Influence of maturity on alfalfa hay nutritional fractions and indigestible fiber content

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ABSTRACT

This study focused on changes in fibrous and protein fractions, changes in fiber digestibility and amount of indigestible neutral detergent fiber (NDF) as a consequence of increased maturity in alfalfa. A total area of 720 m² was divided in 18 blocks randomly assigned to 3 treatments, differing in cutting intervals. Treatment 1 was harvested with a 21-d cutting schedule, at a pre-bloom stage; treatment 2 with a 28-d schedule, at about first-bloom stage; whereas a full bloom was observed in treatment 3, harvested with a 35-d cutting schedule. Treatments were replicated 4 times through the spring-summer period for 2 subsequent years, 2011 and 2012. Statistical differences were observed for crude protein [treatment 1: 20.8%, treatment 2: 17.3%, and treatment 3: 17.0%; standard error of the mean (SEM) = 0.83], soluble protein, and nonprotein nitrogen among treatments on a dry matter basis. Similar results were observed for acid detergent lignin (6.3, 6.9, and 7.3%, respectively; SEM = 0.39), lower in treatment 1 compared with others, and in vitro NDF digestibility at 24 or 240 h. Indigestible NDF at 240 h resulted in lower values for treatment 1 compared with treatments 2 and 3 (15.5, 17.2, and 18.3%, respectively; SEM = 1.54). Moreover, the indigestible NDF:acid detergent lignin ratio varied numerically but not statistically among treatments, being as much as 9% greater than the 2.4 fixed value applied for rate of digestion calculation and Cornell Net Carbohydrate Protein System (Cornell University, Ithaca, NY)-based model equations. Assuming the diet composition remained unchanged, treatment 3 (35-d cutting interval) would be expected to yield 1.4 kg less milk per day based on energy supply, and 2.8 kg less milk daily based on protein supply than treatment 1.

Key words: alfalfa, maturity, indigestible neutral detergent fiber

INTRODUCTION

Alfalfa hay, one of the most utilized forages in Italy, is particularly important in the areas of Po Valley involved in production of Parmigiano Reggiano cheese, where silage feeding is prohibited to prevent environmental clostridia contamination (Parmigiano-Reggiano Cheese Consortium, 2011) in milk. Thus, hay is the main source of forages for these dairy rations. In northern Italy, 4 or more cuttings of alfalfa can be harvested annually. Numerous studies have shown that forage quality is affected by growth stage (Nordkvist and Åman, 1986; Yu et al., 2003), species, cultivar (Griffin et al., 1994), and growing conditions (e.g., rainfall, temperature, and soil composition; Minson and McLeod, 1970; Cox et al., 1994; Mathison et al., 1996).

Alfalfa (*Medicago sativa* L.) is a perennial legume with a unique anatomy comprising relatively distinct protein-containing leaves and fibrous stems. Maturity influences both fiber digestibility and protein fractions in alfalfa through increasing the leaf:stem ratio and increasing lignification of stems, which, in turn, alters fiber digestibility (Weir et al., 1960; Yu et al., 2003). Lignin negatively affects forage fiber digestion degree and rate (Albrecht et al., 1987; Van Soest, 1994; Sewalt et al., 1997b), the extent of which depends on its concentration, composition (Buxton and Russell, 1988; Buxton and Brasche, 1991), tissue distribution (Akin, 1989), and phenolic functionality (Sewalt et al., 1997a). Lignin, due to its peculiar phenolic composition, cannot be digested in anaerobic environments, and is able to reduce the proportion of potentially digestible fiber fraction in forages (Jung and Allen, 1995), representing the indigestible NDF (iNDF). In Cornell Net Carbohydrate Protein System (CNCPS; Cornell University, Ithaca, NY; Sniffen et al., 1992)-based models, such as Cornell-Penn-Miner (CPM) Dairy software [version 3.0.10; Cornell University (Ithaca, NY), University of Pennsylvania (Philadelphia), The William H. Miner Agricultural Research Institute (Chazy, NY), and the University of Maryland (College Park); Boston et al., 2000], the iNDF is calculated as ADL times a fixed value (2.4), without differentiation among forages, nor between early cut or late cut (Sniffen et al., 1992; Van

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Soest et al., 2005). Moreover, iNDF is essential in the rate of digestion (kD) equations, in order to calculate the amount of NDF that could be digested and its rate. The objective of this study was to determine the influence of maturity and cutting treatment on alfalfa chemical composition and, in particular, in vitro fiber digestibility and the iNDF fraction.

MATERIALS AND METHODS

Experimental Design

This study was conducted using a native alfalfa variety, called Romagnola, sown in 2007 in an experimental plot close to the Department of Veterinary Medicine of Bologna University (Bologna, Italy). No insecticides or herbicides were applied, and no soil fertilization or irrigation was conducted. The field had a clay soil with medium porosity, and its relative average humidity and pH were recorded at the beginning of each trial.

A total area of 720 m² was divided in 18 plots randomly assigned to 3 treatments, differing in cutting intervals. Treatment 1 (**PreB-21dd**) involved harvesting every 21 d at about prebloom, treatment 2 (**FrsB-28dd**) every 28 d at about first bloom, and 35 d at about full bloom for treatment 3 (**FulB-35dd**). Treatments were replicated 4 times through the spring-summer period in 2 subsequent years (2011 and 2012). All plots were cut at the beginning of the third week of April to obtain a common starting date for all treatments. Plots were harvested approximately at 0900 h, 7 to 10 cm above the soil to aid plants regrowth and avoid soil contamination.

Chemical Analysis, In Vitro Fermentation, and Rationing

Immediately after harvesting, a representative (1.0-kg) sample, randomly obtained by hand from each block of each treatment, was collected and immediately dried in a forced-air oven at 65°C for 48 h to determine DM content. From each block, forage was sampled and dried following the same procedure as above. Once dried, samples were ground through a 1-mm screen in a Cyclone mill (model SM100; Retsch GmbH, Haan, Germany). The samples were analyzed for CP (AOAC, 1990; methods 976.06 and 984.13), NPN, soluble protein, neutral detergent-insoluble protein, acid detergent-insoluble protein (Licitra et al., 1996), amylase-treated NDF (Mertens, 2002) with addition of sodium sulfite, ADF (AOAC, 1990; method 973.18), and ADL (AOAC, 1990; method 973.18). Briefly, the ADF residue was suspended within the crucible in 40 mL of sulfuric acid (72% concentration) for 3 h. Residues were then washed

with boiling water and dried overnight at 65°C. Once weighed, crucibles were placed in a muffle furnace for 4 h at 490°C to incinerate the residue. Ash content was then subtracted from the initial weight to calculate the ADL content. In vitro NDF digestibility at 24 and 240 h was determined using the Tilley and Terry modified technique (Tilley and Terry, 1963; Goering and Van Soest, 1970; Robertson and Van Soest, 1981). Rumen fluid was collected by sampling rumens of 2 lactating cows (fed a hay-based diet; Table 1; milk production = 31.8 ± 2.3 kg/d; DIM = 274 ± 2), using an esophageal tube, mixed and placed in a thermostatic bottle. Under insufflation of O₂-free CO₂, liquor was filtered through 4 layers of cheesecloth. A 10-mL volume of rumen fluid was inoculated in each Erlenmeyer flask already placed in a heated water bath under CO₂-positive pressure to ensure anaerobiosis. Flasks contained 40 mL of the solutions described by Goering and Van Soest (1970) and 0.50 g of sample. Each sample was analyzed in triplicate in 2 different in vitro fermentations. For both, sample preparation and diet was the same as for the donor cows. At the end of the fermentation, the content of each flask was analyzed to determine NDF content of the residue. Digestibility was calculated as the difference between 100 and 100 times the residual NDF:initial NDF ratio. Other fermentations were conducted for 240 h to estimate the amount of iNDF. We based our choice about fermentation length on previous studies indicating 240 h as the maximum extent of fiber digestion in an anaerobic environment (Chandler, 1980; Van Soest, 1994). For these fermentations, both rumen fluid and buffer were reinoculated after 120 h to preserve the microbial activity during the whole process. A final volume of 100 mL, containing 0.50 g of sample, was treated for NDF content determination as described above.

To predict RDP, NE_L, and possible milk production due to MP and ME, each hay corresponding to the 3 treatments was used as the main forage source in CPM, a dairy ration software based on the CNCPS. Diet compositions are listed in Table 2. Cows were assumed to weigh 620 kg, have a BCS of 3.00, be in second lactation, be at 120 DIM, and produce 35.00 kg of milk/d with 3.60% fat and 3.30% total protein concentration.

Environmental Conditions

During the trial, the total amounts of rainfall and temperatures were recorded daily and results were summarized for each cutting schedule treatment. The weather station was located within 1,000 m of the experimental area. Data were collected from the official website of the Environmental Protection Regional Agency (ARPA Emilia Romagna, Bologna, Italy).

Table 1. Rumen fluid donor cows diet composition

Diet composition	Amount
Ingredient, % of DM	
Grass hay	30
Alfalfa hay (first cut)	20
Corn grain (finely ground)	20
Sorghum grain (finely ground)	12.5
Barley grain (flaked)	5
Soybean meal (48% CP)	10
Cane molasses	2.5
Chemical composition, % of diet DM	
aNDF ¹	31.2
ADF	20.5
ADL	3.5
CP	14.2
Ether extract	3.1
Ash	9.1
Starch	23.1

¹Amylase- and sodium sulfite-treated NDF.

Statistical Analysis

Statistical analysis was conducted for each fraction obtained from each block using a two-way ANOVA (Statistica software; StatSoft Inc., Tulsa, OK) with repeated measures for samples within the same treatment. Specific relationship between fractions and environmental data were analyzed with reg. proc. (regression procedure) of the software. The statistical model was based on analytical fractions as dependent variables, and treatment, year of the experiment, and their interaction as fixed variables. As no significant effects were observed for the treatment $\times$ year interaction, only means of tested main effects (i.e., treatment and year of experiment) were reported in tables.

RESULTS AND DISCUSSION

Environmental Conditions

The first cut of alfalfa is usually a mixture of alfalfa and native grasses, due to crop management and agronomical procedures that provide no addition of herbicides or pesticides. But because of their biology and the climatic conditions, grasses regrow slower than alfalfa, reducing their proportion in subsequent cuttings. Thus, alfalfa becomes predominant usually between the second and third cut. Recorded soil moisture was $68.8 \pm 2.09\%$ and the pH was slightly basic (7.2 ± 0.1).

Temperature and rainfall data are descriptively presented in Table 3, and could be considered in line with the traditional summer conditions of this geographical area, which usually lacks precipitation from July to mid August. No differences were observed ($P = 0.86$) among years. The average temperature among different cuts increased to a maximum in August, and then decreased between late August and early September. No relationship was found between analytical fractions and temperature or rainfall, with the exception of moisture content with temperature ($R^2 = 0.94$; $P < 0.05$). The moisture content of each forage recorded at every cut is reported in Table 4.

Analytical Fractions

As expected, CP concentrations averaged across cuttings were different among harvesting intervals (Table 5). Crude protein content (expressed as % of DM) differed ($P < 0.01$) between PreB-21dd ($20.8 \pm 1.8\%$) and FrsB-28dd or FulB-35dd (17.3 ± 0.9 and $17.0 \pm$

Table 2. Dietary formulas used in CPM software¹ to predict animal response

Diet ingredient, kg/DM	Treatment ²		
	PreB-21dd	FrsB-28dd	FulB-35dd
Alfalfa hay PreB-21dd	10.0	—	—
Alfalfa hay FrsB-28dd	—	10.0	—
Alfalfa hay FulB-35dd	—	—	10.0
Wheat straw	2.0	2.0	2.0
Soybean hulls	2.0	2.0	2.0
Soybean meal 44%	2.0	2.0	2.0
Corn grain meal	7.0	7.0	7.0
Mineral-vitamin mix ³	0.5	0.5	0.5

¹CPM software = Cornell-Penn-Miner Dairy version 3.0.10 [Cornell University (Ithaca, NY), University of Pennsylvania (Philadelphia), The William H. Miner Agricultural Research Institute (Chazy, NY), and the University of Maryland (College Park); Boston et al., 2000].

²PreB-21dd = 21-d cutting interval, prebloom; FrsB-28dd = 28-d cutting interval, first bloom; FulB-35dd = 35-d cutting interval, full bloom.

³Mineral-vitamin mix composition: 1.4% Ca, 8.3% P, 16% Na, 5.5% Mg, 4,000 mg/kg of Zn, 4,000 mg/kg of Mn, 400 mg/kg of Cu, 400 mg/kg of I, 40 mg/kg of Se, 20 mg/kg of Co, 1,200,000 IU/kg of vitamin A, 200,000 IU/kg of vitamin D₃, and 1,000 mg/kg of vitamin E.

Table 3. Descriptive environmental data recorded during the trial

Measure	Treatment ¹			Year		SEM
	PreB-21dd	FrsB-28dd	FulB-35dd	2011	2012	
Rainfall, mm						
First cut	23.8	23.8	25.2	26.6	25.4	0.2
Second cut	77.4	76.4	92.6	76.1	74.0	0.9
Third cut	14.6	9.2	11.0	12.1	11.3	1.1
Fourth cut	9.2	1.8	43.4	19.8	18.9	3.6
Average temperature, °C						
First cut	18.3	18.4	19.4	18.4	19.2	0.3
Second cut	20.3	21.5	22.4	20.9	22.3	0.5
Third cut	22.5	24.5	25.4	23.8	24.6	0.6
Fourth cut	25.8	25.9	22.0	24.5	26.1	0.8

¹PreB-21dd = 21-d cutting interval, prebloom; FrsB-28dd = 28-d cutting interval, first bloom; FulB-35dd = 35-d cutting interval, full bloom.

2.0%, respectively; SEM = 0.83). The NPN and soluble protein concentrations also were different between the 21- and the 28- or 35-d harvest intervals ($P < 0.01$). These results indicate that maturity influenced both the concentration and solubility of alfalfa protein. No differences ($P = 0.12$) were observed for detergent-insoluble proteins (acid detergent-insoluble protein or neutral detergent-insoluble protein), suggesting that harvest interval had little effect on these fractions. When expressed on a DM basis, Elizalde et al. (1999a) reported similar changes with age in soluble CP and NPN for alfalfa. In a companion paper (Elizalde et al., 1999b), they observed that immature alfalfa contained more ruminal degradable protein than mature alfalfa. Thus, the small changes in protein solubility we observed for alfalfa of similar age to those of Elizalde et al. (1999a,b) may have affected their ruminal degradation. Protein content or composition was not influenced by the year of the trial.

Results regarding the fiber fraction are reported in Table 5. No differences ($P = 0.11$) were observed for NDF or ADF [41.0 ± 4.1 , 40.4 ± 2.7 , and $41.32 \pm 4.3\%$ NDF (SEM = 1.35) and 30.25 ± 2.0 , 30.68 ± 1.4 , 31.15 ± 2.3 ADF (SEM = 1.02) for the PreB-21dd, FrsB-28dd, and FulB-35dd treatments, respectively). Acid detergent lignin was lower for the 21-d harvest interval ($P < 0.01$) compared with the 28- and 35-d intervals

(6.3 ± 0.8 , 6.9 ± 0.5 , and $7.3 \pm 0.9\%$, respectively; SEM = 0.39). Lignification is an irreversible process, and this compound tends to decrease the amount and the surface of cell walls available for microbial attachment and digestion (Åman and Nordkvist, 1983). Considering this fact, it is possible to assess, according to Jung and Engels (2002), that the presence of lignin is critical to the potential degradation of cell walls, but its concentration alone may not explain completely these differences, as increased maturity would affect the portion of potentially digestible fibrous components linked to lignin. As for the protein fractions, even fiber amount and composition were not affected by year of the trial.

Fiber Digestibility and Predicted Animal Response

The in vitro estimation of NDF digestibility at 24 h confirms what was expected in that it decreased with maturity (Table 6); the in vitro NDF digestibility at 24 h of alfalfa harvested every 21 d ($44.0 \pm 2.6\%$) differed ($P < 0.01$) with forages grown for 28 ($37.8 \pm 1.8\%$) or 35 d ($34.1 \pm 2.8\%$). Considering the 240-h in vitro NDF digestibility and thus the amount of NDF that is indigestible, it is possible to observe statistical differences ($P < 0.01$) due to different maturity levels. A lower amount of iNDF appeared within PreB-21dd ($15.5 \pm 0.9\%$) than in other treatments (17.2 ± 1.2 and

Table 4. Moisture content of the harvested fresh alfalfa

Moisture, %	Treatment ¹			Year		SEM
	PreB-21dd	FrsB-28dd	FulB-35dd	2011	2012	
First cut	75.2	75.9	73.8	75.3	74.3	0.21
Second cut	72.9	71.0	65.3	72.2	69.5	2.59
Third cut	65.3	58.3	55.8	60.5	59.4	1.21
Fourth cut	59.0	55.3	70.6	60.0	55.3	5.87

¹PreB-21dd = 21-d cutting interval, prebloom; FrsB-28dd = 28-d cutting interval, first bloom; FulB-35dd = 35-d cutting interval, full bloom.

Table 5. Chemical composition of the harvested alfalfa

Chemical composition, ¹ % of DM	Treatment ²			Year		
	PreB-21dd	FrsB-28dd	FulB-35dd	2011	2012	SEM
CP	20.8 ^A	17.3 ^B	17.0 ^B	18.3	17.6	0.83
SolP	6.3 ^A	4.4 ^B	4.9 ^B	5.5	4.8	0.23
NPN	5.3 ^A	3.5 ^B	3.9 ^B	4.3	3.9	0.30
NDIP	5.2	4.3	4.7	4.8	4.7	0.61
ADIP	1.5	1.3	1.6	1.5	1.4	0.03
aNDF	41.0	40.4	41.3	40.8	41.4	1.35
ADF	32.3	31.6	31.5	31.3	31.6	1.02
ADL	6.3 ^b	6.9 ^a	7.3 ^a	6.8	7.0	0.39

^{A,B}Means within a row with different capital letter superscripts differ ($P < 0.01$).

^{a,b}Means within a row with different lowercase letter superscripts differ ($P < 0.05$).

¹SolP = soluble protein; NDIP = neutral detergent-insoluble protein; ADIP = acid detergent-insoluble protein; aNDF = amylase- and sodium sulfite-treated NDF.

²PreB-21dd = 21-d cutting interval, prebloom; FrsB-28dd = 28-d cutting interval, first bloom; FulB-35dd = 35-d cutting interval, full bloom.

18.3 ± 1.1% for FrsB-28dd and FulB-35dd treatments, respectively; $P < 0.01$). With a similar NDF content among plots, this effect could be due to differences in ADL content. But again, iNDF data are far from being dependent on just the ADL values, indicating the ability of this compound to embed other cell wall components, compromising their potential digestion. Moreover, the ratio between iNDF and ADL appeared to be different than the ratio of 2.4 used in the CNCPS-based models (2.46 ± 0.05, 2.57 ± 0.08, and 2.58 ± 0.08 for PreB-21dd, FrsB-28dd, and FulB-35dd treatments, respectively). Other authors (Cherney et al., 1993; Traxler et al., 1998) observed the same trend in iNDF amount related to plant maturity for perennial forages, but not for alfalfa, as is presented in the current study.

Once tested with CPM, the 3 treatments strongly influenced dietary RDP percentage (11.15, 9.99, and 9.88% for PreB-21dd, FrsB-28dd, and FulB-35dd treatments, respectively) without any major difference in NE_L, expressed as megacalories per kilogram (1.67, 1.64, and 1.62 Mcal/kg for PreB-21dd, FrsB-28dd, and FulB-35dd treatments, respectively). According to

other authors (Llamas-Lamas and Combs, 1990; Allen and Oba, 1998), a greater effect could be expected for animal productive performances. In terms of ME and MP, among PreB-21dd and FulB-35dd treatments, a difference of 1.4 (ME) and 2.8 (MP) kg of milk/d would be predicted. These results were obtained with equations that still consider the iNDF as ADL × 2.4 because CPM currently does not allow input of iNDF values. Nevertheless, we suggest that implementation of such software should prove useful to increase the accuracy of evaluating the nutritional characteristics of forages.

CONCLUSIONS

Maturity influences both the protein and fiber fractions in alfalfa hay, but has its greatest effect on digestibility. Although in this study we tested only one alfalfa variety, not focusing on possible genetic effects, we observed that the maximum extent of digestion differed among treatments, with the indigestible fraction of fiber increasing with maturity, resulting in differ-

Table 6. In vitro NDF digestibility and rates of digestion of the harvested alfalfa

Measure ¹	Treatment ²			Year		
	PreB-21dd	FrsB-28dd	FulB-35dd	2011	2012	SEM
NDF digestibility, % of NDF						
IVNDFD (24 h)	44.0 ^a	37.8 ^b	34.0 ^b	39.1	38.4	1.18
IVNDFD (240 h)	62.2 ^a	57.5 ^b	55.7 ^b	58.9	58.1	1.07
iNDF, % of DM	15.5 ^b	17.2 ^a	18.3 ^a	16.7	17.5	0.91
iNDF/ADL	2.46	2.57	2.58	2.46	2.51	0.02

^{a,b}Means within a row with different superscript letters differ ($P < 0.01$).

¹IVNDFD = in vitro NDF digestibility; iNDF = indigestible NDF.

²PreB-21dd = 21-d cutting interval, prebloom; FrsB-28dd = 28-d cutting interval, first bloom; FulB-35dd = 35-d cutting interval, full bloom.

ent ratios of lignin in iNDF. This effect needs to be considered and implemented into the modern ration-calculating software in order to formulate an optimal diet from different alfalfa hays. Moreover, considering that biological determination of iNDF requires a significant amount of time and chemicals, availability of faster and accurate tools could be extremely useful to predict this fraction.

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