In Vitro Fermentation Vessel Type and Method Alter Fiber Digestibility Estimates¹

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ABSTRACT

Selected vessel types and conditions used for in vitro fermentation were compared to evaluate their effects on determinations of neutral detergent fiber (NDF) digestibility (NDFD) in 2 replicate 48-h fermentations. Treatments included 50-mL polyethylene centrifuge tubes with gas-release valves (treatment 1); 50-mL polyethylene centrifuge tubes with continuous gassing with CO₂ (treatment 2); 50-mL polyethylene centrifuge tubes sealed, oriented horizontally, and shaken continuously parallel to the long axis of the tube, with manual gas release (treatment 3); 125-mL Erlenmeyer flasks with continuous gassing with CO₂ (treatment 4); and 125-mL serum vials sealed with stoppers and crimp seals with (treatment 5) or without (treatment 6) manual gas release. Goering and Van Soest medium and blended ruminal inoculum from 4 lactating cows were used. Substrates were alfalfa hay, corn silage, ryegrass hay, and soyhulls. Gas was released and measured in treatments 3 and 6 at 3.0, 5.5, 9.0, 11.5, 23.5, 29.5, and 47.5 h by using a syringe with a hypodermic needle. Vessels from each treatment were harvested at 0, 6, 12, 24, 30, and 48 h for NDF analysis, with NDFD calculated as $1 - [(\text{residual NDF, g} - \text{residual NDF in fermentation blank, g}) / \text{sample NDF, g}]$. Medium pH did not decline below 6.3 for any treatment. Average values for NDFD for 24 through 48 h were 0.576, 0.639, 0.688, 0.668, 0.679, and 0.681 for treatments 1 through 6, respectively (standard error of the difference = 0.008). The lowest NDFD was noted for treatment 1, which differed from all other treatments; treatments 3, 4, 5, and 6 did not differ by treatment or by the interaction of treatment and substrate. Treatments 1 and 2 gave lower NDFD values than the other treatments, but these differences were not consistent and differed by

substrate, with alfalfa showing the fewest differences among treatments and soyhulls the most. Net energy of lactation values for substrates, as predicted from differences in 48-h NDFD, were 7 to 15% lower for treatment 1 than for the average of all other treatments. Slopes of the gas production per gram of substrate dry matter curves differed between treatments 3 and 5. In conclusion, measured NDFD was altered by fermentation treatment, with polyethylene tubes + gas-release valves giving the lowest values. Consequently, NDFD values may not be comparable across fermentation methods, but the effect will vary among feedstuffs. The combination of methods used for sealing, gassing, or agitating vessels may have a greater impact on NDFD than does vessel type.

Key words: in vitro fermentation, neutral detergent fiber, digestibility, method

INTRODUCTION

Neutral detergent fiber digestibility (NDFD) values are used to estimate the energy content of feedstuffs for diet formulation (NRC, 2001) and for estimates of forage digestibilities for field use. A number of different vessel types and methodologies are used for batch culture in vitro fermentations with mixed ruminal microbes to measure NDFD. Erlenmeyer flasks kept under continuous CO₂ pressure (Goering and Van Soest, 1970), polyethylene centrifuge tubes with gas-release valves (Moore and Mott, 1976), and stoppered serum vials (Pell and Schofield, 1993) are among the most commonly used methods. Necked vessels, such as Erlenmeyer flasks or serum vials, can offer challenges to complete recovery of undigested fiber from the vessel. Centrifuge tubes can allow large numbers of samples to be handled for greater throughput, but may be more difficult to swirl for adequate mixing of sample and medium during fermentation, and may inhibit digestion (Grant and Mertens, 1992). The objective of this experiment was to compare NDFD values determined by using several types of vessels in combination with common and modified in vitro fermentation methods used with those vessels.

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Table 1. Composition of fermentation substrates (analytes other than DM expressed on a DM basis)

Substrate	Alfalfa hay	Corn silage	Ryegrass hay	Soyhulls
DM, %	94.27	93.76	93.28	90.58
Ash, %	8.67	4.23	7.98	4.35
CP, %	20.16	7.68	14.08	12.74
NDF, %	50.7	41.2	44.0	64.9
ADF, %	43.2	23.0	28.0	48.7

MATERIALS AND METHODS

Treatments

Three vessel types with variants in their handling methods gave a total of 6 treatments, tested in a randomized complete block design. Polyethylene centrifuge tubes (50 mL, cat. no. 05-562-13, Fisher Scientific, Atlanta, GA) incubated in a warm room at 39°C were sealed with rubber stoppers fitted with gas-release valves (Nalgene vacuum check valve, cat. no. 15-339-2, Fisher Scientific), or had continuous CO₂ gas pressure applied through a single gas line inserted through a rubber stopper that sealed the tube, or were sealed with rubber turnover septum stoppers (no. 57, cat. no. FB68692, Fisher Scientific), oriented horizontally, and subjected to continuous shaking (60 strokes/min) along the long axis of the tube, with periodic release and measurement of gas produced. Erlenmeyer flasks (125 mL) were incubated in a water bath at 39°C, with preparation and continuous CO₂ pressure maintained as described by Goering and Van Soest (1970). Serum vials (nominal 125 mL, Wheaton, cat. no. 06-406K, Fisher Scientific) sealed with butyl rubber stoppers and crimp seals were incubated in a warm room at 39°C, with or without periodic release and measurement of the gas produced. All stoppers were degreased with a sodium hydroxide solution or with soaking 3× in warm soapy water, followed by extensive rinses and soaks in warm tap water and then in distilled water.

Dried corn silage, alfalfa hay, ryegrass hay, and pelleted soyhulls ground to pass the 1-mm screen of a Wiley mill (Arthur H. Thomas, Philadelphia, PA) were used as substrates (Table 1). Samples were analyzed for DM by drying in a forced-air oven at 105°C, ash by heating samples for 4 h at 512°C, CP as N × 6.25 by combustion analysis (Dumas combustion method, Elementar Rapid N, Elementar Americas Inc., Mt. Laurel, NJ), NDF by using heat-stable α -amylase and sodium sulfite (Mertens, 2002), and ADF (Van Soest, 1973). Air-dried samples (0.5, 0.3, and 0.25 g) were weighed into the Erlenmeyer flasks, serum vials, and centrifuge tubes, respectively. Fermentation blanks containing no substrate were included for each sampling time for each

treatment to allow correction for NDF introduced with the inoculum.

In Vitro Fermentation Method and Sample Analysis

Two replicate fermentation runs, each including all treatments, were performed with substrates run singly in each treatment at each sampling hour. Medium and reducing solution were prepared according to the method of Goering and Van Soest (1970), with CO₂ bubbled through the medium through a diffusing stone for 1 h at ambient temperature (22°C) before it was dispensed into the vessels. Rumen fluid and solids were obtained from 4 ruminally cannulated lactating Holstein cows maintained under protocols approved by the University of Wisconsin Institutional Animal Care and Use Committee. Donor cows were fed a TMR consisting (on a DM basis) of 30% corn grain, 30% corn silage, 30% alfalfa haycrop silage, and 10% soybean meal with supplemental vitamins and minerals to meet NRC (2001) recommendations. Rumen inoculum from each cow was prepared separately before combining the inocula of the 4 cows. Rumen fluid (500 mL) was filtered through 4 layers of cheesecloth and held under CO₂ in a 39°C water bath until inoculation. Rumen content solids (250 g) were combined with warm (39°C) medium (550 mL) and reducing solution (27.5 mL), blended for 15 s on low speed and 45 s on high speed in a Waring blender (model HGB-300, Dynamics Corp. of America, New Hartford, CT) under CO₂, and filtered through 4 layers of cheesecloth. The rumen fluid and filtrate from the blended solids were combined and held under CO₂ in a 39°C water bath until inoculation.

All treatments received medium, reducing solution, and inoculum that were prepared as single batches for each fermentation run. The amounts of each reagent were varied by vessel type to maintain similar proportions of reagent to sample. Accordingly, 30, 18, and 15 mL of medium, 1.5, 0.9, and 0.75 mL of reducing solution, and 20, 12, and 10 mL of blended inoculum were dispensed into the flasks, vials, and tubes, respectively. For vessels in the warm room, medium was dispensed by using a bottle-top dispenser, and the reducing solution and inoculum were dispensed by using an Eppendorf Repeater Plus pipette (Eppendorf North America, Westbury, NY). Reagents were dispensed into flasks in the water bath by using repetitive syringes (Wheaton, Millville, NJ). Portions from the total amount of medium (3 mL for flasks, 1.5 mL for vials and tubes) were dispensed into the vessels to dampen samples before addition of the remainder of the medium. Flasks were gassed continuously with CO₂ from the time the first medium was added. During reagent addition, serum vials and tubes were gassed with CO₂ for several sec-

onds after each addition, and were then sealed with solid butyl rubber stoppers. After addition of inoculum, stoppers and crimp seals were applied to the serum vials, and stoppers were applied to the tubes and flasks. Racks of tubes with septum stoppers were then clamped in place between 2 metal plates in an arm of a bench-top shaker (cat. no. 099A, Glas-Col, Terre Haute, IN), turned to provide shaking parallel to the long axis of the tube, and the shaker was started. A perforated metal plate resting on the septum stoppers allowed manual release of gas without removing the tubes from the clamp. All vessels were maintained at 39°C from the time the first medium was added through the time the vessels were harvested. All stationary vessels were swirled individually by hand at each sampling hour.

Gas was measured and released from one set of vials and from the septum-stoppered tubes at approximately 3.0, 5.5, 9.0, 11.5, 23.5, 29.5, and 47.5 h of fermentation. Gas was released by insertion through the stoppers of 22-gauge (vials) or 27-gauge (tubes) hypodermic syringe needles attached to calibrated disposable syringes (Luer-Lok tip, Becton Dickinson, Rutherford, NJ). Before insertion of the needle, the syringe was set to 1.0 to 5.0 mL to increase the ease of plunger movement. The plunger was not free of friction, which may have limited gas release, resulting in underestimation of gas production. Accordingly, the gas production values are not precise measures, but provide information on the relative differences between the treatments. Time of gas measurement during the fermentation was calculated for each vessel as the time of gas release to the nearest minute minus the time inoculum was added to the vessel. Vessels were harvested at 0, 6, 12, 24, 30, and 48 h and immediately placed on ice. The pH of harvested vessels was measured before storing the samples at 4°C for subsequent analysis.

Vessel contents were analyzed for NDF by using amounts of neutral detergent (Mertens, 2002), sodium sulfite, and heat-stable α -amylase (amylase concentrate provided 17,400 liquefon units/g, 1.17 g/mL from *Bacillus licheniformis*, Ankom, Macedon, NY; used as a 1:10 vol/vol dilution) proportional to the amount of sample in each vessel. One hundred, 60, and 50 mL of neutral detergent; 1.5, 0.9, and 0.75 mL of amylase solution; and 0.50, 0.40, and 0.25 g of sodium sulfite (added in excess) were used to analyze for residual NDF in the contents of the flasks, vials, and tubes, respectively. For NDF analysis, fermentation vessel contents were quantitatively transferred to Berzelius beakers, with rinses of the vessel made with neutral detergent. Septum stoppers were rinsed into the beaker to transfer adhered sample. All samples were extracted for 1 h at boiling and under reflux. Samples were filtered through

coarse-porosity Gooch crucibles with 12 g of silica sand (40 to 200 mesh, cat. no. 84880, Fluka, Buchs, Switzerland) added as a filtering aid. Values for NDFD were calculated as $1 - [(residual\ NDF - NDF\ in\ the\ fermentation\ blank)/NDF\ in\ sample]$ and are expressed on an OM basis.

The relative effects of changes in measured 48-h NDFD on estimates of feedstuff energy concentrations were calculated by using feed values and equations from the Dairy NRC (2001). Estimates of the digestible energy content at 1× maintenance (DE_{1x} , Mcal/kg) of substrates used in this study were selected from published values (Table 15-1, entries 80, 35, 51, and 103 for alfalfa hay, corn silage, ryegrass hay, and soyhulls, respectively). Within a substrate, the digestible NDF ($NDFD \times NDF\% \text{ of DM}$) from the treatment with the smallest NDFD value was subtracted from the digestible NDF values for all other vessels to give a digestible NDF difference value. The relative change in DE_{1x} that the difference in NDFD between vessel types would cause was calculated as

$$DE_{1x} - (digestible\ NDF\ difference/100) \times 4.2$$

(derived from equation [2-8a]; NRC, 2001). The discount factor for DE for a diet with 75% total digestible nutrients was calculated by equation [2-9]. The values for DE_{1x} and the discount factor were used to calculate NE_L by using equations [2-2] and [2-11] (NRC, 2001).

Statistical Analysis

Data were analyzed as a randomized complete block design, with individual fermentation vessel as the experimental unit. Variables in the analyses were as follows: Y = the dependent variable, μ = the overall mean, R_i = the fermentation run ($i = 1, 2$), T_j = treatment (j = fermentation vessel and method), S_k = substrate (k = alfalfa hay, corn silage, ryegrass hay, pelleted soyhulls), H_l = sampling hour, and ε_{ijkl} = residual error; interaction terms are represented as combinations of uppercase and subscript letters used to define the variables.

Effects of treatment and treatment $\times$ time on NDFD and pH, analyzed as hydrogen ion concentration (Murphy, 1982), were evaluated with the model

$$Y_{ijkl} = \mu + T_j + S_k + TS_{jk} + H_l + TH_{jl} + SH_{kl} + TSH_{jkl} + \varepsilon_{ijkl},$$

with the term R_i included in the random statement. All variables were treated as classification variables. Least squares means and associated standard errors of the difference were determined with this model. Differ-

ences among treatments for NDFD and pH were also analyzed for 24 through 48 h. Data were evaluated by using type III sums of squares. Mean separation was performed by using the adjusted *P*-values of the Bonferroni method (SAS Institute, 1999), which evaluated all pairwise comparisons of data, but only those describing treatment or treatment effects in a given substrate are reported. Analyses were performed by using the MIXED procedure of SAS (SAS Institute, 1999). Values are reported as least squares means.

Cumulative gas production data were analyzed as repeated measures for 0 through 48 h by using the model shown above, using hour as a continuous variable, with the term H_iH_j and interaction terms containing it included to describe the quadratic effect of time, and with vessel nested within treatment to describe the experimental unit subject to repeated measurements. Comparison of autoregressive, unstructured, antedependence, 3 spatial structures (spatial power law, Gaussian, and spherical), and heterogeneous compound structure showed the last to be the most appropriate for describing the covariance structure (its Akaike's information criterion and Schwarz's Bayesian criterion were closest to zero of all structures). Data were evaluated by using type I sums of squares to establish the order of the equation and type III sums of squares for statistical evaluation. Analyses to determine the significance of variables and the form of the curves were performed by using the MIXED procedure of SAS (SAS Institute, 1999). The GLM procedure of SAS was used to determine the equations for the regression curves describing cumulative gas production over time.

RESULTS AND DISCUSSION

Medium pH was measured to verify that the medium did not become sufficiently acidic during fermentation to depress fiber digestion. Medium pH of the treatments at 24 through 48 h appeared to be largely a function of gas-release treatment and gassing with CO₂ (Table 2). The pH was lowest for the serum vials from which gas was not released ($P < 0.01$; Table 2), likely the result of retained CO₂ under pressure acting as an acid in the medium. In contrast, tubes with gas-release valves had the greatest medium pH ($P < 0.01$). The pH did not differ between the serum vials and tubes with septum stoppers from which gas was released manually ($P = 0.16$). Medium pH of tubes with continuous gassing did not differ from the Erlenmeyer flasks that were also continuously gassed ($P = 0.36$). Medium pH did not decline below 6.3 in any of the fermentation vessels.

Measures of NDFD among vessel types had the potential to be affected by the incomplete transfer of residual

Table 2. Fermentation medium pH by treatment and hour as averages of all substrates (values are least squares means)

Treatment	Mean separation ¹	Hour		
		24	30	48
Tube				
Gas-release valve	e	7.18	7.07	7.25
Continuous CO ₂	d	7.08	6.94	6.95
Sealed + shaken	bc	6.93	6.80	6.85
Flask				
Continuous CO ₂	cd	6.96	6.86	6.93
Vial				
Gas release	b	6.90	6.75	6.73
Sealed	a	6.73	6.55	6.58
SED ²		0.020	0.025	0.015

^{a-c}Treatments with different letters differ, $P < 0.01$. $P > 0.15$ for treatments that did not differ.

¹Treatments compared for the time period of 24 through 48 h of fermentation.

²SED = standard error of the difference.

substrate from the vessel for NDF analysis and by how the NDF analysis was performed. Media and residue from all fermentation vessels were transferred to beakers for NDF analysis with swirling of media, decanting into the beaker, cleaning the vessel with a rubber policeman, and rinsing of the vessel and policeman with a portion of the neutral detergent. Transfer was deemed complete when visual inspection of the vessel detected no residual substrate. Neutral detergent, thermostable α -amylase, and sulfite were added proportionally to the amount of medium in the vessel so that the analyses would not be altered by the ratios of reagents. With the steps taken to ensure that the NDF analysis was performed equivalently on all treatments and having no basis to suspect that it was not, variation in NDF analysis associated with fermentation vessel was considered to be part of the treatment effect.

The disappearance of NDF across fermentation hours (Figure 1) and, conversely, NDFD followed similar patterns among treatments, but there were differences. Neutral detergent fiber digestibility from 0 through 48 h differed among treatments ($P < 0.01$) and was affected by treatment $\times$ fermentation hour ($P < 0.01$) and by treatment $\times$ substrate ($P = 0.02$) interactions. Closer appraisal of the 24-, 30-, and 48-h time points, which are most commonly used to evaluate NDFD, showed differences among treatments ($P < 0.01$) and in treatment $\times$ substrate interactions ($P < 0.01$) but no effect of the treatment $\times$ hour interaction ($P = 0.38$; Table 3). Overall, vials with or without gas release, Erlenmeyer flasks with continuous gassing, and sealed tubes shaken continuously gave the highest NDFD values and did not differ from each other ($P = 1.0$), but did differ ($P < 0.035$) from tubes that were continuously gassed and tubes with gas-release valves ($P < 0.01$).

Figure 1. Neutral detergent fiber digestibility proportions for 48-h fermentations for all in vitro fermentation treatments across all substrates. Values presented are least squares means. SED = standard error of the difference.

Tubes with gas-release valves gave the lowest values for NDFD compared with other treatments, and they underestimated NDFD by up to 16%, depending on the fermentation hour and substrate. Grant and Mertens (1992) also reported a slower rate of NDF fermentation of alfalfa and bromegrass samples for tubes with gas-release valves than for Erlenmeyer flasks continuously gassed with CO₂ during fermentation. Results similar to those of the current study were reported by Sayre and Van Soest (1972), who found that continuously

gassed, 50-mL glass centrifuge tubes gave lower 48-h true DM digestibility values (72.6%) than did Erlenmeyer flasks continuously gassed with CO₂ (75.0%) and sealed 50-mL screw-capped tubes (74.4%; sealed vessel with no gas release) when orchardgrass, timothy, alfalfa, and bromegrass were used as substrates.

The significance of the treatment $\times$ substrate interaction ($P = 0.0004$) for NDFD in the 24- through 48-h time frame suggests that some substrates may be more sensitive to the effects of fermentation vessel and

Table 3. Neutral detergent fiber digestibility¹ by treatment and by hour of fermentation (values are least squares means)²

Treatment	Hour											
	24				30				48			
	AH	CS	RG	SH	AH	CS	RG	SH	AH	CS	RG	SH
Tube												
Gas-release valve	0.327	0.527	0.566	0.675	0.369	0.585	0.617	0.737	0.393	0.676	0.640	0.801
Continuous CO ₂	0.379	0.583	0.640	0.704	0.392	0.673	0.688	0.743	0.429	0.771	0.752	0.911
Sealed + shaken	0.402	0.629	0.666	0.839	0.428	0.714	0.720	0.897	0.463	0.803	0.762	0.937
Flask												
Continuous CO ₂	0.377	0.637	0.640	0.832	0.383	0.683	0.710	0.873	0.440	0.777	0.745	0.920
Vial												
Gas release	0.397	0.630	0.653	0.830	0.430	0.695	0.695	0.902	0.452	0.790	0.758	0.922
Sealed	0.403	0.650	0.675	0.816	0.428	0.692	0.703	0.888	0.458	0.779	0.760	0.919
SED ³	0.037	0.037	0.037	0.042	0.025	0.023	0.023	0.023	0.013	0.013	0.013	0.013

¹Values expressed as decimals.

²AH = alfalfa hay; CS = corn silage; RG = ryegrass hay; SH = soyhulls.

³SED = standard error of the difference.

method than others. As assessed by the mean separation technique, for alfalfa hay, only vials with and without gas release and sealed and continuously shaken tubes differed from tubes with gas-release valves ($P < 0.03$; $P = 1.0$ for other comparisons). With corn silage or ryegrass as a substrate, tubes with gas-release valves differed from all other treatments ($P < 0.01$ for both substrates; $P = 1.0$ for other comparisons). Soyhulls showed the greatest number of significant differences among treatments, with tubes with gas-release valves differing from all other treatments ($P < 0.01$) except for the continuously gassed tubes ($P = 0.58$), which differed from all other treatments ($P < 0.01$; $P = 1.00$ for all other comparisons). Based on this information, it appears that the ability to detect differences among fermentation systems will vary depending on the substrates fermented, and that all fermentation systems are not equally suitable for evaluation of all substrates.

The differences in 48-h NDFD among the treatments translated into differences in the predicted energy contents of the feedstuffs. Comparison of substrate NE_L estimates based on differences in NDFD showed that NE_L values derived from fermentations performed in tubes with gas-release valves were 0.08, 0.12, 0.14, and 0.21 Mcal/kg lower than the average values of all other treatments for alfalfa hay, corn silage, ryegrass, and soyhulls, respectively. These decreases represent 7, 8, 10, and 15% reductions in the calculated energy values for the respective feeds due solely to the fermentation treatment used. Although 48-h rumen *in vitro* assays are accepted for determination of digestible NDF values for estimating feed energy concentrations (NRC, 2001), the impact of fermentation method on NE_L estimates indicates that specification of methods acceptable for this purpose is needed.

The disposition of substrates in vessels offers insight into why vessel form may affect NDFD. All vessels showed some evidence of floating layers of dry or moistened substrate through most of the fermentation, with the exception of the sealed tubes that were horizontally oriented and shaken continuously. In serum vials and Erlenmeyer flasks, the floating substrate layer was generally thin and covered the entire surface of the medium. In the vertical stationary tube treatments, formation of 1- to 2-cm-thick floating mats of substrate may have been a contributing factor to the lower NDFD in these treatments. Even with swirling at sampling hours, thick layers of substrate filled with gas bubbles tended to reform in the tubes and may have limited interaction of the rumen microbes, medium, and substrate, or may have led to altered pH within the mat. In the case of tubes with gas-release valves, although there was no indication that the medium had become aerobic (the resazurin indicator remained clear to light

yellow in color during the entire fermentation), the tubes had a foul odor of rotting material at sampling. It is possible that air could have been admitted through the gas-release valve, but because of the floating mat of substrate, only part of the mat was oxygenated and not the greater portion of the medium.

The cumulative gas production per gram of substrate $DM \times$ time curves for serum vials or septum-stoppered tubes were quadratic in form ($P < 0.01$ for effects of both hour and hour $\times$ hour; Figure 2). Linear and quadratic sampling hour $\times$ treatment interactions were significant ($P < 0.02$ for both), indicating that the slopes of the lines differed between treatments. The consistently numerically lower yields of gas for the tubes may be related to the lower ratio of headspace to medium volumes for the tubes (1.87:1) as compared with the vials (4.85:1), with a greater proportion of the gas evolved remaining solubilized in the medium in the tubes compared with that in the vials. There was no visible evidence suggesting gas or medium loss from the tubes; however, because the medium in this treatment did not have a lower pH that would be associated with increased retention of CO_2 in the medium, this possibility cannot be discounted.

Sealed tubes that were oriented horizontally and continuously shaken compared favorably with other standard fermentation treatments for assessment of NDFD. This tube treatment did not differ in NDFD from the other treatments that achieved high NDFD (Table 3). Continuous mixing of a sealed vessel may be a key factor contributing to this outcome: medium and substrate did not separate, and a pressure of CO_2 was maintained without the necessity of continuous gassing. This vessel and method also increased the ease of sample handling. Swirling of vessels during fermentation was unnecessary. Larger numbers of samples could be fit in a more compact space than allowed by vials or flasks. The straight-sided, unnecked tubes simplified transfer of vessel contents for NDF analysis, and no residue that dried during fermentation was apparent on the inside of the vessel. The interior of the septum stopper did need to be rinsed into the Berzellius beaker with neutral detergent to assure sample transfer. Even with the tube rack clamped between 2 plates to maintain the seal of the stoppers in the tubes, it was essential that gas be released from the septum-stoppered tubes or the medium would eventually be forced out around the stopper and the sample would be lost (results of earlier trials). The necessity of releasing gases evolved during fermentation is not a problem if gas measurement or sampling is desired, but this could be an inconvenience if gas measurement is not the goal. The greatest volume of gas evolved during the first 24 h of fermentation. Gas release at 3-h intervals through the first 12

Figure 2. Cumulative gas production per gram of substrate DM for serum vials and sealed + shaken polyethylene tubes (average of all substrates). Values presented are least squares means. RMSE = root mean square error.

h, and then again at 24 h prevented an undesirable buildup of pressure and compromise of the seal on the tubes.

In conclusion, vertically positioned tubes with gas-release valves or continuous gassing with CO₂ gave lower values for NDFD than the other methods evaluated. These differences among in vitro fermentation methods may still allow qualitative ranking of feeds for NDFD within method but may not allow direct comparison of NDFD values across methods. The values obtained with centrifuge tubes with gas-release valves were lower than, and not comparable to, those of the other methods. Methods that maintained an increased CO₂ pressure and minimized formation of floating mats of substrate gave the highest fiber digestibilities, regardless of vessel type. Further investigation of in vitro fermentation systems that incorporate these characteristics but allow greater throughput of samples and ease of sample handling could be useful.

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