not significant.

Delaying harvest of alfalfa or red clover from morning to the afternoon to produce a forage with higher TNC concentration provided no major or consistent benefit in terms of protein protection in the silo; however, silage pH tended to decrease and starch levels were higher as cutting time was delayed from 06.00 to 18.00 hours. The pH of alfalfa silage was consistently higher than red clover silage as a result of lower sugar levels available for fermentation and a greater buffering capacity in alfalfa (McDonald *et al*., 1991; p. 31). Extensive protein degradation resulted in significantly higher NPN levels in alfalfa compared with red clover regardless of cutting time. Although delaying harvest did not greatly improve protein protection in the silo, forage harvested in the afternoon may be better preserved and of increased quality because of lower silage pH and higher starch concentrations.

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