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# Neutral Detergent Fiber Disappearance and Gas and Volatile Fatty Acid Production During the In Vitro Fermentation of Six Forages

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**ABSTRACT:** Samples of unfractionated forage and isolated NDF from six forages were fermented in vitro, and NDF disappearance and gas and VFA production were measured over time. Rates based on each of these data sets were calculated using a one-pool logistic model. The rates of NDF disappearance and gas and VFA production did not differ within each forage. Gas and VFA production were linearly related to NDF digestion. Gas yield was .35 mL/mg ( $r^2 = .92$ ) of NDF digested for the isolated NDF. The amount of total VFA produced per milligram of NDF digested

was more variable than gas ( $r^2 = .72$ ), with a slope of .01 mmol VFA/mg of NDF digested. The relationship between gas and VFA production was linear (mean slope of 1.43 mmol gas/mmol VFA,  $r^2 = .69$ ). The ratios of end products (gas and VFA) to NDF digestion and the ratio of acetate:propionate were variable during the first 8 h of fermentation but changed little after this time. Changes in the acetate:propionate ratio explained 23% of the variation in gas produced per millimole of total VFA detected.

Key Words: Gases, Volatile Fatty Acids, Fiber, Forage, In Vitro

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## Introduction

The beef NRC (1996) includes a nutrition model that requires digestion rates of the fiber and soluble carbohydrate fractions to predict animal performance. A complete feed library with digestion rates is needed. Dry matter and NDF digestion rates may be obtained using standard in vitro techniques, but these methods are labor-intensive (Pell and Schofield, 1993; Cone et al., 1996). Gas production is a promising technique to measure the digestion rates of the soluble and insoluble fractions of forages (Menke and Steingass, 1988; Pell and Schofield, 1993; Cone et al., 1996).

Wolin (1960) presented calculations on the relationship among VFA, gas production, and the fermentation of glucose. Although no adjustments are made for microbial yield, these calculations (Wolin, 1960) are often used to predict changes in gas production caused by metabolic shifts during fermentation (Beuvink and Spoelstra, 1992). Gas arises directly from microbial metabolism and indirectly from the reaction of acid end products with bicarbonate, an important component of the buffering system (Beuvink and Spoelstra, 1992).

The use of gas measurements to estimate the rate of forage digestion depends on a close relationship between gas yield and VFA production (Blümmel and Ørskov, 1993). Most results are from late in the fermentation and imply a constant relationship between substrate disappearance and end product formation over time. However, microbial population size and metabolism may vary over the course of a fermentation (El-Shazly and Hungate, 1965; Naga and Harmeyer, 1975; Krishnamoorthy et al., 1991). Changes in microbial metabolism or yield may alter the relationship between substrate digestion and gas production and could affect the estimation of digestion rate from gas measurements. The objective of these experiments was to explore the relationships among NDF disappearance and gas and VFA production over time using an in vitro system.

## Materials and Methods

Six forages were chosen to provide a broad range of NDF content. Two samples each of alfalfa (*Medicago sativa*) and bromegrass (*Bromus inermis*) were included, in addition to corn stover (*Zea mays*) and wheat straw (*Triticum aestivum*). All samples were harvested by hand from central New York. The alfalfa and bromegrass samples contained the entire plant. The corn stover did not contain any grain. Samples were oven-dried at 60°C and ground through a

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1-mm screen (Wiley mill, model 4, Arthur H. Thomas Co., Philadelphia, PA). Soluble CP, CP, NDF, ADF, neutral detergent insoluble protein (**NDIP**), acid detergent insoluble protein (**ADIP**), lignin, and ash were determined for each forage. Fiber and ash analyses were performed as described by Goering and Van Soest (1970) and Van Soest et al. (1991). Crude protein was measured using the macro-Kjeldahl procedure (AOAC, 1990) modified by using boric acid in the distillation process. Protein fractions were determined as described by Licitra et al. (1996).

### *NDF Isolation*

Isolated NDF from each forage was prepared by autoclaving 150-mL serum bottles containing 500 mg of forage and 100 mL of ND solution at 105°C for 1 h (Pell and Schofield, 1993). The isolated NDF from different bottles was combined and rinsed with hot water and 100 mL of ethanol using a 37- $\mu$ m nylon mesh filter (Tetko®, Briarcliff Manor, NY). Residual detergent was removed by soaking approximately 1 g of the isolated NDF overnight at 39°C in a solution of 90 mL of 1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 10 mL of *t*-butanol. The isolated fiber was filtered again and rinsed with hot water followed by 100 mL each of ethanol and acetone, and allowed to air-dry.

### *Experimental Design*

For each forage, samples of the unfractionated forage and its isolated NDF were fermented *in vitro* using the computerized gas monitoring system of Pell and Schofield (1993). Individual samples were removed at 2-h intervals from 2 to 24 h and at 32, 40, and 48 h. At each time, pH, NDF disappearance, gas volume, and VFA production were measured. A sample of each forage and its NDF fraction were fermented on two separate days. Gas production was recorded every 30 min until the fermentation of a sample was halted.

Estimates of the rate of NDF digestion and VFA and gas production were calculated from the measurements made when the fermentations were terminated. In addition, the cumulative recording of the gas volume produced from the 48-h digestions provided a separate estimate of fermentation rate. The rate of gas production during the entire 48-h fermentation was calculated from the complete 48 h of gas production data. The curve subtraction approach was used to estimate the contribution of the neutral detergent soluble fraction (**NDS**; Schofield and Pell, 1995).

### *In Vitro Procedure*

Ruminal fluid was collected approximately 4 h after feeding from a mature, nonlactating Holstein cow maintained on average-quality mixed hay (approximately 65% NDF and 8% CP) in accordance with the Institutional Animal Care and Use Committee pro-

cedure. Ruminal fluid was transferred under CO<sub>2</sub> to Balch tubes and centrifuged at 200  $\times$  *g* for 3 min to remove feed particles. Bacteria were separated from the ruminal fluid by centrifugation at 3,500  $\times$  *g* for 20 min. The supernatant was removed, and the bacteria were resuspended in anaerobic medium (Goering and Van Soest, 1970). The bacteria were centrifuged again and suspended in fresh medium to an optical density of 1.0 at 650 nm.

The bicarbonate-phosphate buffer of Goering and Van Soest (1970) was used for *in vitro* digestions. For these experiments, sodium sulfide was replaced by an equal weight of cysteine hydrochloride. Yeast extract and a VFA mixture (Cotta and Russell, 1982) were added to provide the nutrient requirements of the isolated rumen bacteria normally provided by ruminal fluid (.125 g and .3 mL per 100 mL of medium, respectively). The medium was boiled to remove dissolved gases, cooled under CO<sub>2</sub>, and then the cysteine, yeast extract, and VFA mixture were added. The pH was adjusted to 6.8 with NaOH. At the outset of a fermentation, each bottle contained 8 mL of medium, 2 mL of bacteria suspended in medium, and either 100 mg of unfractionated forage or 50 mg of isolated NDF.

As the fermentation progressed, samples were removed from the incubator at the specified times, pressure was released from the bottles using a needle, the sensors were removed from the bottles, and the pH was measured immediately. A 1.5-mL aliquot of the medium was removed and centrifuged. The supernatant was stored for VFA analysis. The pellet was returned to the bottle, in which all remaining material was subjected to micro-NDF analysis (Pell and Schofield, 1993).

### *Gas Measurement*

Gas measurements were taken every 30 min as previously described by Pell and Schofield (1993). The sensor calibration procedure of Schofield and Pell (1995) was used to improve accuracy. All gas volumes were corrected to standard atmospheric pressure (760 mm Hg) and expressed as the volume of gas produced by 100 mg of sample DM. Methane was assumed to be low due to the bacterial isolation procedure and based on previous methane measurements. Therefore, no correction was made for the presence of water-insoluble gas. The gas contribution of the NDS fraction was calculated by subtracting the cumulative gas curves (from the 48-h sample) of the NDF sample (adjusted to represent the quantity of NDF present in the initial forage sample) from that of the corresponding unfractionated forage (**U**) curve (Schofield and Pell, 1995; Stefanon et al., 1996).

### *VFA Analysis*

At each observation time, a 1.5-mL aliquot of the medium was centrifuged at 4,000  $\times$  *g* for 5 min, and

the supernatant was removed and frozen. After the 48-h incubation period, the samples were thawed and vortexed for 30 s. A 360- $\mu$ L aliquot of each sample was transferred to a microcentrifuge tube containing 40  $\mu$ L of 50 mM H<sub>2</sub>SO<sub>4</sub>. After mixing and standing at room temperature for 10 min, the centrifugation was repeated and the supernatant was analyzed for VFA with the HPLC method of Ehrlich et al. (1981) using a BioRad HPX-87H column (7.8  $\times$  300 mm) at 30°C, isocratic elution with 5 mM H<sub>2</sub>SO<sub>4</sub>, and UV detection at 210 nm. A mixture of succinic, lactic, acetic, propionic, isobutyric, and butyric acids was included as a standard in all analyses. For separation of succinic and lactic acids, a column temperature of 60°C was used. Values have been corrected to exclude the VFA added to the medium initially. Where total VFA production is mentioned, the sum of succinic, lactic, acetic, propionic, isobutyric, and butyric acids is implied.

For a few samples (approximately 5%) pyroglutamate overlapped the isobutyrate peak. Pyroglutamate is formed during the industrial production of the casein acid hydrolysate, which was included in the medium as an amino acid supplement. Pyroglutamate was only of concern early in the fermentation when it had not yet been metabolized by the microbes. When pyroglutamate was a problem, the Peakfit program (Jandel Scientific, San Rafael, CA) was used to distinguish among peaks. Areas under the peaks were determined by fitting the sample and standard peaks to a simple Gaussian profile. The variation in peak area estimates was evaluated based on the individual *t*-values (parameter value/parameter standard error), and the *F*-statistic indicated the overall fit. Estimates of peak area were excluded from consideration if either the *t*-values for the peak parameters were below 10 or if the *F*-value of the curve fit did not exceed 1,000.

For the theoretical prediction of indirect gas yield from VFA production (Beuvink and Spoelstra, 1992), it was necessary to determine the amount of gas released from bicarbonate with the addition of acid. Eight bottles containing 10 mL of the *in vitro* medium were placed in the gas monitoring system. Six of the bottles were titrated using .1 mL additions of 1 M acetic acid. The remaining two bottles served as blanks to which an equal amount of water was added to correct for changing volume. Pressure measurements were taken 15 min after each addition of acid. Gas volumes were corrected to standard atmospheric pressure. The slope of the line describing the relationship between gas volume and added acid was determined with linear regression. Only data from the linear portion of the curve (up to 1.2 mmol acetic acid) were used.

### Rate Calculation

Timed removal of samples and measurement of NDF disappearance and VFA production at only 15

times during the 48-h fermentation provided a relatively small number of data points and restricted our choice of models for curve fitting (Cappio-Borlino et al., 1993). Specific rates were calculated using the one-pool logistic model (Zwietering et al., 1990; Schofield et al., 1994). This model takes the form

$$V = V_f * [1 + \exp(2 - 4S(t - \lambda))]^{-1}$$

where *V* is the gas volume at time *t*; *V<sub>f</sub>* is the maximum volume at *t* = ∞; *S* is a rate constant called the specific rate (*S* = maximum rate/maximum volume); and  $\lambda$  is a constant equivalent to a lag value. This model assumes that VFA production, gas production, and NDF digestion are proportional to the accumulated microbial mass and the remaining digestible substrate (France and Thornley, 1984; Schofield et al., 1994).

Our data generated two kinds of gas production curves. The timed removal of forage subsamples gave 15 time points over the 48-h incubation. The uninterrupted data from a single 48-h incubation (readings at 30-min intervals) provided 96 data points. The larger number of data points allows the use of a more complicated mathematical model for estimation of rates. Forage is not a uniform substrate, and the use of a more complex mathematical model to describe the gas production curves may provide insight into changes in the digestion kinetics of the carbohydrate fractions. Kinetic analysis of the 48-h cumulative gas production data was performed using a two-pool logistic model that includes a second additive logistic function in the original model (modified to have the same lag value) (Schofield et al., 1994). A model with more parameters always increases the fit (*F*-statistic), but the *t*-value of the parameters reflects changing standard errors. Therefore, the two-pool model was chosen if a better fit (*F*-statistic) was combined with *t*-values similar to those obtained with the one-pool model. For cases when low *t*-values (*t* < 12) were obtained, the one-pool logistic model was used. All curves were fitted using Table Curve (version 2.0, Jandel Scientific, San Rafael, CA).

### Statistical Analyses

The timed measurements taken during the fermentations formed a split-plot structure; the forage sample represents the whole plot factor, and time represents the split-plot factor. Timed measurements are fully crossed with the effects of forage, fraction, and day. Fraction and day of fermentation are nested within the forage sample. The resulting model is  $Y = \mu + S_i + D(S_i)_j + F(S_i)_k + F * D(S_i)_{jk} + T_1 + T * S_{il} + T * D(S_i)_{jl} + T * F(S_i)_{kl} + E_{ijkl}$ , where  $\mu$  = the population mean, *S<sub>i</sub>* = sample of forage, *D<sub>j</sub>* = day of fermentation, *F<sub>k</sub>* = fraction of the forage, *T<sub>1</sub>* = time, and *E* = error, for *i* = 1 to 6, *j* = 1 to 2, *k* = 1 to 3, and *l* = 1 to 15.

To avoid confounding estimates of the contribution of NDS to gas production with variation in rumen

fluid, samples of an unfractionated forage and its isolated NDF were fermented on the same day. Each computer system was able to monitor only 16 samples (15 fermentations and a blank), and two systems were available. On a single day, there were only enough places for samples of a single forage (a total of 30 fermentations from unfractionated forage and isolated NDF). The confounding of rumen fluid (day) and forage effect was removed by replicating the fermentation of each forage on two separate days. Thus, fermentations occurred on 12 d (six forage samples, each on 2 d), with each fermentation containing unfractionated forage and ND samples (of a single forage) for 15 time points. This formed the nested structure presented, where the effect of rumen inoculum (day) and forage fraction (unfractionated forage, isolated NDF, or NDS) were nested within forage sample. For direct comparison of some final measurements (NDF digestibility, for example) from the fermentation of the intact forage and its isolated NDF, both values may be considered "fractions" within the forage effect. Forage ( $S_j$ ) and fraction ( $F_k$ ) effects were tested on their respective interaction term with the nested variable "day" ( $D_j$ ). In addition to error the  $E_{ijkl}$  term includes the three-way interaction of day, fraction, and time.

As a supplement to the analysis of time effects with the full model, relationships among VFA production, gas volume, and NDF digestion were studied using regression. For each day, the relationship between VFA or gas produced and NDF digested was calculated. The resulting slopes represent the average response over time for each day and removed time as variable from the model.

Curve fit parameters were treated as discrete variables for the comparisons of different rates. Acetate:propionate ratio was included as a continuous variable when the effects of this variable on the relationship between gas volume and VFA production were assessed. All analyses were performed using the GLM procedure of SAS (1985). A significance of  $P < .05$  is assumed unless otherwise stated.

## Results

### *Chemical Composition of Forages*

The chemical compositions of the forages in these experiments are presented in Table 1. The NDF and lignin contents of the forages indicate a broad range of maturity and composition.

### *Fermentation Characteristics*

The NDF digestibility did not differ between unfractionated forage (U) and NDF samples (Table 2). The NDF digestibilities of both bromegrass samples were higher than those of the other forages,

perhaps reflecting the lower lignin content. The average NDF digestibility and lignin contents of the mature alfalfa and the wheat straw were similar. Corn stover had a greater NDF digestibility than immature alfalfa, although the lignin contents were similar. The increased NDF digestion of the corn stover is related to the greater content of hemicellulose. The wheat straw also contains considerable hemicellulose, but it is apparently less available for digestion. After 48 h of fermentation, the mean sample pH exceeded 6.4 for all samples, indicating that fiber digestion was not limited by pH. This pH was within the linear range of the titration curve for the buffer system so that gas released by the titration of bicarbonate from the buffer was linearly related to acid production (Beuvink and Spoelstra, 1992).

Total VFA production at 48 h among the U samples was not different ( $P > .05$ ). Wheat straw (U) produced less propionate than immature bromegrass. This resulted in a higher acetate:propionate ratio for wheat straw relative to the bromegrass; the other samples were intermediate. On a DM basis, isolated NDF from wheat straw produced less total VFA than the other forages. Compared to the intact forage, the isolated NDF tended ( $P = .10$ ) to have greater acetate and total VFA production.

### *Kinetic Analysis of Gas Production*

Table 3 presents the curve fit parameters for the 48-h cumulative gas production. The improved description of the fermentation curves with the two-pool logistic model implies that gas was being produced at two discernible rates, whereas the fit of a single pool suggests a uniform fermentation rate. Two "pools" may occur from the fermentation of two distinct substrates, from two separate microbial populations, or from a combination of both factors. To simplify comparisons among forages, the amount of gas associated with the faster-digesting pool is presented as a percentage of the total gas produced.

Forages with the highest NDF concentrations (corn stover and wheat straw) had a lower proportion of gas associated with the fast specific rate than the other forages. The U sample of corn stover had two distinct pools, with less gas (32%) associated with the faster specific rate compared to alfalfa or bromegrass. There were no differences in the proportion of gas associated with the fast specific rates of alfalfa and bromegrass for either the U or NDF samples. For wheat straw, the U and NDF samples were best described with the one-pool model. The cumulative gas production of the U and NDF samples of wheat straw were so similar that NDS gas production, the difference between these curves, did not differ from zero. Immature alfalfa NDS fit the two-pool logistic model, whereas the mature sample of alfalfa and the other samples were best described with the one-pool logistic model indicating a more uniform substrate in terms of digestion rate.

Table 1. Chemical composition of experimental forages on a DM basis

Fraction, %	Alfalfa (immature)	Alfalfa (mature)	Brome (immature)	Brome (mature)	Corn stover	Wheat straw
NDF	31.1	47.3	40.6	55.9	72.1	85.2
ADF	22.8	32.2	21.5	31.8	38.1	53.4
CP	24.4	18.8	25.6	14.6	4.8	2.1
Soluble CP <sup>a</sup>	11.2	6.9	12.1	5.2	2.0	.7
NDIP <sup>b</sup>	2.2	4.9	5.9	4.4	1.9	.7
ADIP <sup>c</sup>	1.0	1.1	.4	.6	.6	.5
Lignin	6.3	9.0	2.1	3.8	6.0	9.0
Ash	8.8	7.3	9.2	9.0	5.5	3.5

<sup>a</sup>Determined by procedure of Licitra et al. (1996).

<sup>b</sup>NDIP = neutral detergent insoluble protein.

<sup>c</sup>ADIP = acid detergent insoluble protein.

### Rate Comparisons

For each individual forage sample, a similar digestion rate ( $P = .42$ ) was obtained when calculated based on NDF disappearance, terminal gas volume, or VFA production (Table 4). The absolute ranking among the forages by rate changed slightly between categories (NDF disappearance and gas or VFA production). For the U samples, within each category, immature alfalfa consistently had the fastest rate and corn stover and wheat straw consistently had the slowest rates. For the NDF samples, the ranking of rates was not consistent, but the differences among the forages were small ( $SE = .01$ ).

Wheat straw was the only unfractionated forage to have a lag time significantly different from zero ( $P <$

.05) for all three curve types (neutral detergent, gas, VFA). The wheat straw lag was similar for the U and NDF samples. Lag time for the wheat straw was shortest for VFA production, intermediate for NDF disappearance, and longest for gas production.

### Relationships with VFA Production

Figure 1 presents the VFA (panel A) and gas production (panel B) over time for three U samples (immature alfalfa, mature brome grass, and wheat straw). These grasses and legume were chosen because they provided a wide range of NDF content. The gas and VFA data show the same basic fermentation curves. The change in relative position for the immature alfalfa (exceeding brome grass in VFA

Table 2. Characteristics<sup>a</sup> of the in vitro fermentations at 48 hours

Item	Alfalfa (immature)	Alfalfa (mature)	Brome (immature)	Brome (mature)	Corn stover	Wheat straw	SE
Unfractionated forage							
NDF dig., %	51.0 <sup>i</sup>	51.2 <sup>i</sup>	92.2 <sup>f</sup>	84.6 <sup>g</sup>	62.0 <sup>h</sup>	51.4 <sup>i</sup>	1.8
pH	6.57	6.60	6.46	6.44	6.44	6.56	.05
Gas <sup>b</sup>	21.5 <sup>g</sup>	19.5 <sup>h</sup>	23.8 <sup>f</sup>	25.6 <sup>f</sup>	21.1 <sup>g</sup>	17.2 <sup>i</sup>	.6
Acetate <sup>c</sup>	36.2	36.6	31.0	32.4	31.1	33.3	4.6
Propionate	19.5 <sup>fg</sup>	16.8 <sup>fg</sup>	20.9 <sup>f</sup>	18.0 <sup>fg</sup>	15.2 <sup>fg</sup>	15.0 <sup>g</sup>	1.7
Butyrate	2.5 <sup>g</sup>	4.2 <sup>fg</sup>	7.7 <sup>f</sup>	6.2 <sup>fg</sup>	5.6 <sup>fg</sup>	3.1 <sup>fg</sup>	1.5
VFA total	58.3	57.5	59.6	56.6	51.9	51.4	6.4
A:P <sup>d</sup>	1.85 <sup>fgh</sup>	2.18 <sup>fg</sup>	1.48 <sup>h</sup>	1.78 <sup>gh</sup>	2.04 <sup>fg</sup>	2.22 <sup>f</sup>	.12
Isolated NDF							
NDF dig., %	51.2 <sup>ij</sup>	47.2 <sup>j</sup>	91.2 <sup>f</sup>	82.2 <sup>g</sup>	68.0 <sup>h</sup>	54.0 <sup>i</sup>	1.5
pH	6.67	6.66	6.55	6.58	6.62	6.67	.05
Gas	20.0 <sup>i</sup>	18.7 <sup>i</sup>	31.5 <sup>f</sup>	27.6 <sup>g</sup>	23.2 <sup>h</sup>	18.8 <sup>i</sup>	.8
Acetate	40.6	53.8	42.0	39.4	50.4	33.4	6.2
Propionate	17.3 <sup>gh</sup>	25.0 <sup>f</sup>	23.2 <sup>fg</sup>	20.1 <sup>fgh</sup>	21.3 <sup>fgh</sup>	15.5 <sup>h</sup>	2.3
Butyrate	5.0	5.2	11.0	8.3	9.6	3.6	2.5
VFA total	62.7 <sup>fg</sup>	83.8 <sup>f</sup>	76.2 <sup>f</sup>	67.8 <sup>fg</sup>	81.2 <sup>f</sup>	52.5 <sup>g</sup>	7.1
A:P	2.35	2.18	1.81	1.95	2.37	2.13	.22

<sup>a</sup>Least squares means  $\pm$  SE. Forage values within rows with different superscripts differ ( $P < .05$ ).

<sup>b</sup>Gas (mL/100 mg of DM).

<sup>c</sup>VFA (mmol·L<sup>-1</sup>·100 mg of initial sample DM<sup>-1</sup>).

<sup>d</sup>A:P = acetate:propionate ratio.

Table 3. Curve fit parameters<sup>a</sup> from the cumulative gas production of the 48-hour in vitro digestions<sup>b</sup>

Forage	% Fast <sup>c</sup>	Fast SR	Slow SR	Lag h	Total volume, <sup>d</sup> mL	
					Proportional	100 mg DM
Unfractionated forage						
Alfalfa (immature)	55.5 <sup>f</sup>	.17 <sup>f</sup>	.036 <sup>fg</sup>	1.7 <sup>g</sup>	21.7 <sup>g</sup>	21.7 <sup>g</sup>
Alfalfa (mature)	46.2 <sup>fg</sup>	.16 <sup>f</sup>	.038 <sup>f</sup>	1.9 <sup>g</sup>	19.4 <sup>h</sup>	19.4 <sup>h</sup>
Brome (immature)	51.1 <sup>f</sup>	.12 <sup>g</sup>	.035 <sup>fg</sup>	1.7 <sup>g</sup>	23.7 <sup>fg</sup>	23.7 <sup>fg</sup>
Brome (mature)	50.0 <sup>f</sup>	.10 <sup>g</sup>	.032 <sup>fg</sup>	2.5 <sup>g</sup>	25.6 <sup>f</sup>	25.6 <sup>f</sup>
Corn stover	32.0 <sup>g</sup>	.10 <sup>g</sup>	.030 <sup>g</sup>	3.1 <sup>g</sup>	21.8 <sup>g</sup>	21.8 <sup>g</sup>
Wheat straw	SP	SP	.035 <sup>fg</sup>	9.4 <sup>f</sup>	16.9 <sup>i</sup>	16.9 <sup>i</sup>
SE	4.3	.01	.002	1.6	.6	.6
Isolated NDF						
Alfalfa (immature)	51.5 <sup>f</sup>	.12	.028 <sup>g</sup>	4.9	6.4 <sup>i</sup>	20.5 <sup>i</sup>
Alfalfa (mature)	47.8 <sup>f</sup>	.11	.028 <sup>g</sup>	8.2	8.9 <sup>h</sup>	19.6 <sup>i</sup>
Brome (immature)	49.6 <sup>f</sup>	.11	.037 <sup>f</sup>	6.2	12.7 <sup>g</sup>	31.3 <sup>f</sup>
Brome (mature)	44.8 <sup>f</sup>	.19	.033 <sup>fg</sup>	7.3	15.3 <sup>f</sup>	27.4 <sup>g</sup>
Corn stover	26.1 <sup>g</sup>	.17	.030 <sup>fg</sup>	8.6	16.6 <sup>f</sup>	23.9 <sup>h</sup>
Wheat straw	SP	SP	.030 <sup>fg</sup>	7.9	15.9 <sup>f</sup>	19.1 <sup>i</sup>
SE	4.8	.02	.002	2.2	.4	.7
Neutral detergent solubles <sup>e</sup>						
Alfalfa (immature)	61.6	.24 <sup>f</sup>	.040	1.9	15.3 <sup>f</sup>	NM
Alfalfa (mature)	100	.13 <sup>g</sup>	SP	.8	10.5 <sup>g</sup>	NM
Brome (immature)	100	.13 <sup>g</sup>	SP	.3	11.0 <sup>g</sup>	NM
Brome (mature)	100	.10 <sup>g</sup>	SP	.0	10.3 <sup>g</sup>	NM
Corn stover	100	.14 <sup>g</sup>	SP	.0	5.2 <sup>h</sup>	NM
Wheat straw	100	NM	NM	NM	NM	NM
SE	4.3	.02	.002	.2	.4	—

<sup>a</sup>Least squares means. Forage values within columns with different superscripts differ within forage fraction ( $P < .05$ ).

<sup>b</sup>Abbreviations: SR = specific rate of digestion, SP = single pool, NM = not measured.

<sup>c</sup>Percentage of material that was in the rapidly digested fraction, calculated from the gas volume parameters of the two-pool logistic equation (Schofield et al., 1994).

<sup>d</sup>Total volume calculated by nonlinear curve fit. The proportional volumes are based on 100 mg DM of the original forage. The NDF and NDS gas volumes are adjusted to represent the mass of those fractions present in unfractionated sample.

<sup>e</sup>Calculated by difference between the gas produced by 100 mg forage DM and the amount of gas produced by the isolated NDF in proportion to forage NDF content. 100% = one-pool logistic model.

formation, but intermediate in gas produced) indicates that gas and VFA production are not exactly equivalent measures.

The gas and VFA production were correlated with NDF disappearance using the isolated NDF samples (Figure 2). When gas production from the isolated NDF was regressed against NDF disappearance for each forage, the resulting slopes ranged from .31 mL/mg for mature bromegrass to .37 mL/mg for corn stover (Table 5). The differences among the forages were small but statistically significant. The mean slope (gas yield from NDF digestion) from the NDF data was .35 mL of gas/mg of NDF disappearance ( $r^2 = .92$ ,  $n = 171$ ).

Total VFA production from the NDF samples was also linearly related to NDF disappearance but was more variable than gas production (Figure 2). The  $r^2$  for individual regressions ranged from .60 (mature alfalfa) to .98 (corn stover). The mature alfalfa sample produced significantly more VFA from the NDF digestion (Table 5) than the grass samples. The pooled slope, across forages, was .01 mmol VFA/mg NDF disappearance ( $r^2 = .72$ ,  $n = 166$ ).

Titration of our buffer mixture yielded a slope of .78 mmol indirect gas per mmol of VFA ( $r^2 = .98$ ). Using

the stoichiometric equation of Beuvinck and Spoelstra (1992) (gas mL =  $V_m \cdot \text{acetate} + 2V_m \cdot \text{butyrate} + .78 V_m \cdot \text{total VFA}$ , where  $V_m$  = molar gas volume, 25.6 mL per mmol at 39°C), actual gas volumes and those predicted from total VFA production were compared. Observed gas was 89% (SE = 20) of predicted gas volume across the entire data set. As the actual gas volume measured increased, the ability to predict gas produced improved. This reflects an over-estimation of gas produced early in the fermentations. In terms of gas produced, microbial yield is a pool of carbon that is not measured and may account for some of the discrepancy between predicted and observed gas production.

Figure 3 presents the overall relationships between VFA production, NDF disappearance, and gas production. The data were quite variable before 8 h, but the values stabilized after this time. If the assumption is made that NDF is equivalent to glucose (Wolin, 1960) (MW = (180 - 18) = 162) one might expect 2 mmol VFA per mmol of glucose or .012 mmols VFA per mg of NDF digested. After 8 h, our values were approaching this theoretical value (Figure 3, panel A). However, the initial values (2 to 6 h) of VFA produced were much higher than can be explained by the fermenta-

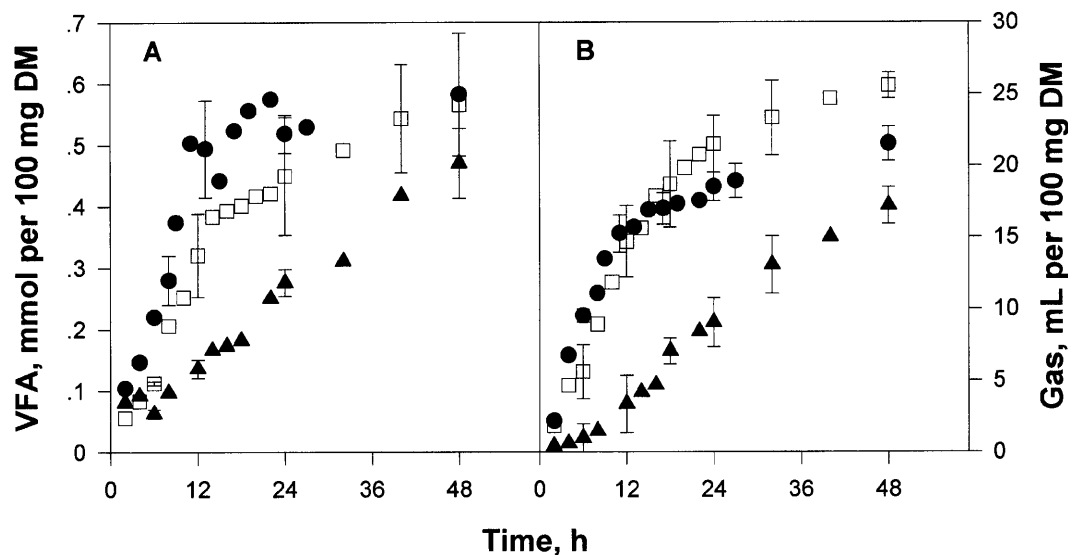


Figure 1. Unfractionated forage VFA (panel A) and gas production (panel B) over time of immature alfalfa (●), mature bromegrass (□), and wheat straw (▲). Values for immature alfalfa and bromegrass are presented with wheat straw to provide examples of legumes and grasses and a range of fiber content. VFA production = acetate + propionate + butyrate. Data were from interrupted fermentations.

tion of glucose. Similarly, more gas was produced early in the fermentation than can be explained by NDF fermentation (Figure 3, panel B). The greatest variability in the relationship between VFA production and gas production was evident in this period (Figure 3, panel C). There were changes in the VFA proportions as well (Figure 4), but these are insufficient to explain the variation in the gas to VFA relationship. Regression of the gas produced per millimole of VFA on the acetate:propionate ratio showed that the acetate:propionate ratio accounted for only 23% of the variation in gas produced from VFA appearance.

## Discussion

### Kinetic Information

The proportion of gas associated with the rapidly digesting portion of the unfractionated forage exhibited the same basic trends that were reported by Schofield and Pell (1995). As the fiber content of the unfractionated forage increased, the amount of gas in the rapid pool decreased. The improved fit of the single pool logistic model for the NDS fraction of the mature alfalfa and bromegrass samples is inconsistent with earlier research. Stefanon et al. (1996) presented parameter estimates from the two-pool logistic model from fermentations of the water-soluble fractions of alfalfa and bromegrass samples of varying maturity. The extraction methods (water vs neutral detergent solution) may account for the differences. Schofield and Pell (1995) also reported parameter estimates from the two-pool logistic model for alfalfa

NDS. Two reasons why the one-pool model better fits the data from this study may be 1) the greater maturity and NDF content of the mature alfalfa sample and 2) the use of washed and resuspended bacteria rather than whole ruminal fluid.

The fast pool of the unfractionated forage is identified from a mathematical analysis of the gas profile rather than from a chemical or physical entity. The values for the specific rates of the rapidly digested pool in the isolated NDF samples (.107 to .194 h<sup>-1</sup>) were similar to the fast rate of the unfractionated forage (.095 to .170 h<sup>-1</sup>) and comparable to rates previously reported (Schofield and Pell, 1995; Stefanon et al., 1996). The gas volume associated with the fast specific rate in the unfractionated forage may be a composite pool originating from both the NDF and NDS fractions and must be interpreted cautiously in relation to chemical composition. Similarly, the slower rates of the NDS fraction presented here for the immature alfalfa (.04 h<sup>-1</sup>) and by Stefanon et al. (1996) for water-soluble components (.03 h<sup>-1</sup> to .07 h<sup>-1</sup>) approach the values of the slower rates within the NDF fraction (.03 h<sup>-1</sup>). This supports the contention that the gas associated with the slower rate in a unfractionated forage may arise from both the NDF and NDS fractions.

### Rate Comparisons

The strong relationship between the rate of NDF disappearance, or *in vitro* organic matter disappearance, and gas production has been documented previously (Blümmel and Ørskov, 1993; Pell and Schofield, 1993; Schofield and Pell, 1995). Our results are consistent with previous research.

Table 4. Parameter estimates<sup>a</sup> from the one-pool logistic model for the in vitro digestions

Forage	Unfractionated forage			Isolated NDF		
	Extent <sup>b</sup>	SR <sup>c</sup>	Lag h	Extent	SR	Lag h
	NDF digestibility, %					
Alfalfa (immature)	50.0 <sup>g</sup>	.76 <sup>d</sup>	1.1 <sup>e</sup>	49.7 <sup>g</sup>	.053 <sup>ef</sup>	.4 <sup>f</sup>
Alfalfa (mature)	47.7 <sup>g</sup>	.052 <sup>ef</sup>	2.1 <sup>e</sup>	43.9 <sup>g</sup>	.063 <sup>de</sup>	4.2 <sup>e</sup>
Brome (immature)	90.3 <sup>d</sup>	.064 <sup>de</sup>	1.1 <sup>e</sup>	86.5 <sup>d</sup>	.074 <sup>d</sup>	3.5 <sup>ef</sup>
Brome (mature)	80.5 <sup>e</sup>	.057 <sup>ef</sup>	1.9 <sup>e</sup>	75.6 <sup>e</sup>	.056 <sup>ef</sup>	2.5 <sup>ef</sup>
Corn stover	60.2 <sup>f</sup>	.044 <sup>fg</sup>	3.0 <sup>de</sup>	61.9 <sup>f</sup>	.045 <sup>fg</sup>	5.6 <sup>de</sup>
Wheat straw	52.4 <sup>g</sup>	.030 <sup>g</sup>	6.2 <sup>d</sup>	52.7 <sup>fg</sup>	.036 <sup>g</sup>	9.0 <sup>d</sup>
SE	1.8	.005	1.2	1.8	.005	1.2
	Gas production, mL/100 mg DM					
Alfalfa (immature)	20.4 <sup>ef</sup>	.082 <sup>d</sup>	.1 <sup>e</sup>	20.1 <sup>fg</sup>	.046	2.4 <sup>e</sup>
Alfalfa (mature)	18.0 <sup>fg</sup>	.056 <sup>e</sup>	.2 <sup>e</sup>	18.6 <sup>g</sup>	.047	6.1 <sup>de</sup>
Brome (immature)	22.7 <sup>de</sup>	.052 <sup>ef</sup>	.2 <sup>e</sup>	30.6 <sup>d</sup>	.056	3.9 <sup>e</sup>
Brome (mature)	24.2 <sup>d</sup>	.049 <sup>ef</sup>	.6 <sup>e</sup>	26.0 <sup>e</sup>	.053	4.4 <sup>e</sup>
Corn stover	21.1 <sup>e</sup>	.037 <sup>f</sup>	.4 <sup>e</sup>	23.0 <sup>ef</sup>	.053	6.4 <sup>de</sup>
Wheat straw	16.6 <sup>g</sup>	.038 <sup>f</sup>	9.1 <sup>d</sup>	18.7 <sup>g</sup>	.044	10.0 <sup>d</sup>
SE	.4	.006	1.6	.4	.006	1.6
	VFA production, mmol/L per 100 mg DM					
Alfalfa (immature)	54.1	.087 <sup>d</sup>	.6 <sup>e</sup>	59.7 <sup>e</sup>	.057	.0
Alfalfa (mature)	49.8	.059 <sup>e</sup>	.0 <sup>e</sup>	88.4 <sup>d</sup>	.031	2.2
Brome (immature)	56.0	.061 <sup>de</sup>	1.4 <sup>de</sup>	66.6 <sup>de</sup>	.046	1.8
Brome (mature)	53.4	.046 <sup>ef</sup>	.8 <sup>de</sup>	57.0 <sup>e</sup>	.066	1.6
Corn stover	52.1	.044 <sup>ef</sup>	.6 <sup>e</sup>	78.9 <sup>d</sup>	.037	1.8
Wheat straw	45.8	.027 <sup>f</sup>	2.3 <sup>d</sup>	49.4 <sup>e</sup>	.057	1.8
SE	5.9	.010	1.0	5.9	.010	1.0

<sup>a</sup>Least squares means. Values within category of measurement and column with different superscripts differ ( $P < .05$ ).

<sup>b</sup>Pool size: NDF disappearance (%); gas production (mL/100 mg DM), VFA production (mmol/L).

<sup>c</sup>SR = specific digestion rate.

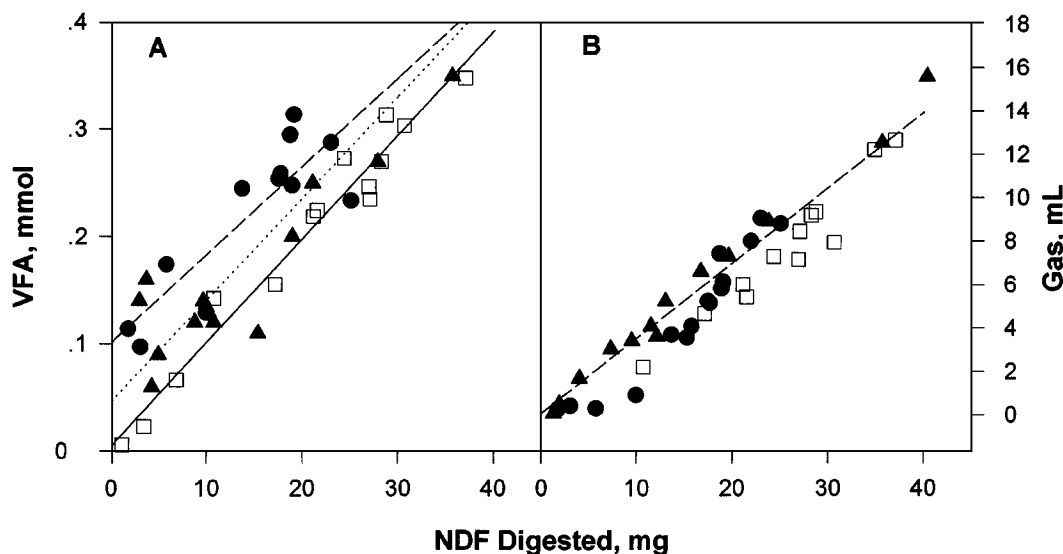


Figure 2. The relationship of VFA production (panel A) and gas production (panel B) to NDF disappearance. Isolated NDF from immature alfalfa (●), mature bromegrass (□), and wheat straw (▲) are presented. Values for immature alfalfa, bromegrass, and wheat straw are shown. VFA production = acetate + propionate + butyrate. Data are from interrupted fermentations. Regression lines presented for the VFA production of immature alfalfa (---)  $y = .0082x + .10$ ,  $r^2 = .84$ , mature bromegrass (—)  $y = .010x$ ,  $r^2 = .96$ , and wheat straw (-.-)  $y = .0095x + .05$ ,  $r^2 = .78$ . Mean gas production, panel B, (---)  $y = .350x$ ,  $r^2 = .92$ .

Table 5. Mean<sup>a</sup> gas and VFA production from the isolated NDF of the experimental forages and the relationship of gas volume to VFA produced for the unfractionated forages

Forage	Isolated NDF			U <sup>b</sup>
	Gas, mL/mg	VFA, mmol/mg	Gas/VFA, mL/mmol	Gas/VFA, mL/mmol
Alfalfa (immature)	.343 <sup>cd</sup>	.0107 <sup>cd</sup>	33.5 <sup>cd</sup>	29.7
Alfalfa (mature)	.366 <sup>c</sup>	.0145 <sup>c</sup>	19.2 <sup>d</sup>	33.1
Brome (immature)	.332 <sup>cd</sup>	.0081 <sup>d</sup>	43.6 <sup>c</sup>	36.8
Brome (mature)	.313 <sup>d</sup>	.0079 <sup>d</sup>	47.6 <sup>c</sup>	47.4
Corn stover	.374 <sup>c</sup>	.0088 <sup>d</sup>	37.8 <sup>cd</sup>	39.3
Wheat straw	.363 <sup>c</sup>	.0077 <sup>d</sup>	40.1 <sup>cd</sup>	37.2
SE	.014	.0015	7.2	5.3

<sup>a</sup>Least square means of the regression slopes.

<sup>b</sup>U = Unfractionated forage samples.

<sup>c,d</sup>Values within columns with different superscripts differ ( $P < .05$ ).

Unexpectedly, there were few differences in rates of NDF disappearance and gas and VFA production for the unfractionated forage samples (i.e., rates for a single forage compared among categories). It is not surprising that the gas and VFA rate estimates are similar because both are measures of the combined digestion of the NDF and NDS fractions. What was not anticipated was that the NDF disappearance rate also would be similar. If the soluble fraction had a significantly greater digestion rate than the fibrous fraction (Stefanon et al., 1996), we would have expected to see a greater rate of gas and VFA production (as an apparent weighted average) than of NDF disappearance.

The data were generally well described by the one-pool logistic model with an average  $r^2 = .96$ ,  $.92$ , and  $.86$  for NDF disappearance and gas and VFA production, respectively. The rate estimates were significant in all cases but have relatively low  $t$ -values (10, 9, and 5, respectively). It should be understood that the small number of points increases the standard error associated with the fit of the various curves (Cappio-Borlino et al., 1993) and limits the conclusions that can be drawn.

The kinetic analysis indicated that the alfalfa and brome samples contained rapidly digesting material in the neutral detergent soluble and insoluble fractions. Because the rates of digestion for the NDF and NDS fractions (.07 to .11 and .10 to .24 h<sup>-1</sup> respectively, using a one-pool model) were of a similar order, there was little difference in rates of NDF disappearance and gas and VFA production for the unfractionated forage samples of the immature alfalfa and brome. The similarity between NDF and NDS rates is probably related to the low lignification of the forages.

#### Effects of VFA Pattern

The quantity of gas produced during a fermentation reflects the amount of substrate digested and the microbial metabolic pathway. Acetate and butyrate,

produced during fermentation, are accompanied by the direct release of CO<sub>2</sub> from microbial metabolism. This direct gas produces an increased slope (1.43 mmol gas/mmol VFA) compared to simple titration of the medium buffer with acid (.78 mmol/mmol).

The variation in gas produced per millimole of VFA formed during the early time points is likely due to an accumulation of errors. The small gas volumes and low concentrations of VFA magnify the error when ratios are used. During the first 4 h of NDF fermentation, the VFA blank (VFA added to the medium initially) made up 80% of the total VFA measured. At 4 h, gas production varied between .01 and .6 mL and VFA concentrations were between .7 and 9 mM. Given that our errors of measurement were  $\pm .2$  mL of gas and  $\pm 1.5$  mM VFA, in several cases our detection errors exceeded the amount of end product measured. The variability in the VFA measurements from multiple in vitro fermentations for this experiment is similar to that reported by other authors (Peters et al., 1989).

The removal of bacteria from the ruminal fluid is likely to have changed the species represented. This alteration may also have led to a period of adjustment to the in vitro conditions requiring the bacteria to utilize internal reserves. Additionally, NDF is a mixture of polymers with variable sugar composition and digestibility. Thus, the mixture of hexoses and pentoses changes constantly over the course of the fermentation (Van Soest, 1994). These factors may partially explain the increased VFA production and low gas volumes early in the NDF fermentations. Because the changing ratios seem to affect only the first 1 to 3 mL of gas produced for any of the samples analyzed, rate calculations are unlikely to be seriously affected.

The elevation in acetate:propionate ratio we found at the early time points was also observed by Joo et al. (1994) for starchy feeds. The elevated early acetate:propionate ratio may be related to either forage

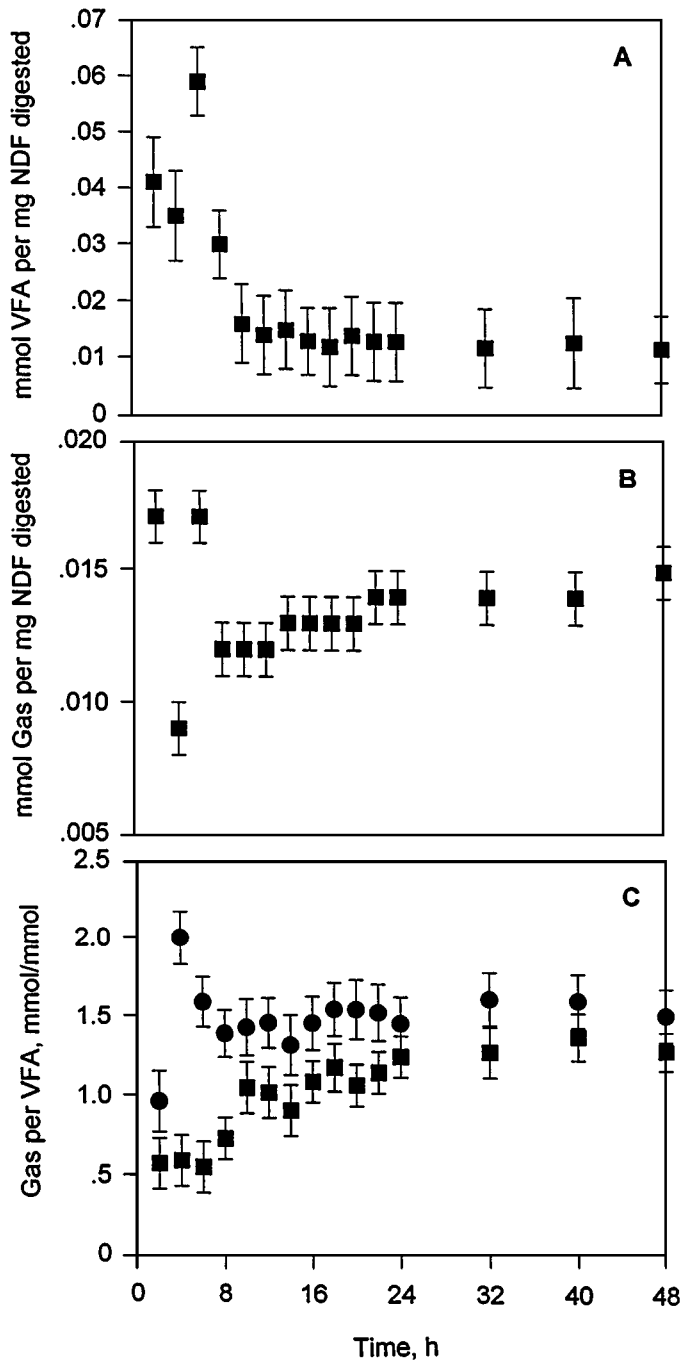


Figure 3. Average ratios presented for each time point for VFA produced per milligram of NDF digested (panel A), gas produced per millimole of NDF digested (panel B), and gas produced per millimole of VFA formed from unfractionated forage (●) and isolated NDF (■).

composition or microbial population and metabolism, or both. However, the change in acetate:propionate ratio early in the fermentations was seen with substrates ranging from unfractionated immature bromegrass to wheat straw NDF. As with the other ratios calculated, acetate:propionate ratio stabilized at approximately 8 h. The mean relationship between actual and predicted gas production was similar to

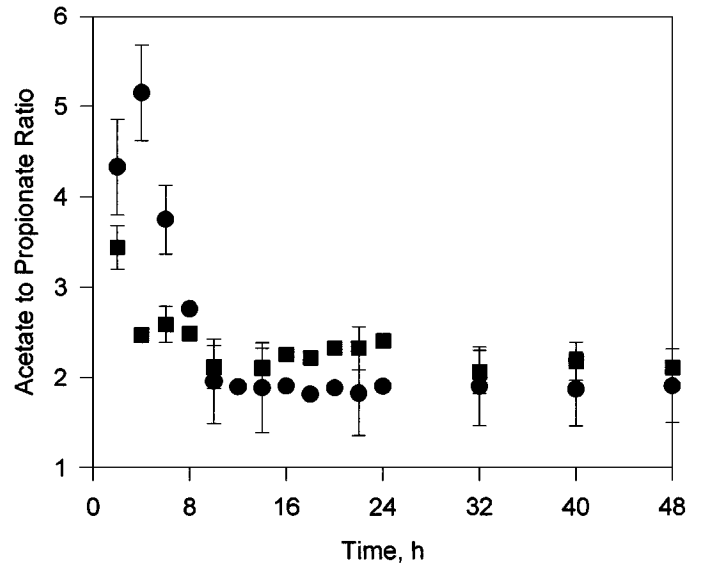


Figure 4. The change in acetate:propionate ratio over time for the unfractionated forage samples (●) and isolated NDF samples (■). Means across all forages samples are presented.

previous studies (Beuvink and Spoelstra, 1992; Blümel and Ørskov, 1993; Pell and Schofield, 1993).

In our experiment, the acetate:propionate ratio was a poor predictor of gas yield per millimole of VFA. With the relatively narrow range in the acetate:propionate ratio during most of the fermentation, but significant variation in gas production per millimole of VFA among forages, it is likely that microbial yield and growth rate were important factors in determining the amount of gas produced per millimole of VFA (Krishnamoorthy et al., 1991; Van Soest, 1994). Krishnamoorthy et al. (1991) documented a curvilinear relationship between total microbial synthesis and the gas production over 2 h. This indicated that smaller amounts of gas were produced per unit of microbial yield at higher rates of fermentation. Thus, the digestion rate and microbial synthesis may affect the balance of metabolic end products between gas and VFA and partially account for the poor correlation between the acetate:propionate ratio and gas produced per millimole of VFA across forages.

### Summary

Similar rates of digestion may be calculated from NDF disappearance and gas and VFA production. Kinetic analysis of isolated NDF and unfractionated forage fermentations with a two-pool model indicates that the faster pool in the unfractionated forage is likely to be a composite of gas produced from NDF and NDS.

The relationship between gas and VFA production is variable early (< 8 h) in the fermentation of forages, but thereafter remains stable. The variation

in gas (mL) per millimole of total VFA produced was only partially explained by changes in the pattern of VFA produced. This indicates that microbial growth and yield need to be studied as additional factors influencing gas production. In general, the variation in the relationship between gas and VFA production affected only the first 2 to 3 mL of gas produced by the unfractionated forages (10%) and is therefore likely to affect lag estimates more than the specific rates calculated from gas production.

### Implications

Digestion rate determines the amount of nutrients supplied to the animal. Conventional techniques for estimating digestion rates are time-consuming and often unsuited to analyzing many samples. Gas production provides some advantage over the traditional techniques because it may be automated. Rate estimation from gas production depends on a predictable relationship between forage digestion and microbial metabolism. This paper provides part of the data needed to validate that the scientific assumptions underlying the use of gas production are correct. Gas production is a promising technique to provide the digestion rates needed by level 2 of the Beef NRC.

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