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Technical Note: Comparison of 4 methods for determining in vitro ruminal digestibility of annual ryegrass

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ABSTRACT

Multiple IVDMD methods exist, but information comparing results obtained by different methods is scarce. This study aimed to compare 3 different IVDMD methods [Daisy^{II} (DY), batch culture (BC), and the Ankom Gas Production System (GP)] at 4 incubation times (IT; 12, 24, 36 and 48 h). Additionally, results obtained at 24 h were compared with those obtained from dual-flow, continuous-culture fermentors (CF). Annual ryegrass at vegetative state was clipped from an ungrazed pasture, dried (60°C, 48 h), and ground in a Wiley Mill (1 mm). Three 48-h periods of each method were conducted using rumen inoculum from a cannulated Holstein cow. Ankom F57 acetone prerinsed bags containing 0.5 ± 0.01 g of sample were used for DY, BC, and GP. Apparent DM digestibility coefficients in CF were estimated in 3 periods (7 d of adaptation and 3 d of collection) started simultaneously with the other methods. Data were analyzed using the mixed procedure of SAS in a model including method and IT as fixed factors and period as a random factor, with IT as a repeated measure. Means within each IT were compared by the PDIFF function. Results indicated that DY predicted greater DM digestibility than GP and BC at IT greater than 12 h. Apparent DM digestibility estimated using CF was similar to that obtained with BC and GP at 24 h but less than DY. We conclude that different IVDMD methods yield different results, and caution should be exercised when comparing data obtained by different methods.

Key words: in vitro digestibility, continuous-culture fermentor, daisy, gas production, annual ryegrass

INTRODUCTION

Although in vivo determinations are considered the standard for DM digestibility (**DMD**) evaluation, they are expensive, time consuming, and labor intensive; therefore, in vitro techniques are widely used and have been observed to be highly correlated with in vivo estimates (Marinucci et al., 1992; Holden, 1999). Multiple in vitro methods exist, but information comparing results obtained by different methods is scarce. The in vitro batch culture (**BC**; Tilley and Terry, 1963) consists of digestion in a glass tube containing buffer and rumen fluid incubated at 38°C. The Daisy^{II} (**DY**; Ankom Technology, Macedon, NY) is a method that allows simultaneous analysis of multiple samples in an incubator at 39.5°C, where 4 glass jars are incubated and rotated continuously (Robinson et al., 1999). The amount of gas produced during fermentation has also been used to estimate digestibility. Menke and Steingass (1988) proposed the use of gas volume and feed composition data to estimate energy content of feeds. Recently, automated methods for measuring gas pressure have been developed, such as the Ankom RF Gas Production System (**GP**; Ankom Technology). In this system, samples are incubated in a shaking water bath for a given time period, and bottle-top sensors measure gas pressure at time intervals, yielding results of total digestibility as well as kinetics of digestion. Finally, rumen continuous-culture fermentors (**CF**; Lee and Jenkins, 2011) are reaction vessels where rumen fermentation runs continuously for longer periods than in the aforementioned methods. This design attempts to mimic in vivo ruminal conditions and allows for natural stratification of feed particles, anaerobiosis, controlled temperature, and salivary buffering, similar to what occurs in the rumen.

These methods are commonly used to estimate IVDMD. Although comparisons between some techniques are available (Holden, 1999; Wilman and Adesogan, 2000), comparisons of the 4 techniques including CF are scarce. Therefore, an experiment was conducted with the objective of determining the reciprocity among these methods (DY, BC, GP, and CF) to estimate rumen DMD. Our hypothesis was that these in vitro methods yield different results, especially at longer incubation times (**IT**).

MATERIALS AND METHODS

All surgical and animal care protocols were approved by Clemson University Animal Care and Use Committee (Protocol 2016–034). The experimental design was a randomized complete block design. The treatments consisted

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of 4 IVDMD methods: DY, BC, GP, and CF. The experiment consisted of 3 periods in which all the methods were started simultaneously. Rumen fluid was collected from a cannulated Holstein dairy cow in mid-lactation fed a TMR diet (34% corn silage, 6% grass hay, and 60% grain mix). For the methods DY, BC, and GP, acetone-rinsed Ankom F57 bags (Ankom Technology) were filled with 0.5 ± 0.05 g of ryegrass and heat sealed. The use of Ankom F57 fermentation bags for BC is a modification from technique originally published by Tilley and Terry (1963). These bags were used to make methods more comparable, because low DM recovery from Erlenmeyer flasks through filtration has previously been reported (Hall and Mertens, 2008). Blended rumen fluid was filtered through 2-layers of cheesecloth and mixed with the prewarmed buffer in a 1:4 rumen fluid:buffer ratio, except for the CF, in which the rumen ratio was 1:1 (Jenkins et al., 2014). For DY, BC, and GP, 4 IT were assessed: 12, 24, 36, and 48 h. In the case of CF, DMD data were compared with those obtained in the other methods at IT 24 h. In DY, a total of 20 bags per run and IT were assessed. Briefly, DY consisted of an incubator at 39.5°C where 4 glass jars rotate by means of gear drives (Robinson et al., 1999). To evaluate each IT, the jar was removed from the incubator when the time was reached. In the case of BC, filter bags containing 0.5 ± 0.05 g of sample were placed into 250-mL Erlenmeyer flasks containing 125 mL of the mix of rumen fluid and buffer, and incubated in a shaking water bath (39.5°C, 40 oscillations/min) during the same IT as in DY. Four flasks per IT were run, and when IT was reached, the 4 flasks were removed at once. At the end of each incubation period, bags were rinsed under cold tap water until water ran clear and dried (60°C, 48 h). In the case of GP, the Ankom RF Gas Production System allows for constant estimation, so the pressures recorded at 12, 24, 36, and 48 h IT were considered. A total of 10 bottles (1 for blank, 1 for standard, and 8 for samples) were run in parallel in each period. Fermentation gases produce pressure changes in the bottle headspace, which were transmitted every 5 min. The readings at each IT were cumulated (ΔP) and converted into gas volume (GV, mL) using the ideal gas law:

$$\mathrm{GV} = (\Delta P/Po) \times Vo,$$

where ΔP is the cumulated pressure change, Vo is the bottle headspace volume (190 mL), and Po is the atmospheric pressure. Blank bottles were used to adjust the baseline by subtraction. Then, DMD was estimated using the Menke equation (Menke et al., 1979), as follows:

$$\mathrm{DMD} = 14.88 + 0.889\mathrm{GV} + 0.45\mathrm{CP} + 0.0651\mathrm{Ash},$$

where DMD is apparent DMD, GV is gas volume (mL), and CP and Ash are the CP and ash content of the grass, respectively.

The dual-flow CF (750-mL volume, n = 5) used in the experiment is a modified version of the design described by Teather and Sauer (1988), with the main modifications being an overflow sidearm angled downward at 45° and a faster stirring rate (45 rpm) to resemble an average rumen solid retention time of 24 h (Lee and Jenkins, 2011). Therefore, particles stratify into an upper mat, a middle liquid layer of small feed particles, and a lower layer of dense particles. Solid passage rate was fixed at 5%/h and liquid dilution rate at 12%/h by regulating the buffer infusion pumps at 90 mL/h. Fermentors were fed 30 g of DM per day, divided into 2 daily feedings at 0800 and 1600 h (15 g of DM/d each). Rumen fluid pH was measured twice daily before feeding and buffer pH adjusted using either HCl or NaOH, to ensure the pH at the fermentors was 7.0 before the morning feeding. The temperature was kept at 39.5°C by a circulating heated water bath (Julabo, Allentown, PA). Fermentors were constantly purged with CO_2 (20 mL/min) to preserve anaerobiosis. Three periods, consisting of 7 d of adaptation and 3 d of sample collection, were run. Total volume and DM content of overflow was measured in the last 3 d. Each period was started with fresh inoculum. Dry matter digestibility was calculated by difference between the amount of DM fed daily and the DM collected from the outflow.

In this study, each technique was run for 3 periods. The CV within replicates for each technique of each sample mean was <5.0%. All methods were evaluated using annual ryegrass (*Lolium multiflorum* 'Enhancer'; Sucraseed, Tangent, OR) harvested from an ungrazed pasture in vegetative stage. Grass was dried at 60°C (48 h) and ground to pass a 1-mm sieve of a Wiley Mill (Thomas Scientific, Swedesboro, NJ). Chemical analysis of the grass used as substrate included NDF with sodium sulfite and α-amylase used in the procedure, ADF (Van Soest et al., 1991), ash content by combustion in muffle furnace (600°C, 2 h), total N by combustion using a Leco CNS Analyzer (Leco Corp., St. Joseph, MI), and water-soluble carbohydrate determination using the phenol and sulfuric acid colorimetric technique (Hall, 2013). Rumen fluid was collected from 5 areas of the rumen (cranial, caudal, dorsal, ventral, and central), strained through 2 layers of cheesecloth, with one handful of unstrained digesta, and kept in a sealed insulated container until being transferred to the laboratory within 20 min from extraction. Rumen fluid and contents were homogenized in a preheated blender while purging with CO_2. A buffer composed of NaH_2 PO_4 × H_2O (5.8 g/L), NaCl (0.28 g/L), $CaCl_2$ (0.02 g/L), MgCl (0.04 g/L), urea (0.3 g/L), and $NaHCO_3$ (3.67 g/L) was used for all the methods (Slyter et al., 1966).

Data were analyzed using the mixed procedure of SAS (SAS Institute Inc., Cary, NC) in a model including DMD method, IT, and their interaction as fixed factors and period as a random factor, with IT as a repeated measure. Denominator df were calculated using the Kenward-Roger method, and a first-order autoregressive covariance structure was used based on low values received for goodness of fit measures for Akaike's information criterion. Means within each IT were compared by the PDIFF function.