DEVELOPMENT OF A RUMEN FLUID PRESERVATION TECHNIQUE AND APPLICATION TO AN *IN VITRO* DRY MATTER DIGESTIBILITY ASSAY

Leslie D. Zeigler, MS, PAS,[1*] Michael L. Schlegel, PhD, PAS,[2] Mark S. Edwards, PhD[2]

[1]Animal & Veterinary Sciences, California State Polytechnic University Pomona, Pomona, CA USA
[2]Department of Veterinary Services, Zoological Society of San Diego, P.O. Box 120551, San Diego, CA 92112-0551 USA

Abstract

The objective of this study was to develop a rumen-fluid freezing and thawing protocol to be used with a two-stage *in vitro* dry matter digestibility (IVDMD) assay. Rumen fluid was collected on three different days (one week apart) from a domestic steer (*Bos taurus*) and preserved via freezing, freezing with 5% glycerol (GLY) or freezing with 5% glycerol plus a buffer solution (BUF). The IVDMD of frozen preparations were compared to those of fresh rumen fluid. Yellow corn, soybean meal, high fiber herbivore pelleted diet and coastal bermudagrass hay were used as reference feed substrates. Frozen rumen fluid preparations were thawed in a warm (39°C) water bath, pre-incubated and infused with CO_2 overnight (12 h) prior to use. Frozen rumen fluid gave similar IVDMD results when compared to fresh samples ($P > 0.05$), whereas IVDMD values determined using GLY and BUF preparations were different from fresh samples ($P < 0.05$).

Introduction

Total tract digestion studies have been considered the conventional and preferred method for determining digestibility of feedstuffs. Unfortunately application of such *in vivo* methods to zoo and free-ranging wildlife populations is challenging. *In vitro* fermentation procedures have been considered practical and accurate for evaluating forage nutritive value.[7] The two-stage Tilley and Terry[13] technique has been widely accepted and implemented as a standard method for evaluating *in vitro* dry matter digestibility (IVDMD).[11] The technique has been modified and adapted to analyze a wide range of feedstuffs in a timely manner.[4,8,15] A disadvantage of the procedure is the requirement for fresh rumen fluid. A method to preserve rumen microorganisms for later evaluation of IVDMD would be extremely useful to wildlife biologists and zoo personnel who seldom have ready access to rumen fluid from their study subjects.

Preserved rumen microorganisms have not been commonly used with *in vitro* procedures due to low viability when compared to fresh inoculum as evident by decreased IVDMD values up to 28%.[11] Lyophilized preparations of rumen fluid have been reconstituted and used to determine IVDMD[12] and *in vitro* ruminal protein degradation.[6] Rumen fluid preserved via freezing also has been used to determine *in vitro* ruminal protein degradation.[5] Luchini et al.[5] reported similar protein degradation rates using fresh rumen fluid and pre-incubated, frozen microorganisms. These methods provided a basis for development of further modifications for preserving and thawing rumen microorganisms to be used in studies of IVDMD.

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Methods

In vitro dry matter digestibility was determined by the two-stage method of Tilley and Terry[13] as modified by Mader and Horn.[8] The statistical design involved a 4 x 4 factorial arrangement of treatments with four rumen fluid preparation methods: fresh strained rumen fluid (FRS), frozen rumen fluid (FZN), frozen rumen fluid with 5% glycerol (GLY), and frozen rumen fluid with 5% glycerol and buffer (BUF) and four reference feeds. Three replicate trials were conducted using rumen fluid collected on three separate days, one week apart. Fresh rumen fluid served as an internal control for freezing treatments.

Rumen fluid collection

Rumen fluid was collected from a five-year old rumen-fistulated Jersey steer (*Bos taurus)* housed at the California State Polytechnic University Pomona Beef Unit. The steer was maintained on an alfalfa-based high fiber herbivore pelleted (1.27 mm) diet (HFH) and coastal bermudagrass hay (CBG) receiving 50% of its daily calories from each component with *ad libitum* access to water (Table 1). The diet was divided into two daily feedings at 0730 and 1730, except on days when rumen fluid was to be collected. On collection days, the animal was fed his morning ration by 0930, after rumen fluid had been collected.

Rumen contents were removed from the steer at 0900 and strained through four layers of cheesecloth into an all-purpose thermos insulated drink container, pre-warmed with 39°C distilled water, and flushed with 100% CO_2 for transportation to the laboratory. At the laboratory, rumen fluid was strained through two layers of cheesecloth and transferred to a pre-warmed round-bottom flask flushed with CO_2 creating the stock strained rumen fluid (SRF) to be used later.

Buffer solution preparation

The buffer solution was prepared according to the formula for “synthetic saliva”.[9] The inoculum-buffer mixture was continuously infused with CO_2 and maintained in a water bath at 39°C prior to being dispensed. A 4% calcium chloride ($CaCl_2$) solution was prepared by adding 4.0 g $CaCl_2$ to 100 mL of distilled water. For each liter of buffer prepared, 1 mL of the 4% $CaCl_2$ solution and 1 g of urea were added to the inoculum-buffer mixture.

Protocol for inoculum preparation, freezing and thawing

The stock SRF fluid was prepared as four treatments:

A. Fresh rumen fluid (FRS)
   Stock SRF (600 mL) was measured into a pre-warmed (39°C) graduated cylinder flushed with CO_2 and added to a pre-warmed round bottom flask containing 600 mL of buffer and 0.6 g urea. The mixture was infused with CO_2 for 10 min in a 39°C water bath to blend before being dispensed.

B. Frozen rumen fluid (FZN)
   Stock SRF (600 mL) was measured into a pre-warmed (39°C) graduated cylinder flushed with CO_2 and placed in a 1-L plastic Nalgene bottle (Nunc International, Rochester, NY). The Nalgene bottle was placed in an ice-water bath and infused with CO_2 for 20 min to blend and then placed in a freezer for 7 d.
C. Frozen rumen fluid with 5% glycerol (GLY)
   Similar to technique previously described (FZN treatment, see above B) except 32 mL of glycerol (G-7757, Sigma Chemical Co., St. Louis, MO) was added to the SRF before being placed into the ice-water bath and infused with CO_2 for 20 min to mix. The mixture was placed in a freezer for 7 d.
D. Frozen rumen fluid with 5% glycerol and buffer (BUF)
   Similar to technique previously described (FZN treatment, see above B) except 64 mL of glycerol was added to the SRF and 600 mL buffer in a round-bottom flask before infusing with CO_2 for 20 min to mix. The mixture was divided into two, 1-L plastic Nalgene bottles and placed into the –20°C freezer for 7 d.

Frozen rumen fluid preparations (FZN and GLY) were thawed in a 39°C water bath for 2 h. Simultaneously in two separate round-bottom flasks, 600 mL of buffer, 0.6 ml $CaCl_2$ and 0.6 g urea were combined and placed in a 39°C water bath to warm and gently infused with CO_2 for 2 h. Thawed rumen fluid was combined with buffer solution and infused with CO_2 for 12 h overnight.

Frozen rumen fluid for the BUF samples were thawed in a 39°C water for 2 h. Simultaneously a round-bottom flask was placed in a 39°C water bath to warm and gently infuse with CO_2 for 2 h. The thawed BUF sample was added to the pre-warmed round-bottom flask and infused with CO_2 for 12 h overnight.

Reference feed preparation

Four reference feeds, ranging in fiber and protein content, were used in the IVDMD procedure to compare rumen-fluid preparation procedures. The four reference feeds were a high fiber herbivore pelleted diet (HFH), coastal bermudagrass hay (CBG), soybean meal (SBM) and yellow corn (YC) (Table 1). All samples were ground through a 1-mm screen of a Thomas-Wiley mill (Thomas Co., Philadelphia, PA). Each of the four reference feeds plus blanks were digested in six replicate tubes for a total of 30 test tubes per rumen-fluid preparation. Blank tubes containing only the rumen fluid inoculum-buffer mixture were included to correct for dry matter (DM) content in the inoculum.

The percent IVDMD was determined by Eq. [1]:[13]

$$\left(\frac{[(\text{sample, g}) \times (\text{sample DM,\%}) - (\text{dried residue, g}) - (\text{avg dried blank residue,g})]}{[(\text{sample, g}) \times (\text{sample DM,\%})]}\right) \times 100$$

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Replicate IVDMD values that were +/- 2.00 standard deviations from the mean were discarded to determine an adjusted mean.

Statistical analysis
Data were analyzed with the analysis of variance procedure of StatView (SAS Inst. Inc., Cary, NC). Variances were tested for heterogeneity using Hartley's f-max test.[3] Dunnett's test was used to compare frozen preparations to the FRS (control) preparation. Differences between feeds and treatments in regards to IVDMD were compared using Tukey's test at the 5% significance level.

Results and Discussion

Data for IVDMD measurements were expressed as adjusted means. The analysis of variance based on IVDMD demonstrated a difference among reference feeds and rumen fluid preparations ($P < 0.0001$). Variances were found not to be heterogeneous ($P > 0.05$).

There were no treatment preparations by reference feed interactions ($P = 0.440$). The mean IVDMD values of FRS and FZN samples were similar ($P > 0.05$), whereas GLY and BUF preparations were 31.8 % units and 31.3 % units lower, respectively than FRS ($P < 0.05$; Table 2). Higher energy and protein reference feeds (YC and SBM) were similar with regards to IVDMD ($P > 0.05$) across all treatments, but were consistently greater ($P < 0.05$) than higher fiber containing feedstuffs (CBG and HFH).

Differences between feeds in regards to IVDMD are most likely attributed to nutrient composition. Concentrate feeds (YC and SBM) had higher IVDMD values using FZN rather than FRS, while HFH and CBG were consistently lower. *In vitro* dry matter digestibility results using preserved rumen fluid were consistent with those of Takatsu et al.[12] Results indicate amylolytic bacterial populations were not impacted by preservation, but cellulolytic populations were, although there were no differences detected between FRS and FZN samples. The CBG had 50% more ADF and NDF than did HFH, so if the cellulolytic bacterial populations were impacted during the freezing and thawing processes this might be an explanation for the reduction in IVDMD values and the increase in variability. Future studies should consider viability of preserved microorganisms in rumen fluid samples, as well as cold shock etiology.

The utilization of a rumen fluid preservation method to be used with an IVDMD assay is dependent on ease of the technique. The method should be implemented using a minimal number of steps. The FZN preservation method contained the least number of steps and gave IVDMD results similar to or higher than samples digested using FRS contrary to a previous report.[11] The mean IVDMD values for FZN and GLY preparations were 70.2% and 44.5%, respectively indicating that the addition of 5% glycerol had a negative effect on IVDMD. In contrast to previous findings,[6] glycerol was not effective as a cryoprotectant in this study. Luchini et al.[6] primarily focused on preserving the proteolytic bacteria population, whereas in this study it was of interest to preserve a variety of bacteria. The exact effect of glycerol addition to rumen fluid prior to freezing was not examined in this study. Glycerol may impact fiber-digesting bacteria differently than protein- and starch-digesting bacteria. Literature regarding the use of glycerol on preserving mixed ruminal microorganisms is scarce and more research needs to be conducted

to determine the optimal amount of glycerol to be added to rumen fluid samples to protect cells from freezing and thawing injuries. Samples digested using the BUF preparation were not different from GLY and suggest that the addition of buffer prior to preserving via freezing will not aid in preserving the viability of the microbial population.

Variation within and between runs should be kept to a minimum to ensure the integrity of the *in vitro* technique. The CV between collection days using FRS was lower than that of FZN in three out of the four reference feeds (Table 3). When FZN was used, IVDMD of higher fiber feedstuffs, such as CBG, were impacted with particularly lower digestibility values, although not different, through the duration of the study. From the first to the second collection, the IVDMD of CBG decreased from 57.0% to 33.4%. Furthermore, IVDMD decreased from 33.4% to 3.3% from the second to the third collection using FZN, while this decrease in digestibility was not apparent with control (FRS) samples. The statistical tests used to analyze the data in this study were unable to detect differences between FRS and FZN samples. Large variations among both fresh and frozen runs could be attributed to the donor animal, donor animal diet, or maintenance of anaerobic conditions during sample preparation. Of the three possible sources of variation mentioned, the presence or lack of CO_2 would have had a greater impact upon frozen samples than fresh samples due to the 12-h overnight pre-incubation period. The lack of a consistent anaerobic environment could have sacrificed the integrity of the microbial population.[14,16]

Sources of error within and across runs were examined, as they are an indicator of consistency in methodology. A SE was calculated to be 7.3 using the residual error from the ANOVA procedure. Ayers[2] reported a SE of 2.0. The large SE in this study might explain why differences between FRS and FZN were not detected. Also, effect size should also be taken into consideration. No data were discarded on the basis of high variability. Future research should establish criteria for rejecting values due to high variation between samples.

Dependability of an IVDMD method is based on the correlation with *in vivo* digestibility values.[1,10,13,15] Perhaps the next step in evaluating the use of a rumen fluid preservation method would be to test the ability of frozen rumen fluid to predict *in vivo* digestibility using high fiber feedstuffs.

These results indicate that rumen fluid can be preserved via freezing and incorporated into a two-stage IVDMD procedure resulting in IVDMD values similar to those obtained with fresh rumen fluid.

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