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Technical note: Evaluation of an alternative automatic heating-stirring system in the determination of *in vitro* ruminal dry matter digestibility of forages using the Tilley and Terry method

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ABSTRACT. This study evaluated an alternative heating-stirring system in the determination of *in vitro* ruminal digestibility of dry matter (IVRDMD) of forages using the Tilley and Terry (TT) method. For this purpose, the IVRDMD of three forage species (Marandu, Tifton 85, and Mombasa) was determined by incubating 500 mg of each dried and ground (1 mm) forage in 50-mL rumen inoculum during 48h, followed by quantification of the incubation residue. Two heating-stirring systems were used: i) heating in a water bath at 39°C with manual stirring every two hours (i.e., traditional system); and ii) heating in an oven with controlled temperature at 39°C and automatic agitation (44 rpm; alternative system); there was no effect of the interaction between the heating-stirring system and the type of forage ($p = 0.829$) on the IVRDMD of forages. The type of heating-stirring system ($p = 0.422$) did not affect the IVRDMD of forages. Nevertheless, the IVRDMD values of Marandu grass (system i = 598.7 g kg^{-1} vs system ii = 599.4 g kg^{-1}) were greater ($p < 0.001$) than Tifton 85 (system i = 392.1 vs g kg^{-1} vs system ii = 370.7 g kg^{-1}) and Mombasa (system i = 397.4 g kg^{-1}; system ii = 369.7 g kg^{-1}) grasses. In conclusion, the obtained data indicate that the alternative heating-stirring system produces similar results to those obtained using the traditionally heating-stirring system during the determination of the IVRDMD of forages.

Keywords: *in vitro* system; nutritive value; pasture; rumen degradation of feed; ruminant.

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Introduction

Determination of the feed digestibility in ruminants can be done under three conditions: *in vivo*, *in situ*, and *in vitro* (Di Marco, Ressia, Arias, Aello, & Arzadúnc, 2009). However, since determination of *in vivo* digestibility is laborious, expensive, and impractical for routine laboratory operations, alternative methods have been developed, under *in vitro* conditions. The *in vitro* methods simulate the digestion processes in which the feed goes through rumen when ingested by ruminants (Mabjeesh, Cohen, & Arieli, 2000), as well as being economic and easy to execute (Vargas & Olivera, 2017). Therefore, they are widely used in feedstuff analysis laboratories, to provide a quick response to the nutritionist in the field.

Tilley and Terry (1963) and Daisy are the most used *in vitro* methods around the world to determine the ruminal digestibility of feedstuffs in laboratory analysis, being the last one developed by the ANKON® company (Mabjeesh et al., 2000). Both methods consist of fermentation in ruminal inoculum (Ruminal fluid mixture: McDougall (McDougall, 1948) or Kansas (Camacho et al., 2019) buffers) of the feed at 39°C, with periodic agitation. Particularly in the TT method, heating is performed using a thermostatic water bath and stirring is manual (Vargas & Olivera, 2017). However, alternative heating-stirring systems in the TT method were not tested until now. Hence, this study evaluated an alternative heating-stirring system (i.e., heating in an oven with mechanical stirring at constant frequency) versus the traditionally one used (i.e., heating in a thermostatic water bath with manual stirring every two hours) during determination of the *in vitro* ruminal dry matter digestibility (IVRDMD) of forages using the TT method.

Material and methods

Ethical considerations

All procedures with animals were developed in accordance with the Animal Welfare and Ethics Committee – CEBEA of the Federal Rural University of the Amazon, Parauapebas Campus (Protocol # 5417130520), in which the experiment was conducted.

Chemical analyses of forages

Samples of pre-grazed Marandu (*Urochloa brizantha* cv. Marandu), as well as of post-grazed Tifton 85 (*Cynodon spp.* cv. 85) and Mombasa (*Megathyrsus maximus* cv. Mombasa) forages were collected using the Hand Plucking method (Cook, 1964) in February 2021, at the facilities of the Federal Rural University of the Amazon, Parauapebas Campus, Pará, Brazil (6° 4′25.53″S; 49°48′54.57″W). Forages were dried in a forced-air oven at 55°C for 72h (Solab SL 100/80, Piracicaba-SP, Brazil) and milled (1-mm sieve size; model: #108, De Leo, Porto Alegre-RS, Brazil). Subsequently, dry matter (method: G-003/1), mineral matter (method: M-001/1), crude protein (method: N-001/1), neutral detergent fiber (method: F-001/1), acid detergent fiber (F-003/1), and lignin (F-005/1) contents were determined using the methods proposed by Detmann et al. (2012; Table 1).

Table 1. Chemical composition of the forages used in the study

Item	*Marandu*	*Tifton 85*	*Mombasa*
Dry matter, g kg^{-1}	248.3	282.5	262.6
Mineral matter, g kg^{-1} DM	76.4	75.2	115.8
Crude protein, g kg^{-1} DM	107.6	57.1	52.1
Neutral detergent fiber, g kg^{-1} MS	735.1	753.0	747.6
Acid detergent fiber, g kg^{-1} MS	600.4	576.5	546.7
Lignin, g kg^{-1} MS	136.2	131.7	147.3

Procedures for *in vitro* ruminal incubation

A fistulated Nellore bull grazing on Marandu grass (*Urochloa brizantha* cv. Marandu) was used as ruminal fluid donor. Ruminal fluid was collected at 7 am, filtered through two cheese-cloth layers and collected in a thermal bottle previously conditioned at 39°C; thereafter, the collected rumen fluid was gassed with CO_2. Incubations were performed in 100 mL glass tubes, containing 500 mg (W_1) of dried and ground forage and 50 mL of ruminal inoculum (1:4 ruminal fluid: McDougall buffer (McDougall, 1948); pH = 6.9 – 7.2). Each tube was gassed with CO_2 and closed with a one-hole rubber cap to regulate internal pressure. Sequentially, the tubes were incubated at 39°C for 48h, using two different heating-stirring systems: i) conventional heating in a thermostatic water bath at 39°C with manual agitation every two hours (Figure 1); and ii) heating in an oven with controlled temperature at 39°C and mechanical agitation at 44 rpm (Figure 2). In each heating-stirring system, nine tubes (three repetitions per forage) were used as incubation systems (experimental unit), totalizing 18 tubes for two heating-stirring systems in each incubation run. Additionally, there were three tubes containing 50-mL inoculum without forage addition (i.e., blank tubes) per incubation run. Two incubation runs were developed in different days.

Figure 1. Conventional heating system of incubation tubes in a thermostatic bath at 39°C with manual agitation every two hours, using Tilley and Terry (1963) method.

Figure 2. Alternative heating system of incubation tubes in an oven with a temperature controlled at 39°C and constant mechanical stirring at 44 rpm, using the Tilley and Terry (1963) method.

At the end of 48h, the incubation process was stopped, and the tubes were transferred to an ice bath. Thereafter, the tube's content was filtered using a PYREX™ Gooch Type Filtering Crucible and a vacuum filtration equipment, and the residue was dried until constant weight at 55°C in a forced-air oven (Solab SL 100/80, Piracicaba, São Paulo State Brazil). The amount of residue obtained after filtering was determined (W_2) to calculate the IVRDMD of the evaluated forages, using the following equation:

$$\text{IVRDMD} = 1 - \left(\frac{W_2 - W_3}{W_1}\right),$$

in which, W_1 was corrected using DM value for forages, W_3 corresponds to the average mass of residue in the blank tubes for each type of system and incubation run, and IVRDMD was expressed in g kg^{-1} DM.

Statistical analysis

Differences between the type of heating-stirring system and the type of forage, as well as the possible interactions between these factors, were determined with a completely randomized design of 3 × 2 factorial structure (type of forage × type of heating-stirring system) by using the PROC MIXED of SAS 9.4. The statistical model was the following:

$$y_{ijkl} = \mu + \tau_i + \beta_j + \gamma_{ij} + \pi_k + \varepsilon_{ijkl},$$

in which y is the response variable (i.e., IVRDMD), μ is the mean, τ is the type of forage (i = 3; fixed effect), β is the type of heating-stirring system (j = 2; fixed effect), γ corresponds to the interaction between the type of forage and the type of heating-stirring system (fixed effect), π corresponds to replications (k = 2; random effect; performed on different days), and ε corresponds to the residual error. The significance level adopted was 0.05.

Results and discussion

In vitro methods are widely used to assess the digestibility of animal feedstuffs (Mabjeesh et al., 2000), since they are faster and more economical than the *in vivo* methods (Forejtová et al., 2005). Among the *in vitro* methods, TT is a reference for evaluating the ruminal forage digestibility in analytic laboratories, due to its high precision (Van Soest, 1994) and the high correlation of IVRDMD predictions with the *in vivo* digestibility of diverse forages used in ruminant feeding (Tilley and Terry, 1963). Thus, this study evaluated an alternative heating-stirring system compared to the one traditionally used in the TT method during the determination of the IVRDMD of forages. The current results indicate that the alternative heating-stirring system produces statistically results equal to those obtained using the traditional system.

It was observed that there was no effect of interaction between the heating-stirring system and the type of forage ($p = 0.829$) on the IVRDMD (Figure 3). This condition suggests that the factors of heating-stirring system and forage type are independent of each other, therefore, these should be analyzed separately. Data showed that type of forage influenced the IVRDMD, in which the mean value for Marandu grass (599.6 ± 45.2 g kg^{-1}) was greater ($p < 0.01$) than those of Tifton 85 (381.4 ± 45.2 g kg^{-1}) and Mombasa (383.5 ± 45.2 g kg^{-1})

grasses, in which the latter two were statistically equal (p = 0.932: Figure 3) between each other. The values found for the IVRDMD are in accordance with several studies, which reported that the total *in vitro* DM digestibility of Marandu, Tifton 85, and Mombasa grasses ranged from 350 to 750 g kg^{-1} (Castagnara et al., 2012; Maia et al., 2014). Variability in the *in vitro* DM digestibility of forages may be associated with several factors, such as cutting time and maturity. These factors lead to changes in the chemical composition of forages (Table 1), mainly caused by the increase in the cell wall constituents with a concomitant reduction in the cell content (Van Soest, 1994).

The heating-stirring system did not influence the IVRDMD (*P* = 0.422; Figure 3). Data revealed that IVRDMD averages determined for Marandu, Tifton 85, and Mombasa grasses using the traditional heating-stirring system were 598.7 ± 48.4, 392.1 ± 48.4, and 397.4 ± 48.4 g kg^{-1}, respectively; whereas those obtained using the alternative heating-stirring system were 599.4 ± 48.4, 370.7 ± 48.4, and 369.7 ± 48.4 g kg^{-1}, respectively (Figure 3). These values indicate that the alternative heating-stirring system produced forage values of IVRDMD statistically equal to those obtained using the traditional heating-stirring system. This condition constitutes as an operational and analytical advantage, as the implementation of an automatic heating-stirring system in the TT method can reduce the working hours of the analyst, as well as reduce the potential errors associated with the determination of IVRDMD of forages in laboratories, due to a reduction of operator intervention in the process.

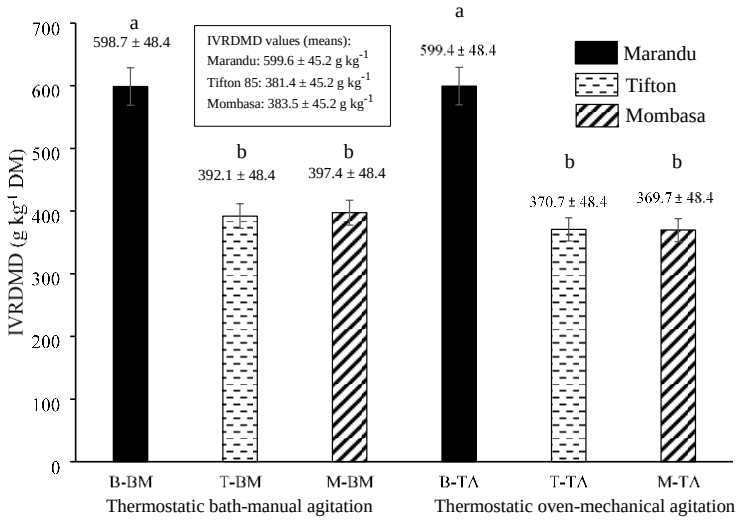

Figure 3. *In vitro* ruminal dry matter digestibility (IVRDMD; g kg^{-1} DM; mean ± SEM) of Marandu (B; *Urochloa brizantha* cv. Marandu), Tifton 85 (T; *Cynodon spp.* cv. 85), and Mombasa (M; *Megathyrsus maximus* cv. Mombasa) grasses, using as a heating-stirring system: i) Thermostatic bath-manual agitation (BM), ii) Oven-mechanical stirring (TA). Forage type × heating-stirring system: p = 0.829; forage type: p < 0.001; heating-stirring system: p = 0.422. Means with different letters indicate that they are statistically different.

Thermostatic water bath-heating with manual stirring is the most used system during the determination of the IVRDMD of forages by the TT method (Mabjeesh et al., 2000; Forejtová et al., 2005; Russouw, Raffrenato, Chaucheyras-Durand, & Chevaux, 2016). However, although the temperature is adequately controlled with that system (heating to 39ºC), the manual stirring system may not be efficiently replicated between incubations, due to a potential non-homogeneous agitation of tubes between incubations. In contrast, the automatic thermo-stirring system (alternative system), offers an appropriate temperature regulation (39ºC) with constant and homogeneous agitation, which can allow the standardization of this step in the TT method (Camacho et al., 2019).

The efficient simulation of ruminal physiological processes and the widespread use of the TT method in the evaluation of IVRDMD in feedstuffs for ruminants have stimulated the evaluation of critical "points" from this method. One of them is the composition of the buffer used in the incubation. Russouw et al. (2016) evaluated the effects of different types of buffers (Kansas State (KS); McDougall (MD); Goering and Van Soest (GV)) on the *in vitro* ruminal digestibility of neutral detergent fiber (NDF) in forages, revealing that the MD buffer produces higher values of NDF digestibility than those obtained when using KS and GV buffers. Additionally, the exploration of critical points in the TT method stimulated the development of the Daisy system (Ankom®; Camacho et al., 2019). In this system, the substrate (diet) is inserted into type F57 *filter bags* (Ankon®), which are incubated into a ruminal inoculum heated in a controlled temperature oven and at

constant mechanical agitation. The heating-stirring system of the Daisy method is similar to that proposed in this study; therefore, innovations in the TT technique are always welcome, given the ease implementation of this method in laboratories when compared to others.

Conclusion

The proposed automatic heating-stirring system produces similar IVRDMD values, when compared to those obtained using the traditional system in the TT method. However, there is a need to develop additional studies that evaluate the proposed heating-stirring system using different agitation speeds, a greater variety of forages, and different diets for ruminants. The improvement of the system proposed in this study may lead to a better simulation of the ruminal physiology conditions, obtaining more realistic values for the IVRDMD of feedstuffs, on a laboratory scale.

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