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Protein Degradation and Fermentation Characteristics of Red Clover and Alfalfa Silage Harvested with Varying Levels of Total Nonstructural Carbohydrates

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ABSTRACT

Extensive degradation of protein during fermentation of high-protein crops reduces efficiency of dietary N utilization in ruminants. Evidence suggests that enhanced levels of fermentable carbohydrates can reduce proteolysis. Our objective was to evaluate whether delaying daily cutting time, to allow total nonstructural carbohydrates (TNC) to accumulate, would inhibit protein degradation by way of greater acid production in the silo. Red clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.) were harvested at 0600, 1000, 1400, and 1800 h in 1993, 1994, and 1995 and wilted to a dry matter (DM) content of 350 g kg^{-1} before ensiling. The level of TNC in fresh forage of both species increased throughout the day. Starch accounted for most of the daily change in TNC in fresh alfalfa, whereas in red clover, quantitative increases in sugar and starch impacted TNC similarly. Level of TNC at initiation of ensiling did not consistently affect protein degradation during fermentation as confirmed by generally insignificant correlation coefficients. The extent of proteolysis in the silo was consistently greater in alfalfa than red clover. Silage pH typically decreased and starch increased as cutting time was delayed from 0600 to 1800 h. While the extent of proteolysis was largely unaffected by inherent increases in TNC, lower silage pH and higher starch concentrations indicate that silage from the afternoon cuttings may be better preserved and higher in quality.

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Published in Crop Sci. 39:1873–1880 (1999).

A KEY OBJECTIVE of forage preservation as hay or silage is to minimize quality and dry matter (DM) losses. A major disadvantage to preserving forage as hay is the risk of exposure to adverse weather conditions since several days are usually required for drying. Much of the forage grown in humid regions of the USA, where the climate is not conducive to hay making, is preserved as silage. Harvest and quality losses associated with inclement weather are usually lower in silage because drying time is typically reduced by at least 50% compared with making hay. Quality reductions due to detrimental microbial activity are minimized in silage by excluding oxygen from the silo and rapidly attaining a pH of 3.8 to 5.0 (Pitt, 1990).

Preservation of high quality legumes as silage is limited by extensive protein degradation that occurs in many forage species during fermentation (McKersie, 1985; Charmley and Thomas, 1987; Albrecht and Muck, 1991). Of the total N (TN) in fresh forages, 75 to 90% is protein N (PN), and the remainder is nonprotein N (NPN) (Oshima and McDonald, 1978). After ensiling, NPN may account for as much as 80% of TN (Papadopoulos and McKersie, 1983; Albrecht and Muck, 1991). Waldo (1985) concluded that extensive protein degradation in the silo may result in lower dry matter intake by ruminants and reduce the efficiency with which N is

Abbreviations: NPN, nonprotein nitrogen; PN, protein nitrogen; TN, total nitrogen; TNC, total nonstructural carbohydrates.

utilized. Inefficient forage N utilization by ruminants also poses potential environmental problems since excess N is excreted in urine (Tamminga, 1992).

Protein degradation in the silo is influenced by several factors including forage species (Papadopoulos and McKersie, 1983; Albrecht and Muck, 1991), pH (Brady, 1961; Finley et al., 1980; Scalet et al., 1984; McKersie, 1985), DM content of the crop at ensiling (Carpintero et al., 1979; Muck, 1987), and temperature (Brady, 1961; Muck and Dickerson, 1988). Albrecht and Muck (1991) evaluated several legumes and detected a significant negative relationship between tannin concentration and NPN in the silo. Red clover and cicer milkvetch (*Astragalus cicer* L.) consistently underwent less protein degradation than alfalfa during fermentation although neither of these species contains measurable levels of tannins. Other researchers have also noted that the increase in NPN during ensiling is lower in red clover than alfalfa (Papadopoulos and McKersie, 1983; McKersie, 1985; Jones et al., 1995c).

The pH of well fermented silage should rapidly fall to 5 or less to maintain forage quality and limit the extent of protein degradation. While not completely prevented, the activity of plant proteinases decreases linearly between pH 6 and 4 (McKersie, 1985; Jones et al., 1995a). Management practices used to promote a rapid pH decline during ensiling include rapid filling, good packing, and good sealing (Muck, 1988). In addition, it is critical that an anaerobic environment be achieved as quickly as possible and that sufficient substrate be available for fermentation by lactic acid bacteria. The amount of substrate necessary for successful fermentation depends primarily on buffering capacity (defined as the amount of acid needed to reduce forage pH from 6 to 4 per unit DM) and DM content of the crop at ensiling (Muck, 1988). The buffering capacity of legumes is typically higher than that of 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 affects the extent of pH decline, which in turn may affect proteolysis in the silo. Naturally available substrate for fermentation varies diurnally. Total nonstructural carbohydrates (TNC), including sugar and starch, are lowest early in the morning and increase through mid to late afternoon (Holt and Hilst, 1969; Lechtenberg et al., 1971). We hypothesized that forage legumes harvested later in the day would contain greater levels of available substrate for fermentation, thus reducing the extent of protein degradation in the silo. Therefore, our objective was to determine whether delaying cutting time during the day, to enhance TNC accumulation, would inhibit protein degradation as a result of greater acid production 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 and 'Dart' alfalfa 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. Eptam (*S*-ethyl dipropylcarbamothioate) was applied preplant incorporated to control weeds during establishment, and malathion (*O*,*O*-dimethyldithiophosphate of diethyl mercaptosuccinate) was used as needed to control alfalfa weevil (*Hypera postica* Gyll.) and potato leafhopper (*Empoasca fabae* Harris).

Harvesting and Sample Preparation

Wilted silage was made from forage harvested on 24 Aug. 1993 (growth cycle succeeding new seeding growth), 27 May 1994 (first growth cycle), and 26 June 1995 (second growth cycle). Plants were clipped to a 5-cm stubble height at 0600, 1000, 1400, and 1800 h in 1993 and 1994. In 1995, forage was harvested at 0600, 1000, and 1400 h on 26 June, and the 1800-h cutting was taken the next day (27 June) because of cloudy and rainy weather the afternoon of 26 June. 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 in 1993 and 1994 and three samples in 1995 (each representing a replicate for statistical analyses) were randomly taken from the bulk forage of each species at each cutting time. Each of the fresh forage samples was further divided into two portions: (i) approximately 900 g (wet weight) was immediately placed on wire screens elevated approximately 4 cm off the ground and allowed to wilt in the field to a DM content of 350 g kg^{-1}; and (ii) 500 g (wet weight) was used for analysis of fresh herbage characteristics. A microwave oven was used to determine rapidly the DM content of 100-g fresh forage in order to calculate the amount of water loss required to reach the targeted DM level.

The 500-g fresh plant portions were immediately placed on ice and taken into the lab where they were chopped into 1-cm pieces with a paper cutter and then thoroughly mixed. Approximately 100-g 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 TN and 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. Wilted forage samples were taken into the lab, chopped into 1-cm pieces, and thoroughly mixed. Two 50-g subsamples (one for TN and NPN and one for sugar and starch) were treated in the same manner as fresh forage. A separate 100-g subsample was dried in a forced-air oven for 48 h at 60°C for DM determination.

Ensiling

Duplicate 50-g subsamples of wilted forage were ensiled using methods described previously (Owens et al., 1999). Briefly, forage was ensiled in 100-mL polypropylene centrifuge tubes after inoculating with appropriate lactic acid bacteria. Silos, with gas traps attached, were placed in a 30°C water bath for 35 d and then stored at −20°C until further chemical analyses were performed.

Characterization of Fresh Forage, Wilted Forage, and Wilted Silage

Ten grams of the frozen fresh forage, wilted forage, and wilted 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, then the homogenate was prepared for analysis of NPN and fermentation products (Owens et al., 1999). Two 20-mL aliquots were dispensed into separate 50-mL polypropylene centrifuge tubes. Five milliliters 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 by 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 determinations in 1993. In 1994 and 1995, unmacerated fresh forage, wilted forage, and wilted silage were lyophilized for DM determination due to concerns with the loss or transformation of carbohydrate or N components during drying at 60°C (Raguse and Smith, 1965). We 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 with an intermediate Thomas-Wiley mill. Total N was measured on these samples by 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 from the subsamples that had been lyophilized and stored at −20°C.

TNC Analysis

Sugar Extraction and Measurement. Sugar and starch were determined by 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), centrifuged, and the supernatant decanted into a graduated test tube. 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 with 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, 1 U amyloglucosidase (Sigma Chemical product A3514) and 40 U α-amylase (Sigma Chemical product A2643) in 200 mM acetate buffer (pH 5.0) were added to each tube and 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 with glucose Trinder reagent (Sigma Chemical Diagnostic kit No. 315). Absorbance was read at 505 nm and the results compared to glucose standards containing 0, 5, 10, 20, 40, 60, and 80 μg glucose mL^{-1} 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 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 analyzed across years. Some of these interactions resulted in changes in order of treatments; therefore, data were analyzed by individual years. Analysis of variance was used to test statistical significance of species, cutting time of day (CT), and species × CT interaction using the general linear model (GLM) of SAS (SAS Institute, Cary, NC). Species and cutting time were treated as fixed effects in the analysis of variance model. Means were separated by Fisher's protected least significant difference (LSD $P = 0.05$) when *F*-tests were determined to be significant. The CORR procedure of SAS (SAS Institute, Cary, NC) was used to generate Pearson correlation coefficients to evaluate the relationship of sugar and TNC concentrations of wilted 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, Wilted Forage, and Wilted Silage

Forage was cut when the stage of maturity was typical of what would be harvested and preserved as silage in the North Central USA. Red clover was harvested at about 33% bloom stage in 1993, at early bud stage in 1994, and at 50% bloom stage in 1995. Alfalfa was harvested at early-bud stage in 1993, at late-bud stage in 1994, and at 10% bloom stage in 1995. Total N for fresh red clover ranged from 27 to 35 g kg^{-1} DM and for alfalfa ranged from 29 to 49 g kg^{-1} DM, and was lowest in 1995 because both species were harvested at more advanced stages of maturity (Tables 1 to 3). The concentration of TN in both species tended to decrease slightly during the day, in agreement with findings by Youngberg et al. (1972) with alfalfa. The decrease in TN is likely attributed to dilution of N compounds during daytime fixation of carbon into nonstructural carbohydrates. Loss of TNC during wilting caused the level of TN in wilted forage to be more similar across cutting times within each harvest, although a trend of decreasing TN with later cutting times was still present (Tables 1 to 3). Dry matter losses associated with respiration and fermentation resulted in slightly higher levels of TN in silage. The observed changes are comparable to those reported by Albrecht and Muck (1991) for unwilted red clover and alfalfa silage.

The DM content of fresh forage was lowest at 0600 h and tended to increase to a maximum by early after-

Table 1. Means for dry matter (DM) content, total N (TN), nonprotein N (NPN), sugar (primarily glucose, fructose, and sucrose), starch, total nonstructural carbohydrate (TNC), and pH in fresh and wilted forage and wilted silage harvested 24 Aug. 1993.

Cutting Time (CT)	Fresh forage							Wilted forage							Wilted silage						
	DM	TN	NPN	Sugar†	Starch	TNC	pH	DM	TN	NPN	Sugar	Starch	TNC	pH	DM	TN	NPN	Sugar	Starch	TNC	pH
	g kg^{-1}	g kg^{-1} DM	g kg^{-1} TN	— g kg^{-1} DM —				g kg^{-1}	g kg^{-1} DM	g kg^{-1} TN	— g kg^{-1} DM —				g kg^{-1}	g kg^{-1} DM	g kg^{-1} TN	— g kg^{-1} DM —			
Red clover																					
0600	131	33	122	80	4	84	6.52	340	33	198	69	2	71	6.52	337	34	333	3	1	4	4.43
1000	155	35	129	86	6	91	6.62	348	35	186	71	2	73	6.64	340	36	335	3	1	4	4.41
1400	171	31	150	81	11	92	6.64	338	34	198	71	4	75	6.56	327	35	376	4	1	5	4.27
1800	167	32	144	91	18	109	6.50	370	34	180	71	3	74	6.60	372	35	366	4	1	6	4.29
Mean	156	33	136	84	10	94	6.57	349	34	190	71	3	74	6.58	344	35	353	4	1	5	4.35
Alfalfa																					
0600	135	49	142	75	2	77	6.42	371	50	281	57	1	58	6.45	361	50	624	8	0	8	5.05
1000	162	47	142	84	5	89	6.51	340	50	279	59	2	61	6.35	332	50	627	8	1	9	4.81
1400	183	43	149	100	29	129	6.49	361	48	260	85	6	91	6.32	351	49	335	11	4	14	4.39
1800	181	42	136	99	42	141	6.36	320	47	199	83	8	91	6.39	313	47	314	8	4	12	4.40
Mean	165	45	142	90	20	109	6.44	348	49	255	71	4	75	6.38	339	49	625	9	2	11	4.66
Cutting time means																					
0600	133	41	132	77	3	81	6.47	356	42	239	63	2	65	6.48	349	42	479	5	0	6	4.74
1000	158	41	136	85	5	90	6.57	344	42	232	65	2	67	6.49	336	43	481	6	1	7	4.61
1400	177	38	150	91	20	111	6.56	350	41	229	78	5	83	6.44	339	42	505	7	2	10	4.33
1800	174	37	140	95	30	125	6.43	345	40	189	77	6	83	6.49	342	41	490	6	3	9	4.35
LSD‡																					
Species	5						0.06		1					0.07			15				
CT	8						0.08		NS					NS			NS				
S × CT	NS	3	15	9	4	10	NS	22	NS	26	7	1	7	NS	28	2	NS	1	1	1	0.10

† 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.

noon, except in 1995 when forage harvested at 1000 h was at the highest DM content. High temperatures and low rainfall for several weeks preceding the 1995 harvest probably accounted for the lack of change in DM levels. Fresh herbage, harvested on days with clear skies and then wilted in the field, required 8.5 to 29.5 h to reach a DM concentration of 350 g kg^{-1} (Table 4). While producers are generally encouraged to harvest earlier in the day to shorten drying time and reduce the risk of exposing the forage to adverse weather, drying time actually decreased in red clover as cutting time was delayed. Drying time also tended to be shorter in after-

Table 2. Means for dry matter (DM) content, total N (TN), nonprotein N (NPN), sugar (primarily glucose, fructose, and sucrose), starch, total nonstructural carbohydrate (TNC), and pH in fresh and wilted forage and wilted silage harvested 27 May 1994.

Cutting Time (CT)	Fresh forage							Wilted forage							Wilted silage						
	DM	TN	NPN	Sugar†	Starch	TNC	pH	DM	TN	NPN	Sugar	Starch	TNC	pH	DM	TN	NPN	Sugar	Starch	TNC	pH
	g kg^{-1}	g kg^{-1} DM	g kg^{-1} TN	— g kg^{-1} DM —				g kg^{-1}	g kg^{-1} DM	g kg^{-1} TN	— g kg^{-1} DM —				g kg^{-1}	g kg^{-1} DM	g kg^{-1} TN	— g kg^{-1} DM —			
Red clover																					
0600	129	31	185	130	6	136	6.22	310	30	207	121	2	123	6.12	310	33	494	20	1	21	4.13
1000	139	32	168	121	7	127	6.16	326	31	208	120	1	122	6.04	320	33	510	18	1	19	4.14
1400	139	31	186	133	13	146	6.13	328	31	203	138	3	141	6.11	322	31	526	28	3	31	4.11
1800	140	30	194	138	17	155	6.05	254	30	175	148	4	152	6.22	255	32	519	17	3	20	4.07
Mean	137	31	183	131	11	141	6.14	304	31	198	132	3	134	6.12	302	32	512	21	2	23	4.11
Alfalfa																					
0600	182	32	160	81	9	90	6.35	341	34	160	64	4	68	6.45	341	34	672	10	3	13	4.38
1000	194	32	166	82	10	92	6.54	331	32	189	60	2	62	6.37	333	33	680	10	1	12	4.35
1400	192	32	164	81	17	98	6.52	373	30	184	77	6	83	6.40	371	32	666	17	5	22	4.19
1800	195	32	171	85	29	113	6.33	339	31	171	82	10	92	6.38	334	32	697	14	7	21	4.22
Mean	191	32	165	82	16	98	6.43	346	32	176	71	6	76	6.40	345	33	679	13	4	17	4.28
Cutting time means																					
0600	156	32	173	105	7	113	6.28	325	32	184	93	3	96	6.28	326	33	583	15	2	17	4.25
1000	167	32	167	101	8	110	6.35	329	31	198	90	2	92	6.21	326	33	595	14	1	15	4.25
1400	166	32	175	107	15	122	6.32	350	30	193	108	4	112	6.25	346	31	596	23	4	26	4.15
1800	167	31	183	111	23	134	6.19	296	31	173	115	7	122	6.30	294	32	608	15	5	21	4.15
LSD‡																					
Species	4	NS	15	6		6				13	6		6			NS	12	3		3	0.06
CT	6	NS	NS	NS		8				18	8		9			1	16	4		5	0.08
S × CT	NS	NS	NS	NS	4	NS	0.13	28	2	NS	NS	2	NS	0.09	30	NS	NS	NS	1	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. Means for dry matter (DM) content, total N (TN), nonprotein N (NPN), sugar (primarily glucose, fructose, and sucrose), starch, total nonstructural carbohydrate (TNC), and pH in fresh and wilted forage and wilted silage harvested 26 June 1995.

Cutting Time (CT)	Fresh forage							Wilted forage							Wilted silage						
	DM	TN	NPN	Sugar†	Starch	TNC	pH	DM	TN	NPN	Sugar	Starch	TNC	pH	DM	TN	NPN	Sugar	Starch	TNC	pH
	g kg^{-1}	g kg^{-1} DM	g kg^{-1} TN	— g kg^{-1} DM —				g kg^{-1}	g kg^{-1} DM	g kg^{-1} TN	— g kg^{-1} DM —				g kg^{-1}	g kg^{-1} DM	g kg^{-1} TN	— g kg^{-1} DM —			
										Red clover											
0600	201	29	110	99	7	105	6.02	349	30	157	91	2	93	6.03	343	30	398	15	2	17	4.14
1000	215	29	115	103	7	110	6.03	399	26	163	103	3	106	6.01	397	27	396	34	3	37	4.07
1400	211	27	116	122	14	135	6.01	390	26	157	105	4	109	6.01	388	28	410	27	4	31	4.10
1800	197	27	130	99	22	121	6.03	333	29	130	91	4	95	5.99	326	29	412	13	4	17	4.12
Mean	206	28	118	106	12	118	6.02	368	28	152	97	3	101	6.01	364	29	404	22	3	26	4.11
										Alfalfa											
0600	220	32	132	66	15	80	6.02	367	34	188	56	6	62	6.14	364	33	698	14	4	18	4.78
1000	239	31	130	68	25	94	6.06	358	33	193	61	7	68	6.14	357	32	687	15	6	20	4.46
1400	235	30	136	71	38	109	6.05	359	32	189	61	7	68	6.15	354	32	674	13	6	19	4.46
1800	236	29	150	66	48	114	6.07	345	32	184	58	8	65	6.13	341	33	707	13	5	18	4.77
Mean	233	30	137	68	32	99	6.05	357	33	189	59	7	66	6.14	354	33	691	14	5	19	4.62
										Cutting time means											
0600	210	30	121	82	11	93	6.02	358	32	172	73	4	78	6.09	353	32	548	14	3	17	4.46
1000	227	30	122	85	16	102	6.05	378	30	178	82	5	87	6.07	377	30	541	24	4	29	4.27
1400	223	28	126	96	26	122	6.03	375	29	173	83	5	88	6.08	371	30	542	20	5	25	4.28
1800	216	28	140	83	35	118	6.05	339	31	157	74	6	80	6.06	333	31	559	13	4	18	4.45
										LSD‡											
Species	7	1	9	7		7	NS		1	11	4	1	5	0.02		1	15		1		0.01
CT	10	NS	13	10		10	NS		2	NS	6	NS	6	NS		1	NS		NS		
S × CT	NS	NS	NS	NS	4	NS	NS	30	NS	NS	NS	NS	NS	NS	30	NS	NS	5	NS	7	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.

noon cuttings of alfalfa, except in 1993 and 1994 when forage cut at 0600 h reached the desired DM on the day of harvest. Cutting later in the day may allow producers to obtain higher TNC yields in wilted silage if fresh forage harvested in the afternoon can consistently dry at an equal or faster rate than forage harvested in the morning.

The pH of fresh red clover and alfalfa varied little from year to year, ranging from 6.01 to 6.64 in both species (Tables 1 to 3). Wilting did not significantly alter the pH of either species. Fermentation caused the pH to fall 1.87 to 2.31 units in red clover silage and 1.36 to 2.04 units in alfalfa silage.

Nonprotein N in Fresh Forage, Wilted Forage, and Wilted Silage

The NPN (on a TN basis) concentration of fresh red clover ranged from 110 to 194 g kg^{-1} TN and fresh alfalfa 130 to 171 g kg^{-1} TN. The level of NPN in fresh forage was not consistently affected by species or cutting time. In 1993, NPN levels were similar between species (Table 1), in 1994, red clover was higher in NPN than alfalfa (Table 2), and in 1995, alfalfa contained more NPN than red clover (Table 3).

Protein hydrolysis by plant enzymes (Kemble, 1956; Oshima and McDonald, 1978) generally resulted in greater levels of NPN in wilted than in fresh forage (Tables 1 to 3). The increase in NPN during wilting was usually greater in alfalfa than in red clover, indicating that protein in red clover is protected from degradation soon after harvest. These results concur with Jones et al. (1995b) who found that red clover extracts browned (an indication of the reaction believed to be responsible for the inhibition of proteolysis) very quickly.

Level of NPN in wilted material was affected by cutting time in 1994 (Table 2), and there was a species × cutting time interaction for NPN in wilted forage in 1993 (Table 1). This interaction was the result of the low level of NPN found in wilted alfalfa from the 1800-h cutting time compared with all other alfalfa cutting times. In contrast, NPN levels in wilted red clover were similar across cutting times in 1993. In 1994, wilted alfalfa cut at 0600 and 1800 h and red clover cut at 1800 h contained less NPN than the other cutting times (Table 2). It is likely that differences in wilting time were the cause of variation in NPN in these samples since they reached the targeted DM content 3 to 12.5 h sooner than the other forage samples (Table 4). In 1995, fresh herbage from all cutting times required similar wilting periods, resulting in comparable NPN levels in wilted forage (Table 3).

Averaged over years and time of day at harvest, proteolysis in the silo caused NPN levels to increase by 136% in red clover and 232% in alfalfa. Most of the protein

Table 4. Time (h) required for red clover and alfalfa to reach a DM content of approximately 350 g kg^{-1}. Forage was hand-clipped at 0600, 1000, 1400, and 1800 h at each cutting time and immediately placed on wire screens in full sunlight to dry.

Cutting time	24 Aug. 1993		27 May 1994		26 June 1995	
	Red clover	Alfalfa	Red clover	Alfalfa	Red clover	Alfalfa
	Drying time (h)					
0600	29.5	8.5	29.5	12.0	27.5	26.5
1000	25.5	26.5	28.0	24.5	25.0	23.5
1400	24.0	25.5	24.0	22.0	23.5	24.0
1800	21.5	22.3	21.0	19.0	23.0	22.0

is degraded in 1 to 2 d (Oshima and McDonald, 1978), and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), the most abundant protein in the plant, is particularly susceptible to degradation (Fairbairn et al., 1988). The lower level of NPN in red clover compared with alfalfa silage may be a direct or indirect result of the action of polyphenol oxidase on plant proteinases in red clover (Jones et al., 1995b).

Increasing the level of TNC at ensiling by harvesting later in the day did not significantly decrease final silage NPN concentrations in either species. In 1994, there was a significant effect of cutting time, but the concentration of NPN was actually higher in silage from the late afternoon harvest. Furthermore, there was no difference in NPN of silage harvested at 0600, 1000, and 1400 h (Table 2). The absence of a strong correlation of silage NPN with wilted sugar and TNC levels provides further evidence that protein degradation in wilted silage is not greatly affected by cutting time (Table 5). McKersie (1985) stated that the amount of protein hydrolyzed during ensiling is largely dependent on the rate of pH decline and the "proteolytic potential," i.e., the total proteinase activity and the availability and susceptibility of protein to degradation. We speculated that a higher rate of pH decline would have occurred in forage harvested later in the day (by increasing the level of TNC), thus inhibiting protein degradation in the silo. Since delaying cutting time did not result in large increases in sugar, we did not eliminate the possibility that addition of a rapidly fermentable substrate could further increase the rate of pH decline and reduce protein hydrolysis during ensiling. McKersie (1985) also proposed that proteolytic potential might be different between years, cuts, harvest dates, and cultivars. Our data support this hypothesis since substantial variability for NPN exists over the three harvest years, which included different growth cycles, harvest dates, and species.

Total Nonstructural Carbohydrates, Sugar, and Starch in Fresh Forage, Wilted Forage, and Wilted Silage

Level of TNC in fresh forage varied significantly with species and cutting time in 1994 (Table 2) and 1995 (Table 3). A greater increase in TNC in alfalfa compared with red clover resulted in a species × cutting time interaction in 1993 (Table 1). The concentration of TNC in fresh forage increased in both species to a maximum at 1800 h, with the exception of red clover in 1995 where the highest level of TNC was observed in forage harvested at 1400 h. Red clover contained more TNC than alfalfa except in the afternoon cuttings from 1993.

Year-to-year variation in sugar concentration was greater than cutting time differences, although sugar levels tended to increase throughout the day of each harvest. Starch had a greater effect than sugar on daytime changes in TNC in fresh alfalfa, whereas quantitative increases in sugar and starch concentrations affected TNC similarly in fresh red clover (Tables 1 to 3). These results for alfalfa concur with those of Lechtenberg et al. (1971) who reported that starch was responsible for 65% of the daily increase in TNC in first-growth alfalfa. It is apparent from these data and those of Lechtenberg et al. (1971) that excess photosynthate production late in the day results in starch synthesis and storage in leaves.

Wilted red clover contained 2 to 32% and wilted alfalfa 16 to 43% less TNC than fresh forage. As a result of these changes, the effect of cutting time on TNC concentration was generally less pronounced in wilted forage (Tables 1 to 3). Close examination of the data in Tables 1 to 3 reveals that a greater proportion of starch than sugar was lost during wilting. In fact, the concentration of sugar in wilted forage was comparable to that found in fresh herbage. Loss of original sugar from fresh forage may have been masked by the release of glucose from starch hydrolysis. In 1993 and 1994 starch decreased by 1.7 to 15.0 g kg^{-1} DM in red clover and 1.2 to 34.5 g kg^{-1} DM in alfalfa during wilting. Nonetheless, wilted forage from the afternoon cuttings continued to maintain a higher concentration of starch than wilted herbage from the morning harvests. In 1995, the starch concentration of wilted forage was similar across cutting times because of extensive starch hydrolysis during wilting of all samples.

Cutting time had a significant effect on TNC in wilted silage from each harvest. There was a species × cutting time interaction in 1993 and 1995, although from a practical perspective the low level of TNC remaining in silage made this interaction less consequential.

Sugar content of wilted silage, ranging from 3 to 34 g kg^{-1} DM, was not consistently affected by cutting time (Tables 1 to 3). A species × cutting time interaction in 1993 (Table 1) and 1995 (Table 3) resulted in changes in silage sugar level rankings across cutting times. Sugars remaining in silage could be derived from the hydrolysis of structural carbohydrates (Dewar et al., 1963) or starch (Melvin, 1965; Muck, 1990).

Table 5. Simple correlation (r) of sugars and total nonstructural carbohydrates (TNC) of wilted forage with nonprotein N (NPN) and pH of wilted silage and silage pH with silage NPN.

Variable		Red clover			Alfalfa		
		1993	1994	1995	1993	1994	1995
		r					
Sugar†	NPN‡	0.11	0.55*	0.03	−0.04	0.21	−0.34
TNC	NPN	0.21	0.54*	0.10	−0.06	0.29	−0.31
pH	NPN	−0.55*	−0.31	0.07	0.03	−0.30	0.49
Sugar	pH	−0.19	−0.62*	−0.38	−0.94***	−0.36	−0.22
TNC	pH	−0.25	−0.61*	−0.37	−0.95***	−0.43	−0.36

*, **, *** Significant at 0.05, 0.01, and 0.001 level of probability, respectively. Values followed by no indication of significance are not significant.

† Data for sugar and TNC are taken from wilted forage.

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

pH and Fermentation Products of Wilted Silage

The pH of silage from red clover ranged from 4.07 to 4.43 and alfalfa from 4.19 to 5.05. A species × cutting time interaction in 1993 (Table 1) indicated a differential response to cutting time for each species. The trend of decreasing pH at each cutting time was similar (Table 1); however, a greater reduction in silage pH levels was

observed in alfalfa compared with red clover between 1000 and 1400 h. Red clover consistently attained a lower pH than alfalfa during fermentation as a result of higher levels of rapidly fermentable sugars and a lower buffering capacity. Because alfalfa tends to be lower in sugar content at a given cutting time and has greater buffering capacity than red clover, delaying cutting time had a greater impact on pH decline in alfalfa in the silo, however.

The pH of wilted silage was usually higher in forage harvested in the morning than the afternoon, particularly in alfalfa. The relationship between sugar or TNC and silage pH was weak for both species, except for alfalfa in 1993 ($P < 0.001$) and for red clover in 1994 ($P < 0.05$) when significant negative relationships of sugar and TNC concentrations with silage pH were observed (Table 5). Cutting time resulted in greater differences in sugar concentration in wilted alfalfa in 1993 (Table 1) and in wilted red clover in 1994 (Table 2), and was the likely cause of higher correlation coefficients. In 1995, the pH values of alfalfa silage from the 0600 and 1800 h harvests were nearly equal because of similar levels of TNC in wilted alfalfa from these cuttings (Table 3). The pH of alfalfa silage was >4.5 in several instances (harvests taken at 0600 and 1000 h in 1993 and 0600 and 1800 h in 1995), yet a lactate-to-acetate ratio >2.0 and a favorable odor indicated that all silages were well preserved. Other workers have also reported well preserved silage with pH values >4.5 (Kung et al., 1984; Muck, 1987; Fairbairn et al., 1988).

Cutting time did not consistently affect lactate and acetate concentrations, although there was a trend of increasing lactate and decreasing acetate as time of harvest went from 0600 to 1800 h (Table 6). This is reflected by silage pH values which were usually lower in silage made from forage harvested in the afternoon (Tables 1 to 3). A species × cutting time interaction for lactate (Table 6) was present in the first 2 yr because of greater increases in lactate production in alfalfa than red clover silage in forage harvested later in the day. In 1995, red clover silage contained more lactate than alfalfa silage; however, a cutting time effect was not detected.

Cutting time had a greater and more consistent enhancing effect on lactate production in alfalfa than red clover silage. This is not surprising since 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. In 1993 and 1994, alfalfa silage from the afternoon harvests contained a greater concentration of lactate than the morning cuttings as a direct result of higher TNC levels. Acetate did not consistently decrease with later cuttings, however. Cutting time had no effect on lactate levels in either species in 1995 because there was very little difference in TNC concentration of the wilted forage.

CONCLUSIONS

Delaying harvest from morning to the afternoon to produce a higher TNC forage provided no major or consistent benefit in terms of lactate production or in protein protection in the silo; however, silage pH tended

Table 6. Concentration of fermentation products in wilted red clover and alfalfa silage. Forage was harvested at 0600, 1000, 1400, and 1800 h and wilted to a DM content of approximately 350 g kg^{-1} before ensiling.

Cutting Time (CT)	1993						1994						1995						
	Suc†	Lac	For	Ace	Pro	Eth	Suc	Lac	For	Ace	Pro	Eth	Suc	Lac	For	Ace	Pro	Eth	But
	g kg^{-1} DM																		
Red clover																			
0600	3.7	85.8	0.6	21.1	1.1	0.2	3.8	105.1	1.4	12.3	4.4	0.9	1.5	89.3	4.8	9.5	0.7	1.3	ND
1000	3.3	86.8	1.6	19.2	1.0	ND‡	2.3	109.9	2.1	16.3	2.8	1.7	1.1	76.9	3.8	6.5	0.2	1.0	ND
1400	2.3	95.6	1.6	12.7	0.1	0.1	3.9	102.0	2.1	11.2	3.8	2.6	1.4	85.5	4.1	7.7	1.4	1.4	ND
1800	2.4	89.2	1.6	13.9	0.4	0.4	6.5	123.8	1.9	23.0	0.8	1.4	1.2	79.9	2.7	11.0	0.9	1.8	ND
Mean	2.9	89.4	1.4	16.7	0.7	0.2	4.1	110.2	1.9	15.7	3.0	1.6	1.6	82.9	3.9	8.7	0.8	1.4	ND
Alfalfa																			
0600	5.7	61.9	0.2	28.0	0.2	1.4	4.2	70.6	0.6	15.8	0.9	2.8	6.8	54.1	1.2	19.8	TR	2.2	0.4
1000	4.4	69.1	0.4	24.4	0.2	1.6	4.1	65.9	ND	11.8	0.9	2.6	2.6	59.0	1.4	11.6	0.3	1.9	ND
1400	3.3	87.4	0.5	15.0	0.1	1.6	2.7	80.2	0.2	8.8	0.5	2.1	2.7	63.4	1.6	14.8	0.1	1.8	ND
1800	4.2	89.7	1.3	16.1	0.3	2.1	5.3	84.5	0.7	12.2	6.6	4.5	8.0	53.8	1.0	21.9	0.9	3.1	1.1
Mean	4.4	77.0	0.6	20.9	0.2	1.7	4.1	75.3	0.4	12.1	2.2	3.0	5.0	57.6	1.3	17.1	0.3	2.2	0.4
Cutting time means																			
0600	4.7	73.9	0.4	24.5	0.6	0.8	4.0	87.8	1.0	14.0	2.7	1.8	4.2	71.7	3.0	14.7	0.4	1.8	0.2
1000	3.9	78.0	1.0	21.8	0.6	0.8	3.2	87.9	1.1	14.0	1.8	2.1	1.8	68.0	2.6	9.0	0.2	1.4	ND
1400	2.8	91.5	1.1	13.9	0.1	0.9	3.3	91.1	1.2	10.0	2.2	2.4	2.0	74.5	2.9	11.2	0.8	1.6	ND
1800	3.3	89.5	1.4	15.0	0.4	1.2	5.9	104.1	1.3	17.6	3.7	3.0	5.1	66.8	1.9	16.5	0.9	1.4	0.6
LSD§																			
Species	0.8		NS	2.7	NS	0.3	NS		0.8				2.2	10.5	0.5	2.2	NS	0.6	NS
CT	1.1		NS	3.8	NS	NS	NS		NS				NS	NS	0.7	3.1	NS	NS	NS
S × CT	NS	5.9	NS	NS	NS	NS	NS	10.9	NS	6.3	4.3	1.3	NS	NS	NS	NS	NS	NS	NS

† Suc = succinate, Lac = lactate, For = formate, Ace = acetate, Pro = propionate, Eth = ethanol, and But = butyrate.
‡ ND = none detected; TR = trace; Note — no butyrate was detected in any silage in 1994 and 1995.
§ 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.

to decrease and starch increase as cutting time was delayed from 0600 to 1800 h. The pH of alfalfa silage was consistently higher than red clover silage as a result of lower sugar levels for fermentation and a greater buffering capacity (McDonald et al., 1991, p. 31) in wilted alfalfa. Extensive protein degradation resulted in significantly higher NPN levels in alfalfa than in red clover regardless of cutting time. Forage harvested in the afternoon frequently required less time to dry than herbage from morning cuttings (Table 4). Consequently, while delaying harvest did not improve protein protection in the silo, forage harvested in the afternoon did not necessarily lead to delayed ensiling, and may be better preserved and of increased quality because of lower silage pH and higher starch concentrations.

ACKNOWLEDGMENTS

The authors are grateful to Ed Bures for excellent technical assistance provided.

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