Tedeschi, L. O., P. Schofield, and A. N. Pell. 2008. Determining feed quality for ruminants using in vitro gas production technique. 2. Evaluating different models to assess gas production measurements. Page 15 p. in The 4th Workshop on Modeling in Ruminant Nutrition: Application of the Gas Production Technique, Juiz de Fora, MG, Brazil. EMBRAPA.

Determining feed quality for ruminants using *in vitro* gas production technique.

2. Evaluating different models to assess gas production measurements

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Introduction

The *in vitro* gas production technique has been frequently used to assess biological values of feeds based on their pattern of accumulated gas when incubated with rumen fluid under anaerobic conditions. The technique was initially proposed by Menke et al. (1979) to assess digestibility and metabolizable energy (**ME**) content of feeds commonly fed to ruminants. Empirical equations using gas production and chemical components of the feeds were used to predict ME (Menke and Steingass, 1988). In that system, fermentation of 200 mg of feed was carried out in 150-ml glass syringes containing 10 ml of ruminal fluid and 20 ml of buffer. This system is still used to determine fermentation dynamics, microbial biomass, and short chain fatty acids (Blümmel et al., 1997). Theodorou et al. (1994) described a variation of the *in vitro* gas production technique in which a pressure transducer was used to read and release the accumulated gas pressures from the incubating syringes. A similar system was described by Cone et al. (1996). These systems were likely derived from the work of Beaubien et al. (1988). Pell and Schofield (1993) designed a closed system with 16 50-ml Wheaton flasks, each connected to a sensor. Data are sent to an IBM-PC via an analog-to-digital converter (**ADC**) card to be stored for further analysis. A more detailed discussion was provided by Schofield (2000).

After data is collected, kinetic parameters that precisely describe the pattern of fermentation can be obtained. It is important that these parameters have biological interpretations. The fractional degradation (or fermentation) rate indicates the proportion of feed matter that disappears per unit of time (usually hours), the extent of digestion informs the proportion of the feed matter that has disappeared due to the fermentation (usually within 48 h), the lag provides information about the time required to commence fermentation usually it is related to particle hydration and microbial attachment processes but it could also indicate technical problems with the fermentation. Some techniques to fit data to kinetic models were discussed by Mertens (2005), including curve peeling, logarithmic transformation and regression, and non-linear least squares regression. Several models have been described and used to fit *in vitro* gas production data to nonlinear functions (López et al., 1999).

The objective of this paper was to describe the procedures of an *in vitro* gas production technique using a fermentation chamber as previously describe by Tedeschi et al. (2008) and to compare the parameter estimates of *in vitro* gas production of alfalfa hay to several nonlinear functions that have been commonly used to describe gas production profile of feeds for ruminants.

Material and Methods

Feed samples. Alfalfa hay (*Medicago sativa*) was used as an internal standard feed for all fermentations that were performed using the fermentation chamber (Tedeschi et al., 2008). The alfalfa hay was dried at 60 to 65^{o}C in a forced-air oven and then ground to pass through a 2-mm screen using a Wiley mill (Model 3375-E25, Thomas Scientific, Swedesboro, NJ 08085). In this paper, we compared the fermentation pattern of the standard alfalfa hay that was used in four projects either adjusted (n = 32) or not adjusted (n = 36) for the gas production of blank flasks. The chemical composition of the alfalfa hay is shown in Table 1. The standard alfalfa hay contains 89.3% DM, 33.9% ADF, 50.8% NDF, 19.4% CP, and 56.9% *in situ* dry matter (**DM**) digestibility.

In vitro anaerobic fermentation chamber. The *in vitro* anaerobic fermentation chamber as described by Tedeschi et al. (2008) uses the same principle to that described by Pell and Schofield (1993) and Schofield and Pell (1995). The fermentation chamber included an incubator (chamber) with a multi-place stirrer, pressure sensors attached to incubation flasks (125-ml Wheaton flasks), an analog-to-digital converter card, and an IBM-PC provided with appropriate software (Pico Technology, Eaton Socon, Cambridgeshire, UK). The software was set to collect pressure signal every 5 minutes for 48 h. Strict anaerobic technique was employed in all transfers (Bryant, 1972; Hungate, 1950) by venting all containers with CO_2 for at least 5 minutes using low to medium flow rate of CO_2. The CO_2 used was medical grade with more than 99.8% of purity. The CO_2 was not passed through a column of hot reduced copper for CO_2 purification (Hungate, 1966) because Menke and Staingass (1988) reported that small amounts of air (5 and 30 ml of air) can be tolerated by the mixed ruminal bacteria without affecting the fermentation of hay or glucose; respectively.

Preparation of the rumen fluid. The ruminal fluid inoculum was obtained from nonlactating, rumen-cannulated Jersey cows, which had free access to medium quality mixed forages (mostly grasses) and was fed once daily with a commercial ration for nonlactating cows. The collected ruminal content was transported in a pre-warmed with hot water, closed plastic container (Thermos) completely full of ruminal content to the Ruminant Nutrition Laboratory. Immediately upon arrival, the rumen content was filtered through four layers of cheesecloth and then through glass wool into an Erlenmeyer flask with a O_2-free headspace. The ruminal fluid was mixed continuously with CO_2 to minimize changes in microbial populations and to avoid O_2 contamination, and was maintained at 39 ^{o}C at all times.

Preparation of the medium. The *in vitro* medium used was the phosphate-bicarbonate medium and reducing solution of Goering and Van Soest (1970) (trypticase was not added). The medium flask was ventilated with CO_2 all the time; no CO_2 was added in the medium. The medium was heated separately to just below boiling temperature and then cooled to room temperature. At this point, cysteine hydrochloride was added. The medium pH and CO_2 saturation was controlled by color change of resazurin indicator from purple to pink/colorless; the optimum pH utilized was between 6.8 and 6.9.

Preparation of feed samples and incubation. Feed samples (200 mg) were transferred to 125-ml Wheaton flasks, which contained a small Teflon-covered stir inside. Inside the flasks, feed samples were wetted with 2.0 ml of boiled, double-distilled water that had been previously cooled to room

temperature; the water was used to avoid particle dispersion, and discounted by the media. Each flask was filled with 14 ml of media as described above, closed with previously unused, lightly greased with petroleum grease base (Lubriseal; stopcock grease, Thomas Scientific, Swedesboro, NJ 08085) butyl rubber stoppers (Geo-Microbial Technologies, Ochelata, OK 74051), and crimp sealed with Aluminum caps. All flasks were placed in the fermentation chamber and the respective sensor for each bottle was inserted using needles. When the fermentation chamber reached 39 ^{o}C, 4 ml of the filtered mixed ruminal bacteria inoculum was injected into the Wheaton flasks. The fermentation chamber was closed and when the internal temperature reached 39 ^{o}C, pressure inside each bottle was zeroed by puncturing the stopper with a needle for 5 seconds. The fermentation chamber was closed and when the temperature reached 39 ^{o}C, the recording of the pressure was initiated. The atmospheric pressure was recorded at the beginning and at the end of all rounds.

Preparation of fermentation residue. After 48 h of fermentation (2880 data points per sample were collected), each flask was depressurized, the pH and oxidation/reduction (redox) potential were measured, and 40 ml of neutral detergent solution (Van Soest et al., 1991) was added to each Wheaton flask to determine neutral detergent residue (**NDR**; (Mertens, 2002)); sodium sulfide and amylase were not used. Wheaton flasks were crimp sealed and cooked in an autoclave for 60 min at 105 ^{o}C to determine the undegraded fiber, filtered by gravimetric method using a Whatman 54 filter paper, and dried in oven using the micro-method for determination of residual fiber (Pell and Schofield, 1993). The NDR was determined gravimetrically.

Statistical Analyses

Statistical analyses were performed with R 2.7.2 (R Development Core Team, 2008) and with SAS (SAS Inst. Inc, Cary, NC) packages as specified below.

Nonlinear fitting. The `nls` function (Bates and Chambers, 1993) of R was used to fit the data to nonlinear functions using the "port" algorithm (Fox et al., 1978; Gay, 1990). The following R code was used to obtain the parameter estimates of the nonlinear functions (the exponential with lag is shown).

```
> f_exponential_1lag<-function (t,a,b,c) ifelse((t-c)<0,0,a*(1-exp(-b*(t-c))))
> ssExponential_1lag<-function(p) sum((g-f_exponential_1lag(t,p[1],p[2],p[3]))^2)
> grid<-expand.grid(a=seq(a_lr, a_ur, a_by),b=seq(b_lr, b_ur, b_by),c=seq(c_lr, c_ur, c_by))
> idx<-which.min(apply(grid,1, ssExponential_1lag))
> startval<-grid[idx,]
> results<-nls(g~f_exponential_lag(t,a,b,c), data=data, start=startval, algorithm="port")
> sse<-results$m$deviance()
> df<-length(data$g)-3
> mse<-sse/df
> pred<-as.matrix(results$m$pred())
> res<-as.matrix(results$m$resid()/sqrt(mse))
> a<-results$m$getAllPars()[1]
> b<-results$m$getAllPars()[2]
> c<-results$m$getAllPars()[3]
```

Several sets of initial estimates were generated by the `expand.grid` function and the one with the lowest sum of squares of errors (**SSE**), computed by the `ssExponential_1lag` function, was used as the initial values in the `nls` function. The selection of the best nonlinear function was performed based on the SSE.

Categorical analysis. The frequency analysis was performed using the PROC FREQ of SAS package (SAS Institute Inc., Cary, NC) using the χ^2 and the likelihood ratio χ^2 (**Lχ^2**) tests to check for independency of categorical variables (Agresti, 2002).

Variance and regression analyses. The PROC GLM and PROC MIXED of SAS package (SAS Institute Inc., Cary, NC) were used for ordinary least square (**OLS**) and generalized least square (**GLS**) analyses; respectively. The PROC MIXED was used for the random coefficients model analysis, assuming variance component for the variance-(co)variance matrix (Littell et al., 2006). The least-square means test was used to compare the levels of significant ($P < 0.05$) factors for the OLS and GLS analyses.

Nonlinear functions. Mertens (2005) has detailed graphical representations and differential equations of several digestion models that can be used to determine the disappearance of matter in the rumen through anaerobic fermentation, including single or multiple compartments, single or multiple reactions, with or without indigestible pool, and with or without microbial contamination. These models can be modified for *in vitro* gas production. Several nonlinear functions have been discussed and adapted for estimating parameter coefficients for gas production profiles. Equation [1] shows the linear relationship between gas volume (Y) and substrate (S); it is assumed a proportionally constant conversion between degraded substrate and gas production (ρ, ml/g) (France et al., 2000; Schofield et al., 1994) and S depends on a fractional rate of degradation (μ) as shown in Equation [2].

$$Y = \rho \times (S_{t=0} - S_t) \quad [1]$$

$$\frac{dS}{dt} = -\mu \times S \quad [2]$$

Equation [3] has the basic form of the exponential nonlinear function without a discrete lag (Schofield et al., 1994) and Equation [4] represents Gompertz nonlinear function. In these equations, the parameter b represents the fractional degradation rate (h^{-1}).

$$Y = a \times (1 - \exp(-b \times t)) \quad [3]$$

$$Y = a \times \exp(-\exp(-b \times t)) \quad [4]$$

Equations [5], [6], and [7] represent the exponential, Gompertz, and logistic nonlinear functions with discrete lag time; respectively, based on the derivations of Schofield et al. (1994). In these equations, parameters a represent the asymptote (ml), parameters b represent the fractional degradation rate (h^{-1}), and parameters c represents lag time (h) (Schofield et al., 1994). Other derivations based on Gompertz and logistic nonlinear functions have been proposed (France et al., 2000; France et al., 1990). Dhanoa et al. ((2000)) indicated that Equations [6] and [7] unavoidably yields a gas volume at time zero.

$$Y = \begin{cases} a \times (1 - \exp(-b \times (t - c))); \forall t \geq c \\ 0; \forall t < c \end{cases} \quad [5]$$

$$Y = a \times \exp(-\exp(1 + b \times (c - t))) \quad [6]$$

$$Y = \frac{a}{1 + \exp(2 + 4 \times b \times (c - t))} \quad [7]$$

Equations [8] and [9] represent the exponential (Lopéz et al., 1998; McDonald, 1981; Ørskov and McDonald, 1979) and Gompertz nonlinear function used to compute substrate disappearance on *in situ* experiments. For Equation [8], a represents the very rapidly degradable component, b represents the more slowly degraded pool, and c represents the fractional rate of degradation (McDonald, 1981).

$$Y = a + b \times (1 - \exp(-c \times t)) \quad [8]$$

$$Y = a + b \times \exp(-\exp(1 - c \times t)) \quad [9]$$

Equations [10] and [11] are Gompertz and logistic nonlinear functions for two pools and a single lag time as suggested by Schofield et al. (1994).

$$Y = a \times \exp(-\exp(1 + b \times (c - t))) + d \times \exp(-\exp(1 + e \times (c - t))) \quad [10]$$

$$Y = \frac{a}{1 + \exp(2 + 4 \times b \times (c - t))} + \frac{d}{1 + \exp(2 + 4 \times e \times (c - t))} \quad [11]$$

The so-called Cone nonlinear function (Equation [12]) was discussed by Groot et al. (1996) to describe the sigmoidal kinetic in a gas production profile. When, the parameter c ≤ 1, there is no point of inflection (Equation [13]). The fractional rate of degradation/fermentation can be computed using Equation [14]. Multiple phases can be modeled using Equation [12] as discussed by Groot et al. (1996).

$$Y = \frac{a}{1 + \left(\frac{b}{t}\right)^c} \quad [12]$$

$$t_l = b\left(\frac{c - 1}{c + 1}\right)^{1/c} \quad [13]$$

$$R = \frac{c \times t^{c-1}}{b^c + t^c} \quad [14]$$

Where *Y* is gas produced, ml; *t* is time, h; *a* is the asymptotic gas production, ml; *b* is the time after incubation at which half of the asymptotic amount of gas has been formed, h; and *c* is a constant determining the sharpness of the profile, dimensionless. The t_l is the time at the inflection and R is the fractional rage of degradation/fermentation.

The generalized Mitscherlich nonlinear function (Equation [15]) for gas production was discussed by France et al. (1993). The time needed for fermentation of the proportion (p) is given by Equation [16]. Similar to Equation [12], Equation [15] yields a variable fractional rate of degradation (Equation [17]).

$$Y = \begin{cases} a \times (1 - \exp(-(b \times (t - c) + d \times (\sqrt{t} - \sqrt{c})))); \forall t \geq c \\ 0; t < c \end{cases} \quad [15]$$

$$t_p = \left[\left(\frac{-d}{2} + \sqrt{\left(\frac{d^2}{4} + b \times \left\{ b \times c + d\sqrt{c} - \ln(1-p) \right\} \right)} \right) \Big/ b \right]^2 \quad [16]$$

$$R = b + \frac{d}{2 \times \sqrt{t}} \quad [17]$$

Where Y is gas produced, ml; *t* is time, h; a is asymptote, ml; b is fractional rate, h^{-1}; c is lag time, h; d is time, $h^{-1/2}$; R is fractional rate of degradation, h^{-1}; and t_p is time to obtain p proportion of matter degraded.

Equation [18] has the form of the generalized Michaelis-Menten nonlinear function (France et al., 2000). If b is zero, Equation [18] assumes the form of the Cone nonlinear function (Equation [12]) in which the parameter b of Equation [12] is equal to the parameter d of Equation [18].

$$Y = \begin{cases} \frac{a \times (t-b)^c}{(t-b)^c + d^c}; \forall t \geq b \\ 0; \forall t < b \end{cases} \quad [18]$$

$$t_p = c + d \times \left(\frac{p}{1-p} \right)^{c^{-1}} \quad [19]$$

$$R = \frac{c \times (t-b)^{c-1}}{(t-b)^c + d^c} \quad [20]$$

Models based on compartmental digestion assume the existence of two (or more) compartments (Van Milgen et al., 1991). These age-dependent models contain stochastic elements of the probability theory to enhance predictions of degradation profiles and feed passage out of the rumen (Ellis et al., 1994; Matis, 1984). These models assumed that feed is forced to exit the rumen and while in the rumen feed is digested. Vieira et al. (2008a; b) proposed a model of fiber degradation profiles that are consistent with the theoretical concepts and probability theory to generalize the processes of feed digestion in the rumen. Equation [21] is an adaptation of their model for gas production profile.

$$Y = a \times \left\{ 1 - \left[\left(\frac{\lambda}{\lambda - k} \right)^{n} \times \exp(-k \times t) + v \times \exp(-\lambda \times t) \times \sum_{i=0}^{n-1} \left(1 - \left(\frac{\lambda}{\lambda - k} \right)^{n-i} \right) \times (\lambda \times t)^{i} \Big/ i! \right] \right\} \quad [21]$$

Where Y is gas produced, ml; *t* is time, h; a is asymptote, ml; λ is asymptotic age-dependent fractional availability of the substrate, h^{-1}; k is fractional degradation rate, h^{-1}; n is time dependency related to the preparation of the substrate for digestion (number of compartments), and v is a dummy variable that is zero if n = 0, otherwise, it is one.

The parameter λ is the fractional rate of transit of substrate from one compartment to another and k is the degradation rate of the substrate. When n = 0 or λ approaches infinity, Equation [21] assumes the form of exponential function without a discrete lag (Equation [3]).

An R script was created to perform the fitting of these nonlinear functions to gas production data automatically. It may be downloaded from http://nutritiomodels.tamu.edu/gasfit.htm. There are several other nonlinear functions that can be used to describe the *in vitro* fermentation dynamics using the gas production technique, including Schumacher, von Bertalanffy, Richards (Thornley and France, 2007).

Results and Discussion

The convergence of the nonlinear functions investigated in this study was not always feasible. The initial values used in the `nls` function are of critical importance. Furthermore, as pointed out by Mertens (2005), the determination of rate and extend of fermentation depends on the adequacy of the model to describe the pattern of gas production, data collection, and adequacy of the method used to estimate the parameter coefficients. In the GasFit model, an initial guess value for each parameter is derived from a simple nonlinear function. Even though the parameters of different nonlinear functions may have different biological interpretation and differ; they are high correlated (Fitzhugh, 1976). Therefore, one could use the estimate of a simple nonlinear function as the initial value for another nonlinear function or more complicated nonlinear functions. The time needed for convergence was drastically reduced when "smart" initial values were used. Average convergence time was from 0.057 (exponential function) to 2.47 (Cone function) seconds.

Adjustment for the blank flask gas production. The categorical analysis indicated a non-independency between the selected model (lowest SSE) and with or without adjustment for the gas production of blank flasks (χ^2 = 40.4 and $P < 0.001$; $L\chi^2$ = 52 and $P < 0.001$), suggesting an effect of adjustment on model selection. In fact, when no adjustment was performed, the nonlinear functions with the lowest SSE were Gompertz (Equation [4]; 7 out of 36), Gompertz with two pools (Equation [10]; 9 out of 36), exponential with intercept (Equation [8]; 16 out of 36), and Gompertz with intercept (Equation [9]; 4 out of 36). When the adjustment was performed, logistic with two pools (Equation [11], 3 out of 32) and Gompertz with two pools (Equation [10], 29 out of 32) were selected. This finding has clearly indicated that adjustment for gas production in the blank flasks does impact the fitting outcome. Discussions about adjustment for the gas production of the blank flasks have been summarized by Rymer et al. (2005). Williams (2000) has suggested the gas recorded for the blank flasks be subtracted

from the total gas produced from the flasks with substrate to obtain the total gas derived due to fermentation of the substrate. The gas produced in the blank flasks should not be subtracted from the gas production of the flasks with substrates prior to curve fitting (Williams, 2000).

When the gas production was adjusted, selected models were independent of trials (χ^2 = 2.4 and P < 0.493; Lχ^2 = 3.09 and P < 0.377), when month the fermentation (Feb, Mar, Jun, Jul, Sep, Oct, Nov, and Dec) was conducted (χ^2 = 2.57 and P < 0.921; Lχ^2 = 3.40 and P < 0.845), and flask sets in which the fermentation was conducted (χ^2 = 1.2 and P < 0.273; Lχ^2 = 1.16 and P < 0.282). When the gas production was not adjusted for the blank flasks, the selected models were independent of trials (χ^2 = 10.7 and P < 0.297; Lχ^2 = 13.7 and P < 0.0.133) and flask sets (χ^2 = 5.33 and P < 0.149; Lχ^2 = 5.85 and P < 0.119), but there was a dependency with month of fermentation (χ^2 = 35.9 and P < 0.0221; Lχ^2 = 40.2 and P < 0.007). Most of the profiles that had the nonlinear function exponential with intercept selected were conducted during the spring whereas the other selected models were conducted during the fall.

The OLS analysis indicated that there was no relationship between model selected and ruminal fluid pH (P = 0.24), media pH (P = 0.79), average atmospheric pressure during the fermentation (P = 0.79), pH in the flask at the end of the fermentation (P = 0.71), redox potential of the rumen fluid (P = 0.30), or redox potential of the solution in the flask at the end of the fermentation (P = 0.94) when the gas production was adjusted.

When the gas production was not adjusted for the gas production of the blank flaks; however, there was an effect of ruminal fluid pH (P = 0.0084), redox potential of the rumen fluid (P = 0. 06), or redox potential of the solution in the flask at the end of the fermentation (P = 0.04) on model selection. There was no effect of media pH (P = 0.21), average atmospheric pressure during the fermentation (P = 0.70), pH in the flask at the end of the fermentation (P = 0.21) on model selected. The ruminal fluid pH was the greatest in the nonlinear function exponential with intercept (6.42) compared with Gompertz (6.07; P < 0.0066), logistic with two pools (6.12; P < 0.013), and Gompertz with intercept (6.04; P < 0.017). Even when a GLS analysis was performed with trials as random effects, ruminal fluid pH still had an impact on the selected model (P = 0.0258). This is likely related to the month in which the fermentation occurred as discussed above month of fermentation and model selected were not independent (Lχ^2 = 40.2 and P < 0.007). Therefore, there might be a relationship between when the fermentation occurred and the model that was selected likely due to the quality of the feed that was available for consumption by the cows (ruminal fluid donors) even though the results in the literature are contradictory (Huntington et al., 1998; Nagadi et al., 2000).

There are several factors that affect the *in vitro* gas production technique. The use of a meticulous and standard technique is necessary to ensure adequate repeatability. Rymer et al. (1998) compared the production of CO_2 of four media commonly used for *in vitro* gas production after 12 mmol of propionic acid was added to the media. They reported that while there was no impact of media in the initial pH, the final pH was altered. The volume of gas produced was different among media. The addition of CO_2 decreased medium pH due to the formation of carbonic acid; consequently, the increase in the gas produced was greater with the addition of CO_2. Further factors affecting *in vitro* gas production were discussed by Rymer et al. (2005), including venting, atmospheric pressure, agitation,

sample size and preparation, inoculum, etc. Therefore, *in vitro* gas production conducted in different laboratories or even at different times may not yield similar results because of personnel, technical, and laboratorial variations (and adaptations). Reliability of the technique depends on the repeatability of the results; therefore, each laboratory has to develop its own standards.

Analysis of the selected nonlinear function parameters. Table 2 has the statistics of the SSE of the nonlinear functions. An analysis of the histograms indicated the distribution of SSE was not normal and highly skewed to the right for all nonlinear functions. Based on the average of the SSE and the frequency of model selection, the exponential nonlinear function with intercept (Equation [8]) was selected to compare the function parameters when no adjustment was performed for the blank flasks and the Gompertz with two pools (Equation [10]) was selected when gas production was adjusted for the gas produced in the blank flasks.

Analysis of data without adjustment. Month of the fermentation and trial were not independent ($L\chi^2$ = 61.3 and $P < 0.0001$); a third of the fermentations occurred in October and one trial had two-thirds (n = 19) of the total data points (N = 36). Table 3 contains the estimates of the parameter coefficients. There was no difference ($P > 0.05$) in the slowly degraded pool estimate (weighed average of 0.71 V), but the fast degradable pool and the fractional rate were different among months. The fast degradable pool was lower during months of September and October than the other months and the fractional rate were the fastest on March and slowest on October. This is consistent with Nagadi's et al. (2000) findings in which changing proportion of concentrate to hay affected the dynamics of fermentation. There were positive relationships between the rapidly degraded pool (a) with ruminal fluid pH (P = 0.0043), media pH (P = 0.0145), and redox potential of the solution in the flask at the end of the fermentation (P = 0.0059). There was a positive relationship between the fractional rate and redox potential of the solution in the flask at the end of the fermentation (P = 0.0045).

Analysis of data without adjustment. Similarly, month of the fermentation and trial were not independent ($L\chi^2$ = 51.1 and P = 0.0003). In contrast with the analysis of data without adjustment, month of fermentation had no impact ($P > 0.102$) in the estimates of the parameter coefficients of the Gompertz with two pools nonlinear function. The average of the estimates was 6.99 ± 0.46 ml; 0.0869 ± 0.0066 h^{-1}; 1.57 ± 0.13 h; 13.42 ± 0.64 ml; and 0.324 ± 0.014 h^{-1} for parameters a, b, c, d, and e; respectively. There was a relationship between the lag time (parameter c) with ruminal fluid pH (P = 0.012) and redox (P = 0.017), and a tendency between the asymptotic value (parameter a) with ruminal redox (P = 0.052) and atmospheric pressure (P = 0.069).

Dhanoa et al. (2000) evaluated the fitting properties of Equations [15] and [18], and Gompertz and logistic nonlinear equations, as derived by France et al. (2000), to gas production profile of 216 feedstuffs, including grass, silage, hay, straw, pure substrates, etc. They found that the generalized Mitscherlich and Michaelis-Menten nonlinear functions were mode suited because of their flexibility to represent sigmoidal and non-sigmoidal shapes of gas production.

Our analysis indicated that adjustment for the gas production of the blank flasks does change the pattern of the gas production; consequently, affecting the model that best fits the data.

Acknowledgements

We would like to express our appreciation to the students that have contributed in one way or another with the experimental and laboratorial procedures of these projects. Special thanks for Carolina Monteiro for gathering the database used in this study.

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Table 1. Chemical composition of the standard Alfalfa hay

Item	Unit	Value
Dry matter	% as fed	92.6
Crude protein, CP	% DM	21.2
Neutral detergent insoluble nitrogen, NDIN	% CP	21.7
Acid detergent insoluble nitrogen, ADIN	% CP	6.13
Soluble protein, SP	% CP	37.3
Starch (likely pectin)	% DM	1.1
Sugar	% DM	4.5
Neutral detergent fiber, NDF	% DM	44.3
Lignin	% NDF	17.4
Acid detergent fiber, ADF	% DM	36.3
Fat	% DM	2.0
Ash	% DM	10.7
Ca	% DM	1.45
P	% DM	0.27
Mg	% DM	0.21
K	% DM	3.05
S	% DM	0.28
Na	% DM	0.048
Fe	ppm	104
Mg	ppm	36
Zn	ppm	29
Cu	ppm	11
Cl	ppm	0.77

Table 2. Statistics for the Sum of square of errors of the nonlinear functions with and without adjustment for the gas production in the blank flasks [1]

Nonlinear function	Adjusted for Blank?	N	Mean	SD	Min	Max
Cone (Eq. [12])	Yes	32	48.9	35.5	9.5	174.2
	No	35	0.8	0.2	0.5	1.3
Exponential (Eq. [3])	Yes	32	242.2	190.4	28.4	972.5
	No	36	2.3	0.4	1.9	3.2
Exponential with lag (Eq. [5])	Yes	32	91.8	59.0	23.7	351.4
	No	36	2.3	0.4	1.9	3.2
Exponential with intercept (Eq. [8])	Yes	32	135.6	111.7	24.3	669.4
	No	36	0.1	0.0	0.0	0.2
France (Eq. [15])	Yes	32	85.4	59.6	20.6	351.4
	No	36	0.6	0.1	0.4	0.9
Gompertz (Eq. [4])	Yes	32	2584.8	1053.4	1049.2	5742.5
	No	36	0.1	0.1	0.0	0.5
Gompertz two pools (Eq. [10])	Yes	32	14.5	8.7	2.8	37.0
	No	35	0.1	0.1	0.0	0.3
Gompertz with lag (Eq. [6])	Yes	32	204.2	104.1	30.8	507.1
	No	36	3.7	0.6	2.4	5.0
Gompertz with intercept (Eq. [9])	Yes	32	187.5	101.3	39.9	484.5
	No	36	0.1	0.1	0.0	0.4
Logistic (Eq. [7])	Yes	32	393.5	176.2	17.3	859.1
	No	36	3.7	0.6	2.5	5.1
Logistic two pools (Eq. [11])	Yes	32	27.4	12.1	10.8	60.2
	No	36	0.3	0.1	0.1	0.6
Michaelis-Menten (Eq. [18])	Yes	32	46.1	35.6	9.4	174.2
	No	32	0.8	0.2	0.5	1.3

[1] With adjustment is ml^2 of gas and without adjustment V^2.

Table 3. Estimates (± SE) of the parameter coefficients for the exponential nonlinear function with intercept (Equation [8]) for months of fermentation of alfalfa hay [1]

Months	N	a	b	c
February	4	0.367 ± 0.0113 [a]	0.773 ± 0.0561	0.125 ± 0.0056 [ab]
March	3	0.357 ± 0.013 [a]	0.682 ± 0.0648	0.14 ± 0.0065 [a]
June	5	0.347 ± 0.0101 [a]	0.69 ± 0.0502	0.111 ± 0.005 [bc]
July	2	0.349 ± 0.0159 [a]	0.603 ± 0.0794	0.111 ± 0.0079 [bc]
September	6	0.322 ± 0.0092 [b]	0.688 ± 0.0458	0.112 ± 0.0046 [bc]
October	10	0.323 ± 0.0071 [b]	0.729 ± 0.0355	0.106 ± 0.0035 [c]
November	1	0.345 ± 0.0225 [a]	0.759 ± 0.1122	0.124 ± 0.0112 [abc]
December	5	0.369 ± 0.0101 [a]	0.733 ± 0.0502	0.108 ± 0.005 [c]

[1] a represents the very rapidly degradable component, V; b represents the more slowly degraded pool, V; and c represents the fractional rate, h^{-1} ((McDonald, 1981)).

[a,b,c] Within a column, means without a common superscript letter differ ($P < 0.05$).