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Comparisons of *in vitro* fermentation and high moisture forage processing methods for determination of neutral detergent fiber digestibility☆

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ABSTRACT

Neutral detergent fiber digestibility (NDFD) determined *in vitro* with rumen inoculum is widely used to assess digestibility and potential energy contributions of feedstuffs. An *in vitro* fermentation system (IVFS) with potential to improve sample throughput and ease of handling for NDFD determination was investigated. Additionally, methods for preparing high moisture forages and their effect on *in vitro* NDFD were evaluated. In the IVFS study, a commonly used method that uses Erlenmeyer flasks under continuous CO_2 pressure in water baths (GV) was compared to a system that uses sealed glass tubes in a shaking incubator (TU). Fibrous feeds (alfalfa hay, maize silage, soyhulls, and ryegrass hay) were incubated in duplicate for 24, 30, and 48 h in three fermentation runs (run) in both IVFS. Overall, NDFD was greater for TU than GV at 24 h, and greater for GV than TU at 48 h. Maize silage had lower values with TU than GV, with the difference increasing with fermentation time, possibly due to low pH related to amount of fermentable substrate used. Within-run variability at 48 h was less with GV than TU. Variability of NDFD across runs was or tended to be less for TU at 24 and 30 h, and tended to be less for GV at 48 h. In the forage processing study, silages (alfalfa, maize) and pasture grasses (meadow fescue, orchardgrass, reed canarygrass, ryegrass) were ground with dry ice through a meat grinder to pass 4.5 mm openings. Subsamples were dried in a 55 °C forced-air oven (OD), freeze dried (FD), or retained as undried frozen (UF) material. Samples were fermented in duplicate in TU for 24, 30, and 48 h in two runs. NDFD response to processing varied by forage with FD for orchardgrass and UF for maize silage lower than other treatments for those forages. Overall, NDFD did not differ among processing methods at 24 h, was greatest for OD and UF at 30 h, and for OD at 48 h. Based on NDFD and analytical variability results, TU may be recommended at 24 and 30 h and GV at 48 h, however, substrate amount may need to be restricted in TU to avoid depressing NDFD. Methods of processing high moisture forage samples for NDFD analysis showed no clear advantage for using freeze dried or undried forage over oven dried materials.

Abbreviations: aNDFom, neutral detergent fiber; DM, dry matter; Est. EE+NFC, 1000 – (crude protein + ash + neutral detergent fiber organic matter); FD, freeze dried; GV, flask *in vitro* system; IVFS, *in vitro* fermentation system; NDFD, aNDFom digestibility; OD, oven dried; SD, standard deviation; SEM, standard error of the mean; TU, sealed tube *in vitro* system; UF, undried frozen.

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Table 1
Composition of fermentation substrates (fermentation system comparison).

Analyte[a]	AH[b]	MS[b]	SH[b]	RH[b]
DM, g/kg	943	938	906	933
Ash, g/kg	87	42	44	80
CP, g/kg	202	77	127	141
aNDFom, g/kg	462	375	581	396
Est. EE + NFC, g/kg	249	506	248	383

[a] DM, dry matter; CP, crude protein; aNDFom, neutral detergent fiber; Est. EE + NFC, 1000 – (CP + ash + aNDFom), all as g/kg of DM; all analytes other than DM are expressed on a dry matter basis.
[b] AH, alfalfa hay; MS, maize silage; SH, soyhulls; RH, ryegrass hay.

1. Introduction

Determination of neutral detergent fiber (aNDFom) digestibility (NDFD) by *in vitro* fermentation of feedstuffs with mixed ruminal microbes is used in research and commercially to partially estimate the nutritional value of fibrous feedstuffs for livestock. A commonly used *in vitro* fermentation system (IVFS) employs Erlenmeyer flasks incubated in water baths under continuous CO_2 pressure (Goering and Van Soest, 1970). Challenges with this system include the need for extensive water baths if many samples are run simultaneously. Mixing of samples is accomplished through manual swirling of flasks, as use of mechanical shakers resulted in substrate adhering to walls of the vessels above the level of the media (P.J. Van Soest, personal communication). There is also the potential for unmoistened substrates to float and remain dry at the persistent air/media interface, and thus be inaccessible to rumen microbes for some portion of the fermentation time (Hall and Mertens, 2008). A less space intensive system that required less manual intervention and reliably mixed substrate with media could provide more consistent results and potentially allow greater throughput of samples.

Another challenge in evaluating forage NDFD is how to appropriately process high moisture forage samples for *in vitro* fermentation. Wet, frozen forage samples (Danley and Vetter, 1971) and freeze dried samples (Deinum and Maassen, 1994; Doane et al., 1997) have been reported to have greater *in vitro* digestibility than oven-dried samples. However, these studies applied drying methods before grinding, or used different particle size reduction methods on undried/fresh and dry forage treatments. Grinding size and thus particle size of samples may affect specific rates of cell wall fermentation (Robles et al., 1980), as well as measures of potentially degradable aNDF, indegradable aNDF, and lag time (Bossen et al., 2008). Grinding forages by the same method before applying drying treatments would reduce the likelihood that differences in particle size rather than processing treatment was the basis for differences.

The objectives of this study were to compare NDFD results obtained with the Goering and Van Soest (1970) IVFS (GV) and an IVFS using sealed, continuously mixed glass tubes designed to increase ease of sample handling (TU). Additionally, effects of drying treatments applied to high moisture forage on NDFD results were investigated. The high moisture forages were ground before drying treatments were applied, so samples handled with different processing methods began with comparable particle sizes. Incubation times of 24, 30, and 48 h were selected to reflect those available from commercial laboratories for NDFD analysis.

2. Materials and methods

2.1. Fermentation system comparison

2.1.1. In vitro fermentation methods

Two IVFS (GV and TU) for anaerobic incubation of feed samples with mixed ruminal microbes were compared on the basis of NDFD values and variability of those values. Maize silage (*Zea mays*) dried at 55 °C, alfalfa hay (*Medicago sativa*), ryegrass hay (*Lolium multiflorum*), and pelleted soyhulls (*Glycine max*) all ground to pass the 1 mm screen of a Wiley Mill (Model 4, Arthur H. Thomas, Co., Philadelphia, PA, USA) were used as substrates. The chemical composition of substrates is detailed in Table 1.

The GV IVFS used 125 mL Erlenmeyer flasks maintained under continuous CO_2 pressure and at 39 °C in a water bath (Goering and Van Soest, 1970). Air-dry, ground samples (0.5 g) or fermentation blanks (vessels with no substrate) were incubated with 30 mL of media, 1.5 mL of reducing solution, and 20 mL of blended inoculum. Three milliliters of the medium were dispensed into flasks to moisten the samples 5–10 min before other reagents were added. Flasks were continuously purged with CO_2 and maintained in a water bath at 39 °C from the time of the first medium addition. After inoculation, the gas outlet for each flask was sealed to maintain continuous CO_2 pressure. Flasks were swirled manually to mix at 8:00 and 18:00 daily, and flasks remaining in the incubator were also swirled when other samples were harvested.

The TU IVFS used sealed and shaken borosilicate tubes (121 mm long, 28 mm outer diameter, 2.8 mm wall thickness, open end sealed with a crown cap; custom made by Wilmad-LabGlass, Vineland, NJ, USA). Half the amounts but the same proportions of sample and media reagents were used as for GV: 0.25 g samples or no sample for fermentation blanks, 15 mL of media, 0.75 mL of reducing solution, and 10 mL of blended inoculum. During reagent addition, the headspace of each tube was gassed with CO_2 for several seconds after each addition, sealed with solid butyl rubber stoppers between additions,

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