

***In vitro* digestibility of mountain-grown irrigated perennial legume, grass and forb forages is influenced by elevated non-fibrous carbohydrates and plant secondary compounds**

Running title: Nutritive value and secondary compounds affect *in vitro* forage rumen digestibility

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Abstract

BACKGROUND: Perennial legumes cultivated under irrigation in the Mountain West USA have non-fibrous carbohydrate (NFC) concentrations exceeding 400 g kg⁻¹, a level commonly found in concentrate-based ruminant diets. Our objective was to determine the influence of NFC concentration and plant secondary compounds on *in vitro* rumen digestion of grass, legume and

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forb forages compared with digestion of their isolated neutral detergent fiber (NDF) fraction.

Forages were composited from ungrazed paddocks of rotationally stocked irrigated monoculture pastures between May and August 2016, frozen in the field, freeze-dried, and ground.

RESULTS: The maximum rate (R_{Max}) of gas production was greater for the legumes alfalfa (ALF; *Medicago sativa* L.) and birdsfoot trefoil (BFT; *Lotus corniculatus* L.) than for the legume cicer milkvetch (CMV; *Astragalus cicer* L.) the grass meadow brome (MBG; *Bromus riparius* Rehm.) and the non-legume forb small burnet (SMB; *Sanguisorba minor* Scop.), and intermediate for the legume sainfoin (SNF; *Onobrychis viciifolia* Scop.). The R_{Max} of isolated NDF was greatest for BFT and CMV, intermediate for ALF, SNF and SMB and least for MBG.

CONCLUSIONS: More than 900 g of organic matter (OM) kg^{-1} dry matter (DM) of legumes was digested after 96 h. Across forages, the extent of whole plant digestion increased with NFC and crude protein (CP) concentrations, decreased with NDF concentrations, and was modulated by secondary compounds. The extent of digestion of isolated NDF decreased with concentration of lignin and residual tannins.

Keywords: condensed and hydrolysable tannins; cumulative fermentation gases; irrigated pastures; isolated fiber; non-fibrous carbohydrates; perennial legume forages

INTRODUCTION

Increasing the forage content of beef finishing diets and dairy rations could reduce feed costs and improve the health and longevity of dairy cows ¹. Intake and digestibility of forages by ruminants are a function of forage neutral detergent fiber (NDF) and energy concentration ²; however, the digestibility of NDF and the proportion of NDF that is ultimately not digested in the rumen ³ can limit the rate of passage of forages from the rumen and thereby limit intake ⁴. The concentration and biochemistry of lignin and the localization of its deposition in forage cell walls, constrains ruminant intake and digestibility ⁵, while greater concentrations of non-fibrous carbohydrates (NFC) represent energy for microbial colonization of forages ⁶. These variables are, in part, a function of plant phenology and environment ⁷. For instance, forages grown under irrigation in the high-elevation Mountain West USA have more NFC and less NDF compared with the same forages cultivated in warmer, more humid environments ^{8,9}.

Legume forages such as the true clovers or alfalfa (ALF) are commonly grazed only as components of grass-legume mixtures to avoid issues with pasture bloat ¹⁰ that can result from the rapid digestion of bloat-causing forages ¹¹. Temperate nutrient-dense legumes such as birdsfoot trefoil (BFT) and sainfoin (SNF) are non-bloating due to the presence of condensed tannins (CT) that precipitate excess plant protein in the rumen and reduce protein availability ¹², disrupt biofilms ¹³, or alter rumen microbial ecology ¹⁴. Alternatively, vein structural tissue holds the upper and lower epidermis of CMV leaves together ¹⁵ preventing bloat by slowing rumen microbial access to plant cell contents.

The objective of this study was to compare the rate and extent of digestion of two non-tannin (ALF and CMV) and two CT-containing (BFT and SNF) legumes with a grass (MBG) and a non-legume forb (SMB) when these forages were grown under irrigation in the Mountain West region. Grasses have more fiber and less lignin than legumes ⁵, and the non-legume forb SMB is persistent and palatable to livestock but contains a hydrolysable tannin (HT) that reduces nitrogen excretion in the urine of beef cattle ¹⁶. The *in vitro* gas production technique was used to study the influence of NDF, NFC, crude protein (CP), lignin, CT, HT and other nutrients and secondary plant compounds on the rumen digestibility of these forages.

MATERIALS AND METHODS

Forage collection

The six forage species used in this study were composited from irrigated monoculture pastures (0.365 ha each) that were rotationally stocked with dry beef cows from late May through mid-August of 2016. Pastures were located in Lewiston, Utah (41.95°N; 111.87°W; altitude 1370 m.a.s.l.). Compositated material was collected weekly (CMV MBG and SMB; 11 dates x 5 reps; 1 g DM per sample) or monthly (ALF, BFT, SNF; 3 dates x 9 reps; 2 g DM per sample) between 8.00 h and 12.00 h from paddocks that would be grazed next in rotations by clipping ~10 samples of each species to a 7.6 cm stubble. A total of approximately 250 g fresh weight was collected by walking a corner-to-corner transect of the paddock. Grass pasture regrowth was maintained in the vegetative stage and all forbs were maintained in the flowering stage during

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this period. Forage samples were immediately frozen under dry ice and stored at -20°C until freeze-dried, then milled to pass the 1-mm screen of a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA).

NDF isolation

Neutral detergent fiber of each species was isolated using the standard protocol for the ANKOM A200 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA). Following an acetone rinse, samples were dried at 102°C and weighed to determine NDF concentration. Isolated NDF was soaked overnight in 10% (v/v) tert-butyl alcohol and 90% 1 M sodium sulfate at 39°C and rinsed successively with hot water, 95% ethanol, and acetone¹⁷ to remove traces of neutral detergent solution.

***In Vitro* Rumen Fluid Fermentation**

The *in vitro* methodology of Theodorou et al.¹⁸ was used to determine the kinetics of rumen fermentation. Three runs of the fermentation were carried out over a 3-wk period. Triplicate samples of approximately 0.4 g of each whole plant and NDF isolate sample plus a BFT control and a blank were included in each run. Fermentations were carried out in 125-mL borosilicate glass serum bottles (Wheaton, Boston, MA, USA). A 40-mL aliquot of buffer containing macro- and microminerals, artificial saliva, a reducing solution, and resazurin¹⁹ was added to forage in each bottle, which was flushed with CO_2 and sealed with a butyl rubber septum and aluminum

crimp cap (Wheaton). All reagents were purchased from Sigma-Aldrich (St. Louis, MO). Bottles with forage and buffer were stored overnight at 4°C then warmed to 39°C before 20 mL rumen fluid was added. Blank vials contained buffer and rumen fluid only.

Animal handling was conducted under Utah State University Institutional Animal Care and Use Committee protocol #2834. Rumen fluid was collected approximately 4 h after a meal of ALF hay. Fluid was squeezed from the mat of fermenting forage of a ruminally cannulated Angus cow into a pre-warmed thermal flask, transported 15 min. to the laboratory, strained twice through three layers of cheesecloth and maintained at 39°C under CO₂ gas. The pH of rumen fluid was 6.3 ± 0.4. When fermentation was terminated at 96 h, mean pH of all samples was 6.75 ± 0.05 (SD); the pH of blanks was 7.02 ± 0.20, and the pH of controls was 6.92 ± 0.08.

Gas pressure measurements were made with a needle-equipped pressure transducer (PX409-015GUSBH; Omega Engineering Inc., Stamford, CT, USA) at 1, 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, 72, and 96 hours after inoculation, and accumulated gas was vented after each measurement¹⁸. Fermentation was stopped by cooling serum bottles to 4°C in a walk-in freezer. Undigested fermentation residues were collected in ANKOM *in situ* bags with 50 µm porosity, thoroughly rinsed with distilled water, and dried at 60°C to obtain dry mass.

Gas pressure was transformed to gas volume using the equation gas volume (mL) = 5.3407 × gas pressure (psi)²⁰. A single phasic model (Equation 1) for cumulative gas volume (G) was used to calculate fermentation kinetics parameters where A (mL g⁻¹ organic matter; OM) was the asymptotic (maximum) gas volume, B (hours) was the incubation time (*t*) at which half

the maximum amount of gas had been formed, and C was a constant describing the sharpness of the switching characteristic of the cumulative gas curve ²¹. As the value of C increases, cumulative gas production curves become more sigmoidal and increase in slope,

$$G = \frac{A}{1 + (B/t)^C} \quad \text{Equation (1)}$$

The time when the maximum rate of substrate digestion occurred (t_{RM}) and the maximum rate of substrate digestion (R_{Max}) were calculated from A, B, and C using Equations 2 and 3 ²¹.

$$t_{RM} = B(C - 1)^{1/c} \quad \text{Equation (2)}$$

$$R_{Max} = \frac{1}{B}(C - 1)^{(1-\frac{1}{c})} \quad \text{Equation (3)}$$

Forage Analyses

Dry matter (DM) of forages and isolated fiber was determined by drying three subsamples of each forage for 48 h at 105°C. Digestible dry matter (DDM) was calculated by subtracting DM of undigested fermentation residue at the end of the fermentation from substrate DM and dividing by substrate DM. Residues were ashed at 550°C and subtracted from substrate DM to estimate substrate OM. Undigested OM was calculated by subtracting ash from undigested fermentation residue DM; some minerals may have been solubilized during fermentation²².

Digestible organic matter (DOM) was calculated by subtracting undigested OM from substrate OM and dividing by substrate OM.

Forage composites were analyzed by near infrared spectroscopy (NIRS; FOSS 2500 Feed Analyser, Foss Analytics, Hilleroed, Denmark) to estimate CP, amylase-treated NDF (aNDF), NDF digestibility as a proportion of NDF (NDFD), acid detergent fiber (ADF), NFC, ash, acid detergent lignin (ADL), fat, and total digestible nutrients (TDN) using the March 2018 NIRS Consortium mixed hay equation (<https://www.nirsconsortium.org/>). The calibration set for this equation does not include SMB. The WSC concentration of forage samples was determined by the phenol-sulfuric acid method ²³, and their starch concentration by the glucose oxidase-peroxidase method ²⁴.

Total CT concentrations were determined by the butanol-HCl-acetone method ²⁵. Condensed tannin standards were isolated from BFT and SNF ²⁶. Hydrolyzable tannins were determined for SMB relative to methyl gallate standards and a tannic acid check ²⁷.

Experimental Design and Statistical Analyses

Cumulative fermentation gas kinetics parameters were estimated using PROC NLIN and then analyzed using PROC GLIMMIX (SAS/STAT 14.3, SAS Institute Inc., Cary, NC, USA) with a mixed model in which run was the random factor, and species and material (whole plant or isolated fiber) with their interaction were fixed effects. To account for the correlation of whole plant and isolated fiber materials from the same species, a heterogeneous compound-symmetry

structure (CSH) was included based on AIC and BIC selection. Pairwise differences among the least squares means (LSMEANS) of the kinetics parameters were tested with Tukey-Kramer multiplicity adjustment. PROC CORR was used to determine correlation between whole plant DDM and CP and between ADL and undigested OM. Tests were considered significant at the 0.05 level.

RESULTS

The four legumes used in this study (ALF, BFT, CMV, and SNF) had similar CP, NDF and NFC concentrations (Table 1). The mean CP concentrations of these legumes was approximately twice that of the grass and the non-legume forb, and their NDF concentrations were approximately half that of the grass and the forb (Table 1). The NFC concentration of the non-legume forb was similar to legume NFC concentrations, and the NFC concentrations of all five forbs was more than twice that of the grass (Table 1). Acid detergent lignin concentrations were similar for three of the legumes (ALF, BFT, and CMV), while ADL values were similar for SNF and SMB; the four legumes and the non-legume forb had ADL values 1.4 to 2 times greater than that of the grass. Neutral detergent fiber concentrations estimated by NIRS (Table 1) and NDF concentrations determined by ANKOM digestion (Table 2) were ranked similarly, including that of SMB. Values for NDF by NIRS averaged 7% less than ANKOM analysis.

Water-soluble carbohydrate (WSC) concentrations (Table 2) were greatest for the legumes BFT and CMV and the forb SMB, and were greater for the legume SNF than the grass.

The WSC concentration of ALF was less than that of other forages tested, while starch concentration was greatest for ALF. In whole plant material, the CT concentration of SNF was more than double that of BFT, and the concentration of HT in SMB was intermediate to the CT concentrations of BFT and SNF (Table 2). Isolated NDF of SMB retained about half the HT concentration of whole plant material, while isolated NDF of BFT and SNF retained 28 and 21%, respectively, of the CT in whole plant material of these species (Table 2).

Whole plant DDM and DOM did not differ among legumes and were greater than for the grass; grass values were greater than the non-legume forb SMB (Table 3). There was a positive correlation between whole plant DDM and CP ($r = 0.94$). The concentrations of undigested OM for whole plant material of MBG and SMB were approximately two and three times greater, respectively, than that of the four legumes (Table 3).

The DDM of isolated NDF was greatest for CMV followed by the grass, and least for SMB (Table 3). Acid detergent lignin concentration was positively correlated with undigested OM of isolated NDF ($r = 0.86$). For isolated NDF, more OM remained undigested for the three tannin-containing forages than for other forage species. The NDF of SMB contained the greatest concentration of HT and had the greatest concentration of undigested OM, followed by SNF and BFT.

Forage species had a significant effect on R_{Max} (Table 4) of both whole plant material and isolated NDF. The R_{Max} of ALF and BFT was greater than for other forages except SNF, and the R_{Max} of isolated NDF was greater for BFT and CMV than MBG. Time to reach R_{Max} (t_{RM}) was

less than 8 h for whole plant material of all species and more than 28 h for isolated NDF of all species (Table 4). The R_{Max} of isolated NDF reached values equal to or greater than the rate observed for whole plant material of CMV, MBG, and SMB.

The asymptotic gas volume (Parameter A) is the cumulative gas production at the end of fermentation predicted from analysis of cumulative gas as a function of time (Fig. 1). Asymptotic gas volume of MBG whole plant material was greater than that of CMV and SMB whole plant material, and did not differ from that of ALF, BFT, and SNF whole plant material (Table 5). Asymptotic gas volume of isolated NDF was greatest for CMV and MBG and least for SMB. Acid detergent lignin concentration was negatively correlated with asymptotic gas volume of isolated NDF ($r = -0.93$).

Hours to one-half asymptotic cumulative gas volume (Parameter B; Table 5) is related to both the rate and extent of fermentation; a lower value for Parameter B reflects a more rapid rate of rumen digestion. Among whole plant material, time to one-half gas volume was greatest for MBG and SMB, and did not differ from that of CMV. Parameter B of whole plant CMV did not differ from SNF, and Parameter B of whole plant SNF did not differ from ALF and BFT, which reached one-half asymptotic cumulative gas volume the most quickly; ALF and BFT also had the greatest whole plant R_{MAX} . Time to one-half asymptotic gas volume of whole plant material was negatively correlated with CP ($r = -0.90$). For isolated NDF, time to one-half asymptotic cumulative gas volume was greater for MBG than for ALF, BFT, and CMV and did not differ from SNF and SMB (Table 5).

For whole plant material, Parameter C was greater for ALF and BFT than for CMV, MBG, and SMB indicating a sharper acceleration of fermentation rate following the initial lag phase. Parameter C was greater for whole plant SNF than MBG and SMB and did not differ from ALF, BFT or CMV (Table 5). Values of Parameter C (Table 5) were greater for isolated NDF than for whole plant material ($p < 0.01$) and did not differ among forages (Fig. 1).

DISCUSSION

The NFC concentrations of legumes grown in the Mountain West were comparable to those for corn silage (358 g kg⁻¹ DM) and beet pulp (383 g kg⁻¹ DM)²⁸. The elevated NFC concentrations of forages cultivated in the Mountain West likely result from the greater net photosynthesis of long, sunny days²⁹ combined with cool night temperatures that reduce the rate of respiration that consumes non-structural carbohydrates³⁰.

Starch concentration was least in the cool-season grass, which stores carbohydrates as fructans³¹ in leaf and stem bases below grazing height. Alfalfa starch concentration in this study was greater than values generally reported for forage legumes; however, starch concentration of red clover (*Trifolium pretense* L.) leaves reached 350 g kg⁻¹ DM by the end of the day when grown at day/night temperatures of 19-23°C /14-16°C³². When legume WSC and starch concentrations were summed by species, they accounted for 27% (CMV) to 46% (ALF) of NFC; the balance of NFC consists of organic acids and ND-soluble fiber, including pectins and some hemicelluloses³³.

Increasing the NFC of high-forage diets by adding grain usually decreases fiber digestibility³⁴, augments VFA synthesis and may increase nutrient flow from the rumen^{35,36}. In this study, the undigested OM of isolated legume NDF ranged from 225 to 503 g kg⁻¹ DM after 96 h while legume whole plant undigested OM ranged from 78 to 88 g kg⁻¹ DM. The whole plant undigested OM of SMB, the non-legume forb, was nearly three times greater than for the legumes even though NFC of all five forbs was similar. The reduced digestibility of SMB whole plant and isolated fiber was likely due to the antimicrobial activity of HT, which is known to negatively affect digestion³⁷. These results demonstrate that NFC does not inhibit NDF digestion, and that the extent of forage digestion is influenced by the concentration of NDF and can be modulated by the presence of plant secondary compounds in NDF. Whole plant 96-h DDM of the tannin-containing legumes BFT and SNF was similar to the non-tannin legumes ALF and CMV.

Grass whole plant material produced the greatest asymptotic fermentation gas volume (Parameter A). Diets with greater fiber concentrations result in greater rumen acetate-to-propionate ratios³⁸, and acetate synthesis results in a greater volume of gas production than propionate synthesis^{39,40}. Comparing the gas volume of isolated forage NDF eliminates variation due to acetate and propionate contributions, since non-structural carbohydrates are the main source of propionate synthesis. Cicer milkvetch contains a water-soluble arabinogalactan protein that prevents cellulolytic bacteria from digesting cellulose^{41,42} and cattle gained significantly less on irrigated, rotationally stocked CMV than BFT pastures⁴³. This

arabinogalactan protein may account for the lower R_{Max} and greater Parameter B of whole plant CMV that suggests inhibited short-term digestion compared with ALF and BFT. However, these parameters were not less for CMV isolated fiber. While ALF and CMV had similar whole plant ADL concentrations, ALF has more rigid, erect stems while CMV has less rigid, vine-like stems. These differences in stem structure could result from differences in the amount, type or localization of lignin in stem fiber cell walls⁴⁴ and result in greater isolated NDF DDM of CMV compared with ALF. The grass had the least ADL, which was positively correlated with undigested NDF OM and negatively correlated with isolated NDF asymptotic gas volume. The extent of digestion of SMB isolated NDF was likely constrained by both elevated ADL and the presence of about 50% whole plant HT retained in isolated NDF.

More OM remained undigested in NDF isolates from tanniferous species that retained tannins in the NDF fraction. The HT in SMB bound more dietary N than the CT in BFT or SNF when beef cows were fed tannin-containing hays as their complete diet¹⁶. Here, the NDF of SMB, with half the whole-plant concentration of HT, had the greatest undigested OM. Tannins may bind to structural cell wall proteins and inhibit enzymes secreted by wall-digesting rumen microbes. The concentration of structural proteins in monocots is approximately 1% while structural proteins comprise as much as 10% of the cell walls of dicots, including legumes and forbs⁴⁵. Our demonstration that the NDF fraction of tanniferous forages retains significant CT and HT concentrations suggests tannins could influence the digestion of both the cell contents and the cell wall fraction of forages.

CONCLUSIONS

The perennial legumes used in this study had elevated whole plant CP compared with the grass MBG and the forb SMB. The four legumes and the forb, SMB, had elevated NFC concentrations compared with the grass. The four legumes had the greatest DDM and DOM of the six forages, and the least undigested OM after 96 h. The digestion of isolated NDF, however, was more variable and reduced by the combined concentrations of lignin and residual tannin. Among the legumes, ALF and BFT had similarly elevated maximal rates of digestion, abbreviated times to one-half asymptotic cumulative gas production, and sharp inflection points in their cumulative gas curves. While the CT concentrations of BFT and SNF appeared to affect isolated NDF digestion, they did not reduce the extent of forage digestion measured as DDM. However, some combination of reduced protein concentration, elevated NDF and the presence of HT inhibited both the rate and extent of SMB digestion. While the fiber of CMV was rapidly and more completely digested than that of other forage species, the digestion of CMV whole plant material was extensive but less rapid than other legumes, perhaps due to the presence of a water-soluble arabinogalactan that inhibits fiber digestion. Reducing the concentration of secondary compounds such as the arabinogalactan in CMV or the HT in SMB would be worthy plant breeding goals to enhance forage digestibility and improve the nutrition and productivity of ruminants grazing these forages.

CONFLICT OF INTEREST

The authors have no conflict of interest that might have influenced this study.

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Figure Legend

Fig. 1. Cumulative gas production of whole plant material (A) and isolated neutral detergent fiber (B) for an *in vitro* fermentation carried out for 96 h, expressed as mL gas g⁻¹ digestible organic matter (DOM). Error bars represent SEM; n = 3.

Table 1. Near infrared spectroscopy (NIRS) predictions of for nutritive value composited from pre-grazed rotationally stocked irrigated pastures from May through August of 2016.

	CP	ADF	aNDF	NDFD	NFC	Ash	ADL	Fat	TDN
Species	-----g kg ⁻¹ DM-----			g kg ⁻¹ NDF	-----g kg ⁻¹ DM-----				
ALF	259	198	226	515	441	68.9	50.8	14.5	700
BFT	259	179	192	373	481	62.9	54.2	16.0	822
CMV	253	212	238	671	424	80.2	50.8	14.8	784
SNF	218	233	252	320	476	48.8	73.1	07.0	760
MBG	136	370	588	618	190	80.3	36.8	27.9	604
SMB	122	298	412	487	401	59.8	77.4	34.7	686

CP, crude protein; ADF, acid detergent fiber; aNDF, neutral detergent fiber assayed with a heat-stable α -amylase; NDFD, NDF digestibility; NFC, non-fibrous carbohydrates; ADL, acid detergent lignin; TDN, total digestible nutrients.

ALF, alfalfa; BFT, birdsfoot trefoil; CMV, cicer milkvetch; SNF, sainfoin; MBG, meadow bromegrass; SMB, small burnet.

NIRS Consortium calibration equations do not include small burnet.

Table 2. Fiber, carbohydrate and tannin concentrations of whole plant material and tannin concentrations of isolated neutral detergent fiber (NDF) of forages composited from pre-grazed rotationally stocked irrigated pastures from May through August of 2016.

Species	ANKOM aNDF (SE)	WSC (SD)	Starch (SD)	Whole Plant Tannins (SD)	NDF Tannins (SD)
-----g kg ⁻¹ DM-----					
ALF	260d (0.29)	86.5d (1.36)	67.0a (0.26)		
BFT	245e (0.27)	125.9a (4.20)	36.5b (0.17)	21.2c (1.86)	6.0c (0.41)
CMV	261d (0.15)	130.1a (2.01)	15.3d (0.44)		
SNF	300c (0.24)	110.1b (7.28)	31.8c (0.10)	56.1a (2.07)	11.8b (0.19)
MBG	577a (0.07)	92.3c (4.54)	8.0e (0.30)		
SMB	406b (0.23)	131.1a (5.44)	14.0d (1.57)	39.6b (1.28)	19.9a (1.28)

ALF, alfalfa; BFT, birdsfoot trefoil; CMV, cicer milkvetch; MBG, meadow bromegrass; SMB, small burnet; SNF, sainfoin.

For neutral detergent fiber (aNDF), grass n=46 and forb n=69; aNDF values followed by the same letter are not different at $p < 0.05$ based on the Tukey-Kramer multiplicity adjustment.

Water-soluble carbohydrate (WSC), starch and tannin data are the means of three laboratory replicates. ALF, CMV and MBG contain no condensed or hydrolyzable tannins.

Table 3. Characteristics of whole plant and isolated neutral detergent fiber (NDF) of forages composited from pre-grazed rotationally stocked irrigated pastures from May through August of 2016. Data are the means of three laboratory runs; standard errors in parentheses.

Species	Whole Plant Material			Isolated NDF		
	DDM	DOM	Undigested OM	NDF DDM	NDF DOM	NDF Undigested OM
	-----g kg ⁻¹ initial DM-----					
ALF	833a (9.6)	917a (6.7)	80c (6.4)	626c (9.6)	684b (6.7)	306c (13.8)
BFT	831a (14.0)	916a (11.3)	82c (6.4)	467d (14.0)	532c (11.3)	451b (13.8)
CMV	852a (7.4)	918a (7.4)	78c (6.4)	720a (7.4)	764a (7.4)	225d (13.8)
SNF	848a (13.6)	908a (9.7)	88c (6.4)	432d (13.6)	478d (9.7)	503b (13.8)
MBG	738b (8.2)	796b (5.8)	198b (6.4)	669b (8.2)	706b (5.8)	281cd (13.8)
SMB	693c (9.2)	758c (8.0)	235a (6.4)	347e (9.2)	408e (8.0)	573a (13.8)

DDM, digestible dry matter; DOM, digestible organic matter; OM, organic matter.

ALF, alfalfa; BFT, birdsfoot trefoil; CMV, cicer milkvetch; SNF, sainfoin; MBG, meadow brome grass; SMB, small burnet.

Values within a column followed by the same letter are not different at $p < 0.05$ based on the Tukey-Kramer multiplicity adjustment.

Table 4. Maximum rate (R_{Max}) and time during fermentation at which maximum rate of cumulative gas production is reached (t_{RM}) based on *in vitro* fermentation of whole plant and isolated neutral detergent fiber (NDF) expressed on an organic matter (OM) basis.

Species	ALF	BFT	CMV	SNF	MBG	SMB
R_{Max}						
-----mL gas g ⁻¹ OM h ⁻¹ -----						
Whole	0.071a (0.0052)	0.072a (0.0052)	0.040b (0.0052)	0.053ab (0.0052)	0.032b (0.0052)	0.034b (0.0052)
NDF	0.046ab (0.0022)	0.052a (0.0022)	0.052a (0.0022)	0.047ab (0.0022)	0.040b (0.0022)	0.044ab (0.0022)
t_{RM}						
-----h-----						
Whole	7.3 (2.23)	6.7 (2.23)	7.2 (2.23)	7.7 (2.23)	7.7 (2.25)	3.4 (2.23)
NDF	28.5 (2.41)	30.4 (2.41)	30.4 (2.41)	33.0 (2.41)	33.7 (2.41)	31.7 (2.41)

ALF, alfalfa; BFT, birdsfoot trefoil; CMV, cicer milkvetch; SNF, sainfoin; MBG, meadow brome grass; SMB, small burnet.

Data are the means of three laboratory runs and three replicates in each run; standard errors in parentheses.

Values within a row followed by the same letter are not different at $p < 0.05$ based on the Tukey-Kramer multiplicity adjustment.

Table 5. Kinetics of cumulative gas production from *in vitro* fermentation of whole plant and isolated neutral detergent fiber (NDF)²¹. Parameters include asymptotic cumulative gas production (A) expressed on an organic matter (OM) basis, time to one-half asymptotic cumulative gas volume (B), and a constant (C) describing the sharpness of the sigmoidal inflection point.

Species	ALF	BFT	CMV	SNF	MBG	SMB
Parameter A						
mL g ⁻¹ OM						
Whole	194ab (13.5)	187ab (13.0)	162b (11.3)	171ab (11.9)	207a (14.4)	164b (11.4)
NDF	164b (8.6)	152b (7.9)	188a (9.8)	123c (6.4)	181a (9.4)	104d (5.4)
Parameter B						
hour						
Whole	12.4c (1.34)	12.2c (1.32)	20.1ab (1.44)	15.9bc (1.72)	26.1a (2.82)	26.5a (2.86)
NDF	26.3b (1.65)	26.5b (1.66)	26.4b (1.65)	28.8ab (1.80)	30.9a (1.93)	28.8ab (1.80)
Parameter C						
Whole	2.51a (0.071)	2.48a (0.070)	2.29bc (0.065)	2.40ab (0.068)	2.21c (0.063)	2.13c (0.060)
NDF	3.30 (0.172)	3.57 (0.186)	3.57 (0.186)	3.55 (0.185)	3.33 (0.173)	3.41 (0.177)

ALF, alfalfa; BFT, birdsfoot trefoil; CMV, cicer milkvetch; SNF, sainfoin; MBG, meadow brome grass; SMB, small burnet.

Data are the means of three laboratory runs and three replicates within each run; standard errors in parentheses.

Values within a row followed by the same letter are not different at $p < 0.05$ based on the Tukey-Kramer multiplicity adjustment.

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