LARISSA FROTA CAMACHO

DEVELOPMENT OF A BRAZILIAN STANDARD PROCEDURE FOR *IN VITRO* DIGESTION USING RUMEN FERMENTERS

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Advisor: Edenio Detmann

VIÇOSA - MINAS GERAIS
2021

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
C172d
2021

Camacho, Larissa Frota, 1987-
Development of a brazilian standard procedure for in vitro digestion using rumen fermenters / Larissa Frota Camacho. – Viçosa, MG, 2021.
1 tese eletrônica (37 f.): il. (algumas color.).

Inclui apêndice.
Orientador: Edenio Detmann.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Zootecnia, 2021.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2021.182
Modo de acesso: World Wide Web.

1. Ruminantes - Alimentação e rações. 2. Digestibilidade in vitro. 3. Filtros e filtração. I. Detmann, Edenio, 1974-.
II. Universidade Federal de Viçosa. Departamento de Zootecnia. Programa de Pós-Graduação em Zootecnia. III. Título.

CDD 22. ed. 636.20855

Bibliotecário(a) responsável: Alice Regina Pinto CRB6 2523

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APPROVED: August 6th, 2021

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ACKNOWLEDGEMENTS

To God for giving me strenght to continue on my way.

To my adviser Edenio Detmann for all teachings, patience and for believe that I could do it. In those years as a undergraduated student I had an opportunity to learn not only about Animal Science, but also about to work as a team. And was an amazing team!

To my Family Maurício, Angélica and Mano for every word of encouragement and emotional support, even phisically distant. Thank you for all wonderful vacation provided in family. Those moments were importante to reload my energy to work!

To Arthur, with whom I could share daily challenges and achievements.

To my baby João Pedro. The happiest surprised that I had!

To Universidade Federal de Viçosa for excellent Lab structure and amazing professors and staff.

To my friends from 'salinha': Tadeu, Malber, Amanda, Cris, Aline, William, Dayana, Raphaela, Giselle, João Paulo e Luiz Fernando, for helping me with all work, tests, presentations and great conversations. You were my family in Viçosa. I will miss our coffee time!

To Alininha, João Victor, Izabela and Nicole, that were collaborated with all experiments of this work. You were more than interns; you were part of this team.

To Érica, for your friendship since I arrived in Viçosa. You were the best shoulder and hug in those Years.

To Animal Science Department staff, for all help in laboratory, with animals and documents, in special for Fernanda, Seu Mário, Monteiro and Zezé.

To the members of this committee, for collaboration.

To funding agencies, CNPq, CAPES, FAPEMIG and INCT-CA for financial supports.

The real voyage of discovery, consists not in seeing new sights, but in looking with new eyes. (Marcel Proust)

It’s not how much we do, but how much love we put into what we do. It’s not how much we give, but how much love we put into giving. (Mother Teresa of Calcutta)

ABSTRACT

CAMACHO, Larissa Frota, D.Sc., Universidade Federal de Viçosa, August, 2021. **Development of a Brazilian standard procedure for *in vitro* digestion using rumen fermenters.** Advisor: Edenio Detmann.

This thesis aimed to propose a standard Brazilian procedure for evaluating the *in vitro* dry matter digestibility (IVDMD) for ruminant feeds using artificial fermenters and a machine-rinsing procedure for filter bags after *in vitro* digestion. To recommend a machine-rinsing procedure were used twenty aliquots of for different feeds (Tifton 85 hay, corn silage, soybean meal and soybean hull) incubated in an artificial rumen fermenter for 48 hours. Then, the filter bags were rinsed in a washing machine for seven 1-min cycles. The undigested residues did not decrease ($P>0.05$) from third rinse. The variance among replicates also stabilized from third rinse. We concluded that a minimal of three 1-min cycles of machine rinsing are recommended for ruminal *in vitro* assays using filter bags, which provides stabilized apparently undigested residues and minimized variance among replicates. To propose a standard procedure for IVDMD, a collaborative study was performed with seven Brazilian feed-analysis laboratories and same samples used on machine-rinsing procedure. Two artificial fermenters were evaluated: Daisy^{II} Ankom and TE-150 Tecnal. Each one of the laboratories received 80 sealed filter bags with samples (20 per feed), eight blank filter bags, a plastic bag with buffer solution's reagents and instructions describing how to conduct a 48-h *in vitro* assay using an artificial fermenter and how to collect bovine ruminal inoculum. On average, the contribution of laboratory effect for the total random variance was 24%, being lesser than the contribution of equipment (42%) and random error (34%). The repeatability varied from 3.34 to 5.79% across feeds. The reproducibility varied from 5.93 to 8.94% across feeds, which implied in Horwitz ratios varying from 2.94 to 4.10. Due to specific characteristics of the evaluated analytical entity (i.e., IVDMD), which is defined by the method itself, the prosed method was considered reproducible. In summary, the results highlighted that, if the method is followed exactly recommended, its results are precise and present adequate levels of repeatability and reproducibility.

Keywords: Collaborative study. Horwitz ratio. In vitro digestibility. Rinsing procedure. Thesis.

RESUMO

CAMACHO, Larissa Frota, D.Sc., Universidade Federal de Viçosa, agosto de 2021. **Desenvolvimento de um método brasileiro padrão para digestão *in vitro* usando fermentadores ruminais.** Orientador: Edenio Detmann.

Esta tese teve como objetivo propor um procedimento padrão brasileiro para avaliação da digestibilidade *in vitro* da matéria seca (DIVMS) para alimentos de ruminantes, utilizando fermentadores artificiais e um procedimento de lavagem em máquina para os *filter bags* após a digestão *in vitro*. Para a recomendação do procedimento de lavagem dos *filter bags* em máquina foram utilizadas 20 alíquotas de quatro alimentos distintos (feno de Tifton 85, silagem de milho, farelo e casca de soja) incubados em fermentador artificial por 48 horas. Após esse período, os *filter bags* foram lavados em máquina por sete ciclos de 1-min. Os resíduos não digeridos não diminuiram (P>0,05) após a terceira lavagem. A variação entre as repetições estabilizou após a terceira lavagem. Concluimos que são necessários mínimo de três ciclos de 1-min de lavagem dos *filter bags* em máquina após ensaios in vitro, para obtenção de resíduos aparentemente não digeridos estáveis e minimizar a variância entre repetições. Para propor o procedimento padrão para a DIVMS foi realizado um estudo colaborativo com sete laboratórios brasileiro de análise de alimentos e as mesmas amostras utilizadas no procedimento de lavagem dos *filter bags*. Dois fermentadores artificiais foram avaliados: Daisy^{II} Ankom e TE-150 Tecnal. Cada um dos laboratórios recebeu 80 *filter bags* contendo amostra (20 por alimento), oito *filter bags* em branco, uma bolsa de plástico com os reagentes da solução tampão e instruções para conduzir um ensaio *in vitro* por 48 horas usando um fermentador artificial e como realizar a coleta de inóculo ruminal bovino. Em media, a contribuição do efeito de laboratório para a variância aleatória total foi de 24%, sendo menor que a contribuição do equipamento (42%) e do erro aleatório (34%). A repetibilidade variou de 3,34 a 5,79% entre alimentos. A reprodutibilidade variou de 5,93 a 8,94% entre alimentos, o que implicou em razões de Horwitz variando de 2,94 a 4,10. Devido as características específicas da entidade analítica aqui avaliada (DIVMS), que é definida pelo próprio método, teve o método proposto considerado reproduzível. Assim os resultados evidenciam que, se o método for seguido à risca, seus resultados são precisos e apresentam níveis adequados de repetibilidade e reprodutibilidade.

Palavras-chave: Digestibilidade *in vitro*. Estudo colaborativo. Procedimento de lavagem. Razão de Horwitz. Tese

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GENERAL INTRODUCTION

Initially, *in vitro* digestibility assays were proposed in order to estimate forage *in vivo* digestibility (Tilley and Terry, 1963). However, currently the range of application of those kind of assays has been increased, mainly for screening, discrimination, or direct comparison of feeds and diets (Silva et al., 2017).

The *in vitro* methods to obtain digestibility of feeds/diets for ruminants can be categorized as a type I methods according to Codex Alimentarius. Thus, they are methods which determine a value that can only be arrived at in terms of the method *per se* (Codex Alimentarius Commission, 2018). Because there are no primary reference standards for this type of method, they cannot be validated for accuracy in determining the "true" value for the constituent. To minimize systematic errors (i.e., bias) among laboratories, empirical methods must be followed exactly as described in the standard manuals. Even slight variations in the methodology might result in the measurement of a different constituent (Mertens, 2003).

The *in vitro* digestibility can be affected by several alterations in the standard procedures, such as instruments, vessels and filter bag types, buffer solutions, headspace gas type, the way to incubate samples, analyst working, inoculum sources, inoculum donor acclimation, sample griding, and others (Mould et al., 2005; Hall and Mertens, 2008; Patra and Yu, 2013; Strnad and Makkar, 2014; Silva et al., 2017; Camacho et al., 2019; Castro-Montoya and Dickhoefer, 2019). Any change either in the number of steps or in any parameter of the analysis will result in different *in vitro* methods whose digestibility estimates cannot be directly compared to each other.

Indeed, the among-laboratory variation tends to be larger for empirical methods (i.e., type I methods), because analysts often perform these methods in nonstandard ways that do not follow the official method. In addition, quality assurance programs instituted to verify results in laboratories often are inadequate or even nonexistent. Often the limitations of methods and rationale for specific steps in a method have not been published or have not been properly relayed to the analyst. Most of the among-laboratories variation is associated with the desire of analysts to improve efficiency by shortening times, eliminating steps, or failing to follow the details of a method and assuming that these deviations would not affect the results. These sometimes-well-intentioned deviations ignore the fundamental property of the empirical methods, which requires that they must be followed to the utmost detail (Mertens, 2003).

Despite of those aspects, a Brazilian standard method for evaluating *in vitro* digestibility for ruminant feeds and diets that produces reliable and comparable results is still lacking. Such a proposition in a country level would allow minimizing variability among laboratories and, in a further stage, combining estimates from a broader national dataset in robust statistical equations that would be able to accurately and precisely predict *in vivo* digestion from *in vitro* studies.

In order to do that, the study director's laboratory of the Brazilian National Institute of Science and Technology in Animal Science (INCT-CA) has developed several studies to establish a Brazilian method for *in vitro* digestibility for ruminant feeds or diets (Machado et al., 2016; Silva et al., 2017; Camacho et al., 2019; 2021). However, a joint effort aiming at merging all individual contributions to the method and associating this to a multi-laboratory collaborative study have not been still performed in Brazil.

Therefore, the objective of this thesis was to propose and evaluate a standard Brazilian procedure for evaluating *in vitro* dry matter digestibility for ruminant feeds and diets using artificial fermenters.

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Development of a Brazilian standard procedure for *in vitro* digestion using rumen fermenters: 1. Standardization of a machine-rinsing procedure for filter bags

Desenvolvimento de um procedimento padrão brasileiro para digestão in vitro utilizando fermentadores artificiais: 1. Padronização de procedimento de lavagem por máquina para sacos filtrantes

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ABSTRACT

We aimed to identify a standard machine-rinsing procedure for filter bags after an *in vitro* digestion assay for evaluating ruminant feeds. Twenty aliquots of four different feeds (Tifton 85 hay, corn silage, soybean meal, and soybean hulls) were incubated in an artificial rumen fermenter for 48 hours. Thereafter, the filter bags were rinsed in a washing machine for seven 1-min cycles. The undigested residues did not decrease (P>0.05) from third rinse. The variance among replicates also stabilized from third rinse. We concluded that a minimal of three 1-min cycles of machine rinsing are recommended for ruminal *in vitro* assays using filter bags, which provides stabilized apparently undigested residues and minimized variance among replicates.

Keywords: digestibility, feed analysis, feed evaluation, *in vitro* digestion

RESUMO

Objetivou-se definir um procedimento padrão de lavagem por máquina para sacos filtrantes após ensaio de digestão *in vitro* para avaliação de alimentos para animais ruminantes. Vinte alíquotas de quatro diferentes alimentos (feno de Tifton 85, silagem de milho, farelo de soja e casquinha de soja) forma incubadas por 48 horas em um fermentador artificial. Após a incubação, os sacos filtrantes foram lavados em uma lavadora por sete ciclos de 1 minuto cada. O percentual de resíduo não digerido manteve-se estável (P<0,05) após o terceiro ciclo de lavagem. Similarmente, a variância entre repetições também se estabilizou a partir do terceiro ciclo de lavagem. Conclui-se que o mínimo de três ciclos de lavagem em máquina por 1 minuto cada é recomendado para ensaios de digestão ruminal *in vitro* utilizando-se sacos filtrantes.

Palavras-chave: análise de alimentos, avaliação de alimentos, digestão *in* vitro, digestibilidade

INTRODUCTION

The *in vitro* digestibility assays have been worldwide used to either estimate *in vivo* digestibility (Menke and Steingass, 1988) or discriminate feeds and diets for ruminants (Silva et al., 2017). This technique is mostly used because it is faster, provides precise estimates, and it is relatively inexpensive, compare to both *in situ* and *in vivo* digestibility essays. However, the apparent *in vitro* dry matter digestibility (or indigestibility) is an analytical entity defined by the method itself. As so, the estimates can be affected by a variety of structural factors of the applied methods, such as recipients and equipment (Adesogan, 2005; Silva et al., 2017; Castro-Montoya and Dickhoefer, 2019); buffer solutions (Camacho et al., 2019); management, animal diet, and number of host animals; among others.

Besides structural aspects of the method, evaluations of analytical entities defined by the method are also dependent on the analyst work, which means that the analyst should strictly follow the steps described for the method without any type of alteration. In this sense, subjective interpretations in any step of method must be avoided to assure both repeatability and, reproducibility. One of the steps of *in vitro* assays which is more dependent on analyst work is the rinsing procedures of filter bags. Sometimes, practical recommendations rely on hand-rinsing procedures, whose final point is subjectively defined by the water clarity after washing process (De Boer et al., 1987).

The replacement of the hand-rising by a standard machine-rising procedure may decrease both subjectivity of this method step and variability between and within analysts. Despite some standardizations have been suggested for *in situ* procedures (Coblentz et al., 1997; Vanzant et al., 1998), a machine-rising procedure for filter bags used in *in vitro* assays is not still adequately defined. Thus, we aimed to identify a standard machine-rinsing procedure for filter bags after a digestion *in vitro* assay for evaluating ruminant feeds.

MATERIAL AND METHODS

The study was carried out at the Animal Science Department of the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. All animal procedures were approved by the university's Ethics Committee in Production Animals Use and Care (CEAUP, protocol no. 029/2019).

Four feed samples were used: Tifton 85 hay, corn silage, soybean meal, and soybean hulls. Those feeds were chosen to create a small, but representative, group of feeds commonly used in ruminant feeding in the tropics. Corn silage sample was oven-dried (55°C) and, along with the other samples, was processed in a knife mill to pass through a 1-mm screen sieve. The samples were quantified regarding dry matter (DM) content (oven-drying at 105°C for 16 hours, method G-003/1; Detmann et al., 2012).

The *in vitro* digestibility assay was conducted using an artificial fermenter (TE-150, Tecnal Equipamentos Científicos, Piracicaba, SP, Brazil) (Silva et al., 2017). Twenty 500-mg aliquots per sample were weighed and stored in heat-sealed filter bags (non-woven textile 100 g/m^2; 4 × 4.5 cm; Valente et al., 2011). A rumen cannulated bull, fed with sugarcane and concentrate (220 g of crude protein/kg DM) based diet with a forage-to-concentrate ratio of 80:20, was used as inoculum donor. The animal had free access to water and mineral mixture (90 g/kg of phosphorus), and was adapted to the diet for 14 d prior to rumen inoculum collection (Machado et al., 2016). The ruminal inoculum (liquid and solid digesta) was collected at several points in the rumen shortly before the beginning of incubation. Ruminal inoculum was stored in preheated (39°C) thermal bottles and then mixed for a few seconds using a blender to homogenize liquid and solid phases. The fluid was then filtered through four layers of cheesecloth. The steps from rumen inoculum collection and incubation onset were conducted within 20 min in a climate-controlled room (39°C). The artificial fermenter possessed four jars (3,200 mL), and each jar randomly received all aliquots of each feed and two blank bags. In each jar, 400 mL of ruminal inoculum and 1,600 mL of McDougall`s buffer solution were added. The preparation of buffer solution followed the procedures described by Camacho et al. (2019). Carbon dioxide was flushed into the headspace of each jar, which was closed and placed into the preheated (39°C) artificial fermenter. After 48 h of incubation, the filter bags were superficially washed with distilled water and gently pressed to remove gases.

All bags were placed into a washing machine (model Turbilhão 5 kg, Suggar, Belo Horizonte, Minas Gerais, Brazil). The machine was filled with clean tap water and a rinse cycle of 1 minute of agitation (delicate setting) was used (Vanzant et al., 1998). After that, the residual water was drained and bags were gently pressed to remove excess of liquid, oven-dried (55°C/24 h and 105°C/16 h, sequentially), placed in a desiccator, and weighed. This rinsing procedure was repeated seven times over all filter bags.

The apparently undigested DM residue was estimated as follow:

$$UR = \frac{R-B}{M} \times 100 \quad (1),$$

where UR is the apparently undigested residue (% DM), M is incubated mass of dry matter (g); is R is the undigested residue inside the bag (g); and B is the dry matter mass in blank filter bags (g).

The UR were submitted to an analysis of variance including the fixed effects of feeds and rinsing and their interaction. The sequential rinses were considered as repeated measures. The (co)variance residual matrix was modeled according to a heterogenous compound symmetry structure. Such a choice was based on the Akaike information criterion with correction. The least-square estimates of UR were compared in terms of differences between sequential rinses using the Tukey-Kramer approach according to the follow hypotheses:

$H_0: \mu_i - \mu_{i+1} = 0$ (2a),

$H_a: \mu_i - \mu_{i+1} \neq 0$ (2b),

where i denotes the rinse number.

Degrees of freedom were estimated using the Kenward-Roger approach. Statistical analysis was performed using the GLIMMIX procedure of SAS. Significance was declared at P<0.05.

RESULTS

The analysis of variance indicated an interaction between feeds and number of rinses (P<0.01). However, despite of the interaction effect, all evaluated feeds showed the same pattern, as there was no significant change (P>0.05) in UR after three rinses (Figure 1).

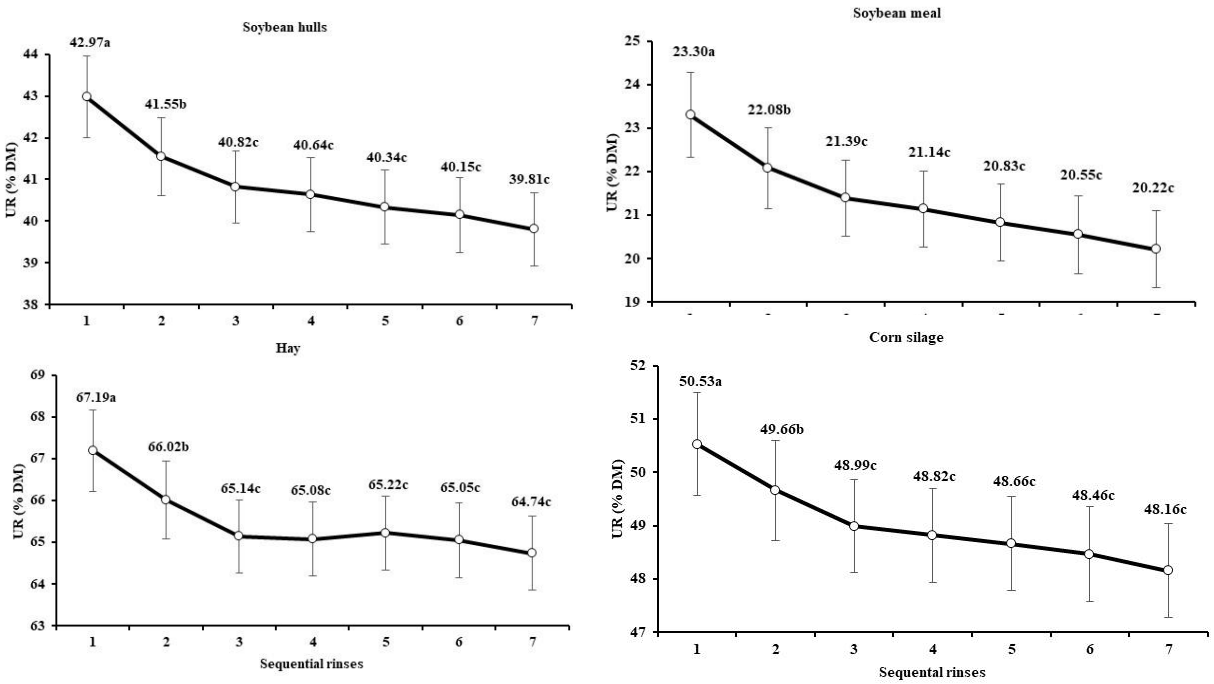

Figure 1. Least square means (± standard errors) for the apparently undigested residue (UR, % dry matter) in the different feeds and according to the numbers of rinses after *in vitro* incubation (means followed by different letters differ from sequential values at P<0.05).

Besides the UR decrease as the number of rinses increased, also the variance among replicates decreased and minimized from the third rinse (Figure 2)

DISCUSSION

In spite of interaction between feeds and number of rinses, the average UR pattern across feeds behaved similar to a first-order kinetics model (Figure 1), with the differences (i.e., decreasing in UR) between sequential rinses becoming smaller as the number of rinses increased. A similar pattern was also observed by Coblentz et al. (1997) when evaluating the quantity of contaminants solubilized in the washing water of filter bags used for an *in situ* degradation assay. According to those authors, the main components of that contamination would include the particles of rumen digesta attached to the bags and the ruminal microbes attached to feed particles.

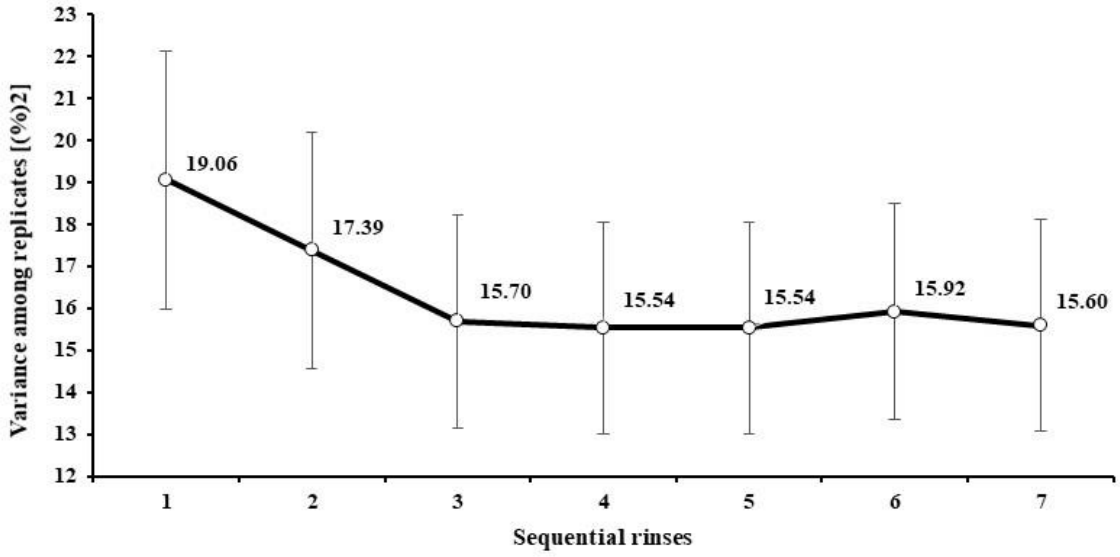

Figure 2. Estimates of variances among replicates (± standard error) for the apparently undigested residue according to the number of rinses after *in vitro* incubation.

Despite of the interaction effect ($P<0.01$), there was no significant change ($P>0.05$) in UR after three rinses for all evaluated feeds (Figure 1). That pattern differs from statements of Vanzant et al. (1998), who recommended a five-cycles (1 minute each) rinsing procedure for bags used for *in situ* incubation in ruminants. However, that disagreement could be supported by differences between incubation environments. The bags used *in situ* are more susceptible to particle attachments caused by the direct interactions with rumen contents, whereas rumen inoculum for *in vitro* procedures is filtered and also diluted in a clean buffer solution. Considering this, it seems logical reasoning that outside-bag contamination should be lesser for *in vitro* procedures, which would demand a lower number of rinses for cleaning when compared to *in situ* procedures.

Besides the UR decrease as the number of rinses increased, also the variance among replicates decreased (Figure 2), which brought evidence for the influence of contaminants on random variation of the results, and that an adequate rinsing procedure can contribute for increasing experimental precision and repeatability as well. General, the variance among replicates became stable and minimized from the third rinse, agreeing with the behavior of UR across sequential rinses (Figure 1). This pattern brings into evidence another operational advantage of a machine-rising procedure. As it does not depend on hand operation, a

standardized mechanical rinsing seems act more homogenous on replicates and so increases precision. It agrees with the statement by Paine et al. (1982), who found smaller standard errors on average DM degradation by using a machine rinsing compared to a hand-rinsing procedure.

CONCLUSIONS

We concluded that three 1-min cycles of machine rinsing are recommended for ruminal *in vitro* assays, which assures obtaining a stabilized apparently undigested residue with a minimized variance among replicates.

ACKNOWLEDGEMENTS

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), and Instituto Nacional de Ciência e Tecnologia de Ciência Animal (INCT-CA), for financial support.

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Development of a Brazilian standard procedure for *in vitro* digestion using rumen fermenters: 2. Collaborative study

Desenvolvimento de um procedimento padrão brasileiro para digestão in vitro utilizando fermentadores artificiais: 2. Estudo colaborativo

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ABSTRACT

Our objective was to propose a standard Brazilian procedure for evaluating the *in vitro* dry matter digestibility (IVDMD) for ruminant feeds using artificial rumen fermenters. A collaborative study was performed with seven Brazilian feed-analysis laboratories and four feeds (Tifton 85 hay, corn silage, soybean hulls, and soybean meal). Two kinds of artificial fermenters were evaluated: Daisy^{II} Ankom and TE-150 Tecnal. Each one of the laboratories received 80 sealed filter bags with samples (20 per feed), eight blank filter bags, a plastic bag with buffer solution's reagents and instructions describing how to conduct a 48-h *in vitro* assay using an artificial fermenter and how to collect bovine ruminal inoculum. On average, the contribution of laboratory effect for the total random variance was 24%, being lesser than both the contribution of equipment (42%) and random error (34%). The repeatability varied from 3.34 to 5.79% across feeds. The reproducibility varied from 5.93 to 8.94% across feeds, which implied in Horwitz ratios varying from 2.94 to 4.10. Due to specific characteristics of the analytical entity evaluated here (i.e., IVDMD), which is defined by the method itself, the prosed method was considered reproducible. In summary, the results highlighted that, if the method is followed exactly as recommended, its results are precise and present adequate levels of repeatability and reproducibility.
Keywords: collaborative study, Horwitz ratio, *in vitro* digestibility, repeatability, reproducibility

RESUMO

Objetivou-se foi propor um procedimento brasileiro padrão para avaliação da digestibilidade *in vitro* da matéria seca (DIVMS) de alimentos fornecidos a ruminantes utilizando fermentadores artificiais com base em estudo comparativo. O estudo colaborativo foi conduzido com sete laboratórios brasileiros de análise de alimentos utilizando-se quatro alimentos (feno de Tifton 85, silagem de milho, casca de soja e farelo de soja). Dois tipos de fermentadores artificiais foram avaliados: Daisy^{II} Ankom e TE-150 Tecnal. Cada um dos laboratórios recebeu 80 *filter bags* selados contendo amostras (20 por alimento), oito *filter bags* em branco, um saco plástico contendo os reagentes para a solução tampão e instruções de como conduzir o processo de incubação por 48 horas utilizando o fermentador artificial e de como coletar o inóculo ruminal. Em média, a contribuição do efeito de laboratório para a variância aleatória total foi de 24%, sendo inferior à contribuição do tipo de equipamento (42%) e do erro (34%). A repetibilidade variou de 3,34 a 5,79% entre os alimentos avaliados. Por sua vez, a reprodutibilidade variou de 5,93 a 8,94% entre alimentos, implicando em razões de Horwitz variando de 2,94 a 4,10. Devido a características peculiares da entidade analítica avaliada (i.e., DIVMS), a qual é definida pelo método em si, o método proposto foi considerado reprodutível. Em suma, os resultados permitiram evidenciar que, caso o método seja seguido exatamente, seus resultados serão precisos e apresentarão valores adequados e repetibilidade e reprodutibilidade.
Palavras-chave: digestibilidade *in vitro*, estudo colaborativo, razão de Horwitz, repetibilidade, reprodutibilidade

INTRODUCTION

Initially, *in vitro* digestibility assays were proposed in order to estimate forage *in vivo* digestibility (Tilley and Terry, 1963). However, currently the range of application of these kind of assays has been increased, mainly for screening, discrimination, or direct comparison of feeds and diets (Silva et al., 2017).

The *in vitro* methods to obtain digestibility of feeds/diets for ruminants can be categorized as a type I methods according to Codex Alimentarius. Thus, they are methods which determine a value that can only be arrived at in terms of the method *per se* (Codex Alimentarius Commission, 2018). Because there are no primary reference standards for this type of method, they cannot be validated for accuracy in determining the "true" value for the constituent. To minimize systematic errors (i.e., bias) among laboratories, empirical methods must be followed exactly as described in the standard manuals. Even slight variations in the methodology might result in the measurement of a different constituent (Mertens, 2003).

The *in vitro* digestibility can be affected by several alterations in the standard procedures, such as instruments, vessels and filter bag types, buffer solutions, headspace gas type, the way to incubate samples, analyst working, inoculum sources, inoculum donor acclimation, sample griding, and others (Mould et al., 2005; Hall and Mertens, 2008; Patra and Yu, 2013; Strnad and Makkar, 2014; Silva et al., 2017; Camacho et al., 2019; Castro-Montoya and Dickhoefer, 2019). Any change either in the number of steps or in any parameter of the analysis will result in different *in vitro* methods whose digestibility estimates cannot be directly compared to each other.

Indeed, the among-laboratory variation tends to be larger for empirical methods (i.e., type I methods), because analysts often perform these methods in nonstandard ways that do not follow the official method. In addition, quality assurance programs instituted to verify results in laboratories often are inadequate or even nonexistent. Often the limitations of methods and rationale for specific steps in a method have not been published or have not been properly relayed to the analyst. Most of the among-laboratories variation is associated with the desire of analysts to improve efficiency by shortening times, eliminating steps, or failing to follow the details of a method and assuming that those deviations should or would not affect results. These sometimes-well-intentioned deviations ignore the fundamental property of the empirical methods, which requires that they be followed to the utmost detail (Mertens, 2003).

Despite of those aspects, a Brazilian standard method for evaluating *in vitro* digestibility for ruminant feeds and diets that produces reliable and comparable results is still lacking. Such a proposition in a country level would allow minimizing variability among laboratories and, in a further stage, combining estimates from a broader national dataset in robust statistical equations that would be able to accurately and precisely predict *in vivo* digestion from *in vitro* studies.

In order to do that, the study director's laboratory of the Brazilian National Institute of Science and Technology in Animal Science (INCT-CA) has developed several studies to establish a Brazilian method for *in vitro* digestibility for ruminant feeds or diets (Machado et al., 2016; Silva et al., 2017; Camacho et al., 2019; 2021). The first objective was to establish a Brazilian procedure that could be incorporated as a national standard to *in vitro* digestion studies with ruminant feeds or diets.

Therefore, a collaborative study for estimating *in vitro* dry matter digestibility for ruminant feeds was conducted following a standard procedure proposed by the Brazilian National Institute of Science and Technology in Animal Science. Those study was complemented by an evaluation the digestibility estimates regarding their precision (i.e., repeatability and reproducibility).

MATERIAL AND METHODS

A collaborative study was perfomed in seven feed-analysis laboratories in Brazil: Universidade Federal de Viçosa, Viçosa, Minas Gerais (director's laboratory); Universidade Federal Rural da Amazônia, Parauapebas, Pará; Universidade Estadual Paulista Júlio de Mesquita Filho, Jaboticabal, São Paulo; Veterinary Medicine College, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais; Animal Science and Veterinary College, Universidade Federal da Bahia, Salvador, Bahia; Universidade Federal de Lavras, Lavras, Minas Gerais; and Agricultural and Environmental Sciences Institut, Universidade Federal de Mato Grosso, Sinop, Mato Grosso.

The laboratories were chosen based on the following criteria: 1. they must be associated with the Brazilian National Institute of Science and Technology in Animal Science; and 2. the following items should be available within the laboratory – at least one rumen cannulated bovine, a CO_2 cylinder, and either a Daisy^{II} (ANKOM Technology Co., Macedon, New York, USA) or a TE-150 (Tecnal Equipamentos Científicos, Piracicaba, São Paulo, Brazil) artificial fermenter.

Four feed samples collected in the Animal Science Department of Universidade Federal de Viçosa, Brazil, were used: Tifton 85 hay (*Cynodon* sp.), corn silage (*Zea mays*), soybean meal, and soybean hulls. Those samples were chosen assuming they would encompass a huge diversity among feeds used for ruminant feeding in the tropics. Corn silage sample was oven-dried (55°C). All sample particle sizes were standardized in a knife mill to pass through a 1-mm screen sieve. After griding, all samples were stored for further chemical analysis and *in vitro* incubations.

Dry matter (DM; dried overnight at 105ºC, method G-003/1), crude protein (CP; Kjeldahl procedure, method N-001/1), and neutral detergent fiber (NDF; method F-002/1) contents were analyzed in the director's laboratory of the Brazilian National Institute of Science and Technology in Animal Science according to its standard analytical procedures (Detmann et al., 2012; Table 1). Particularly, the NDF analysis was performed using a heat-stable α-amylase (Liquozyme Supra 2.2X, Novozymes, Araucária, Paraná, Brazil), omitting sodium sulfite and expressed inclusive of residual ash and protein.

Table 1. Chemical composition of feeds used for evaluating *in vitro* dry matter digestibility

Feed	Dry matter[a]	Crude protein[b]	Neutral detergent fiber[b]
Tifton 85 hay	90.6	6.68	74.5
Corn silage	24.9	6.23	50.1
Soybean meal	88.4	47.9	24.0
Soybean hulls	88.0	15.4	66.4

[a] % as fed. [b] % of dry matter.

For the *in vitro* assay, aliquots of 500 mg of each feed were weighed and stored in heat-sealed filter bags (non-woven textile 100 g/m^2; 4 × 4.5 cm; Valente et al., 2011). Moreover, all reagents necessary to compose 10 L of McDougall's buffer solution (Camacho et al., 2019) were weighed and stored in labeled plastic bags.

Each one of the laboratories received 80 sealed filter bags with samples (20 per feed), eight blank filter bags, a plastic bag with buffer solution's reagents and instructions describing how to conduct a 48-h *in vitro* assay using an artificial fermenter and how to collected bovine

ruminal inoculum. The complete method is described in the Appendix. Briefly, as both types of artificial fermenters possess four jars each, the laboratories were instructed to use one jar for each feed (including two blanks per jar). After *in vitro* incubation, laboratories superficially washed filter bags with distilled water, gently pressed them to remove gases. The bags were then oven-dried (55℃) and sent back to the INCT-CA director's laboratory to estimate *in vitro* dry matter digestibility (IVDMD).

The filter bag rinsing procedure was performed as described by Camacho et al., (2021). The bags were placed into a washing machine (model Turbilhão 5 kg, Suggar, Belo Horizonte, Minas Gerais, Brazil). The machine was filled with clean tap water and a rinse cycle of 1 minute of agitation (delicate setting) was set. The residual water was then drained. This procedure was repeated three times. After that, bags were gently pressed to remove excess of liquid, oven-dried (55°C/24 h and 105°C/16 h, sequentially), placed in a desiccator, and weighed.

The apparently IVDMD was estimated as follow:

$$IVDMD = \frac{M-(U-B)}{M} \times 100 \quad (1),$$

where IVDMD is the *in vitro* dry matter digestibility (% DM), M is incubated mass of dry matter (g); is U is the undigested residue within the filter bag (g); and B is the dry matter mass within the blank filter bags (g).

The initial statistical model used to perform the IVDMD analysis was:

$$Y_{ijkl} = \mu + F_i + E_j + L_{(j)k} + \varepsilon_{ijkl} \quad (2),$$

where Y_{ijkl} is the IVDMD of the aliquot l of feed i, measured in the laboratory k, and using the equipment j; μ is the general constant (fixed effect); F_i is the random effect of feed i, assumed NIID (0, σ^2_F); E_j is the random effect of equipment type j (i.e., artificial fermenter), assumed NIID (0, σ^2_E); $L_{(j)k}$ is the random effect of laboratory k nested to the equipment j, assumed NIID (0, $\sigma^2_{L/E}$); and ε_{ijkl} is the random error, assumed NIID (0, σ^2_ε).

Despite of equipment effect has only two levels (i.e., Daisy^{II} or TE-150), we decided to keep it as a random effect as many other artificial fermenter brands are available in Brazil. Additionally, the laboratory effect was considered a nested effect to equipment in order to absorb the differences among laboratories caused by using different artificial fermenters. Nonetheless, in spite of be a nested effect, that one does represent the variation among laboratories regarding IVDMD.

Initially, we performed an outlier evaluation on the overall dataset. Three different criteria were defined in order to identify outliers: 1. restricted likelihood distance > 0.3, COVRATIO < 0.8, and externally studentized residue (module) > 2.5. An observation was considered as an outlier if it presented at least two of those criteria. After that, only four observations were eliminated from the dataset (Table 2). The residues presented a clear pattern agreeing to the assumption of a normal and homoscedastic distribution (Figure 1).

In order to improve the understanding on the pattern of the results, the IVDMD digestibility was also evaluated for each individual feed according to the model:

$$Y_{ijk} = \mu + E_i + L_{(i)l} + \varepsilon_{ijk} \quad (3),$$

where Y_{ijk} is the IVDMD of the aliquot k, measured in the laboratory j, and using the equipment i; μ is the general constant (fixed effect); E_i is the random effect of equipment type i (i.e., artificial fermenter), assumed to be NIID (0, σ^2_E); $L_{(i)j}$ is the random effect of laboratory j nested to the equipment i, assumed to be NIID (0, $\sigma^2_{L/E}$); and ε_{ijk} is the random error, assumed to be NIID (0, σ^2_ε).

Table 2. Average *in vitro* dry matter digestibility (%) of different feeds according to the laboratories participating in the collaborative study

	Feed[a, b]			
Laboratory	Tifton 85 hay	Corn silage	Soybean meal	Soybean hulls
1	49.1±0.70	58.0±0.96	89.0±0.89	79.6±1.14
2	42.4±0.85	48.5±0.94	86.3±0.61	65.3±1.20
3	45.9±0.57[c]	51.4±0.76	94.2±0.59	73.5±0.87
4	56.2±0.57	63.1±0.46	97.4±0.64	79.6±0.61
5	47.8±0.49	53.2±0.54	85.1±0.78	76.5±0.91
6	51.6±0.66	61.5±0.67	93.2±0.41	75.4±0.74
7	52.6±0.59	64.0±0.65[d]	93.4±0.76 [c]	78.3±0.76
Overall	49.4±0.43[e]	57.1±0.56[f]	91.2±0.43[e]	75.4±0.52[g]

[a] Mean±standard error. [b] Average values were calculated on n = 20, excepting where indicated. [c] n = 19. [d] n = 18. [e] n = 139. [f] n = 138. [g] n = 140.

From the adjustment of the models (2) and (3), the following technical indicators of the method performance were estimated (Horwitz, 1982; Horwitz et al., 1990; Horwitz and Albert, 2006; Mertens, 2003):

$$s_r = \sqrt{\hat{\sigma}_\varepsilon^2} \quad (4),$$

$$r = \frac{s_r}{\bar{Y}} \times 100 \quad (5),$$

$$s_R = \sqrt{\hat{\sigma}_{L/E}^2 + \hat{\sigma}_\varepsilon^2} \quad (6),$$

$$R = \frac{s_R}{\bar{Y}} \times 100 \quad (7),$$

$$s_{Re} = 2 \times C^{0.85} \quad (8),$$

$$Re = 2 \times C^{-0.15} \quad (9),$$

$$HorRat = \frac{R}{Re} \quad (10),$$

where s_r is the standard deviation of repeatability (intra-laboratorial variability), r is the repeatability (%), $\hat{\sigma}_\varepsilon^2$ is the estimate of error variance, $\bar{Y}$ = is the average IVDMD (% DM), s_R is the standard deviation of reproducibility (inter-laboratorial variability), R is the reproducibility (%), $\hat{\sigma}_{L/E}^2$ is the estimate of the variance among laboratories, s_{Re} is the expected standard deviation of reproducibility, Re is the expected reproducibility (%), C is the average IVDMD (g/g DM), and HorRat is the Horwitz ratio.

Moreover, an adapted value of the Z-score (Strnad and Makkar, 2014) was calculated for each level of the random effects within each feed according to the equation:

$$Z = \frac{eBLUP}{SEp} \quad (11),$$

where Z is the adapted Z-score for the respective level of random effect (dimensionless), eBLUP is the empirical best linear unbiased predictor of the respective level of random effect, and SEp is the standard error of prediction associated with the eBLUP.

The laboratory eBLUPs were also used for applying a ranking laboratory performance test by adapting the protocols described by Wernimont and Spendley (1985). It must be noticed the test was applied using eBLUPs instead average IVDMD, as the earlier is adjusted for the effect of different equipment, which could bias the rank of laboratories within different feeds.

All statistical evaluation were performed by using the MIXED procedures of SAS 9.4. The components of variance were estimated according to the restricted maximum likelihood method.

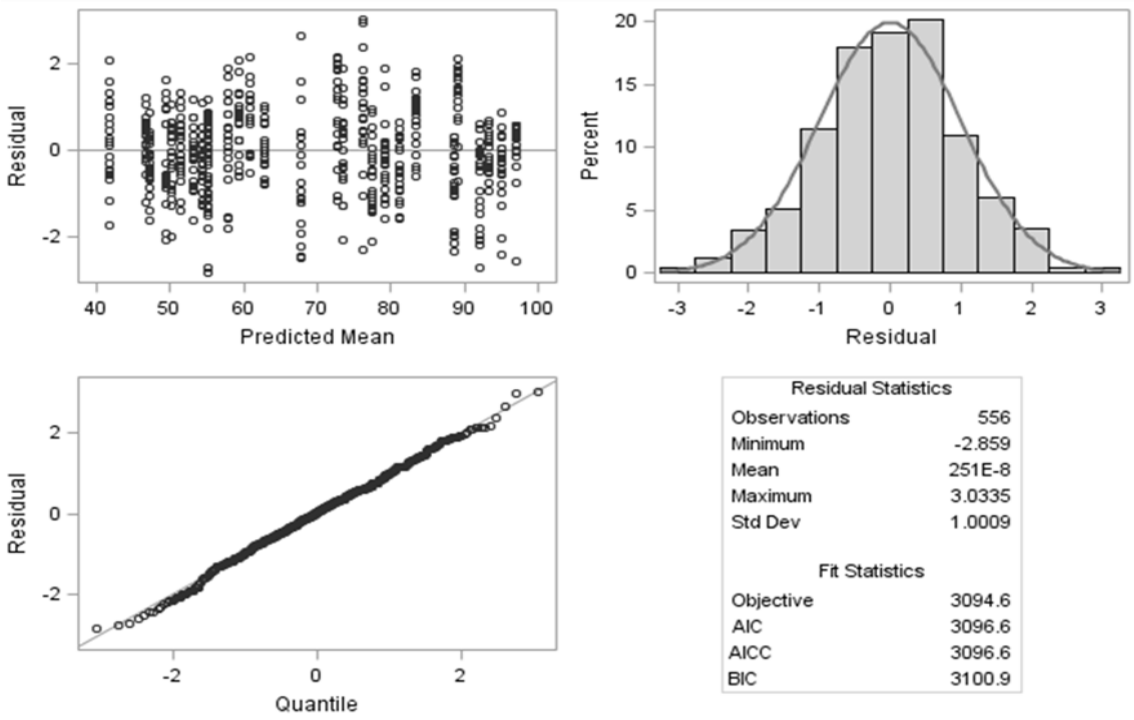

Figure 1. Descriptive pattern of studentized residues for *in vitro* digestibility of dry matter after residual evaluation and outlier elimination.

RESULTS AND DISCUSSION

The total random variance was estimated as the sum of variance components associated with equipment type, laboratories, and random error (Figure 3). Even for the model 2, we did not include variance among feeds as a component of the total random variance. Variance among feeds is expected to occur and it does not influence the performance of the method such as do equipment type or laboratory. On average, equipment type corresponded to 42% of the total random variance. A particular pattern was observed for soybean meal, where the model did not detect a positive variance between equipment types.

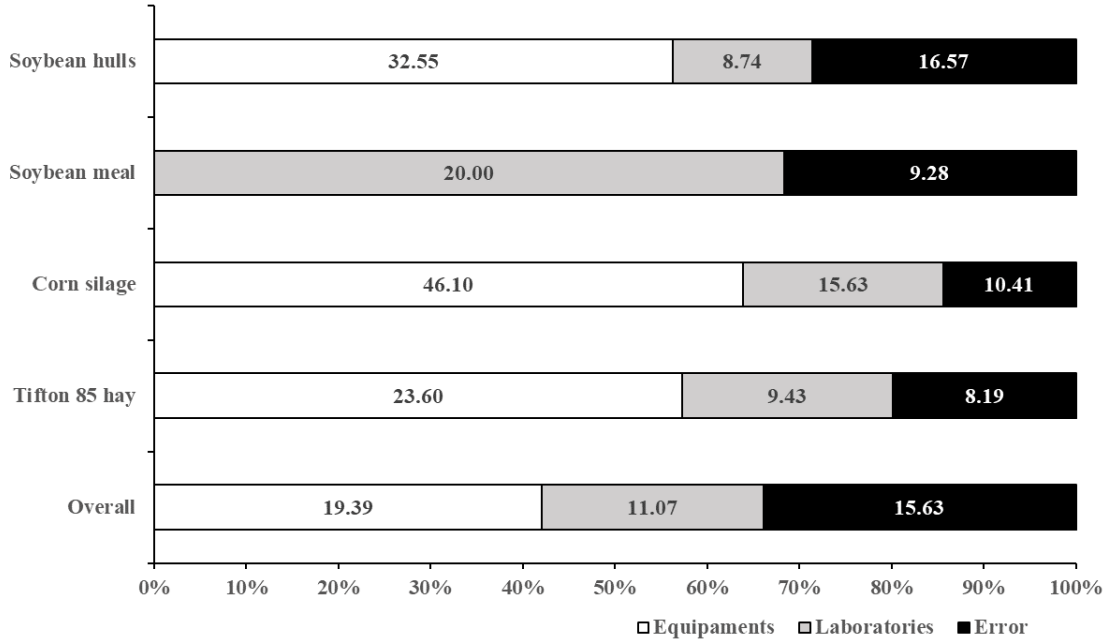

Figure 2. Comparative evaluation between variances for *in vitro* dry matter digestibility associated with equipment type, laboratory, and random error (between replicates) effects according to different evaluated feeds (data label values are expressed as squared percentage units).

This high contribution of equipment for the total random variance shows that equipment features affect *in vitro* digestibility estimates. On the other hand, this is a positive aspect in terms of method standardization and application, because this kind of influence is anticipable and can be used to interpret and adjust the estimates of IVDMD. Overall, the TE-150 presented a positive effect, whereas Daisy^{II} caused a negative effect on the estimates of IVDMD (Figure 3). This pattern agreed with Silva et al. (2017), who found greater IVDMD using TE-150 compared to Daisy^{II}. Those fermenters presented some physical differences, including variations concerning jar rotation rate. This difference is critical, once it may affect the contact between filter bags and inoculum and, as a consequence, altering the IVDMD estimates. On average, the absolute difference between IVDMD obtained with Daisy^{II} and TE-150 was 5.6 percentage points.

On average, the contribution of laboratory effect for the total random variance was 24% (Figure 2), being lesser than the contribution of equipment (42%) and random error (34%). This is a first evidence indicating the method proposed here is reproducible and capable to be applied by different laboratories. It is important to notice that none laboratory behaved as an outlier ($P>0.05$) according to the ranking performance test (Table 3), indicating aspects of robustness of the method, as laboratories did not exhibit a pronounced systematic error (Wernimont and Spendley, 1985).

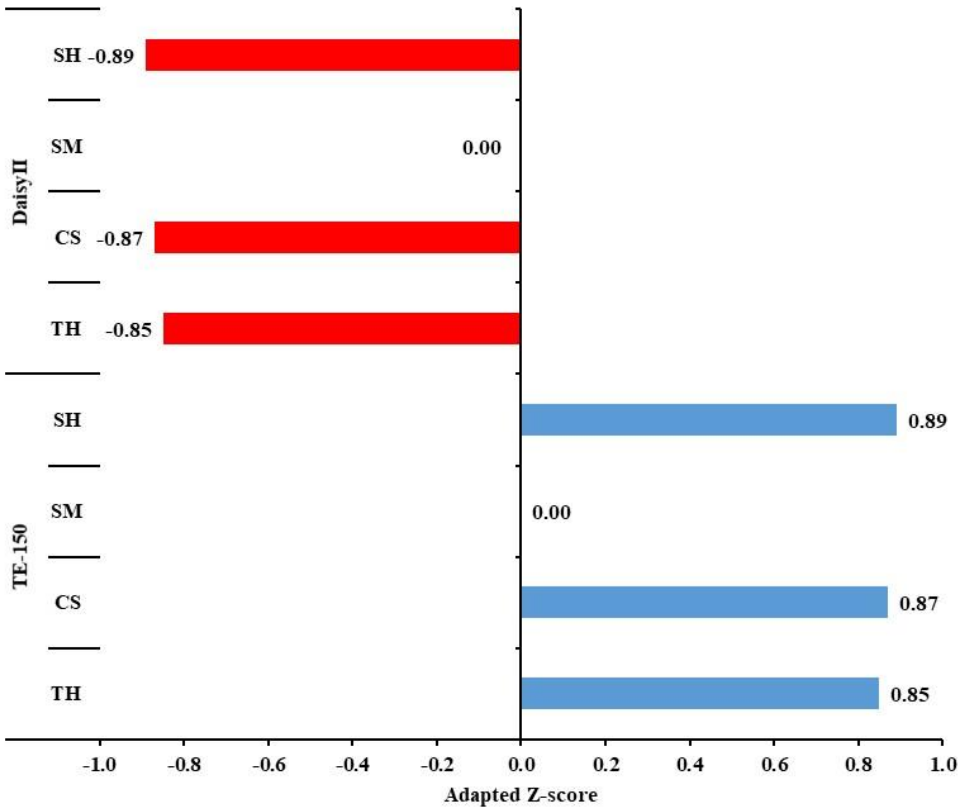

Figure 3. Adapted Z-scores for the *in vitro* dry matter digestibility for the evaluated equipment types with different feeds (TH, Tifton hay; CS, corn silage; SM, soybean meal; and SH, soybean hulls). For details, see Equation (11).

Table 3. Ranking of the empirical best linear unbiased predictors for the effects of laboratories on *in vitro* dry matter digestibility of different feeds

	Feed				
Laboratory	Tifton 85 hay	Corn silage	Soybean meal	Soybean hulls	Sum[a]
1	6	5	5	2.5	18.5
2	5	6	6	7	24.0
3	3	4	2	1	10.0
4	1	2	1	2.5	6.5
5	7	7	7	5	26.0
6	4	3	4	6	17.0
7	2	1	3	4	10.0

[a] Approximate two-tail limits for the sum of ranking scores: 5, 27 (4 feeds, 7 laboratories, $\alpha = 0.05$). For details, see Wernimont and Spendley (1985).

The individual performance of the laboratories was also evaluated using the adapted Z-scores (Figure 4). Normally, the Z-scores are produced from the difference between each laboratory IVDMD and overall mean of IVDMD divided by standard deviation for each feed. However, in our study, there was a second factor contributing for differences among laboratories, which was the two types of artificial fermenters. Then, an adapted Z-score was calculated from eBLUPs, which were previously adjusted regarding equipment type effect, allowing an unbiased comparison among laboratories with regards their performance.

As a general rule in a collaborative study, a satisfactory result is achieved when $|Z| \leq 2$. Moreover, due to inherent and unavoidable variability among laboratories, a frequency of 80% of satisfactory results among laboratories is considered a successful performance (Strnad and Makkar, 2014). However, the number of laboratories was limited in our study. Recommendations regarding the number of laboratories for a collaborative study vary from a minimal of eight (AOCS, 2009), between eight and 15 (Youden and Steiner, 1975), to as many as possible (Wernimont and Spendley, 1985). Nonetheless, due the characteristics of the proposed method, only seven laboratories composed the laboratory sample in our study. Such a low sample size may difficult an adequate evaluation of the distribution frequency of the Z-scores.

Despite of this, the Z-scores exhibited a sigmoidal pattern, which is an inherent characteristic of the normal distribution (Figure 3). Two of the Z-scores assumed marginal values very close to 2 (L5-TH and L7-CS). Assuming that those marginal values can be rounded down to 2, then only five of the Z-scores presented unsatisfactory values. This means that approximately 82% of the Z-scores were found satisfactory, which brought one more evidence towards adequate reproducibility of the evaluated method.

The repeatability varied from 3.34 to 5.79% across feeds (Table 4) and fell within a range similar to what has been observed by other authors in the tropics (Silva et al., 2017; Camacho et al., 2019). A common empirical approach in feed analysis laboratories is to consider that a replicate analysis of IVDMD is acceptable if a maximum difference of 5% between duplicate aliquots is observed. Despite of being a rule of thumb rather than a scientific approach, following this empirical reasoning leads to conclude the observed repeatability for the proposed method is considered adequate in practical terms.

On the other hand, the reproducibility varied from 5.93 to 8.94% across feeds. To our knowledge, there is no Brazilian study on IVDMD where reproducibility was evaluated. However, at first glance, the observed reproducibility was very high when compared to the expected values of R predicted by the Horwitz equation (Horwitz and Albert, 2006).

In a simplified way, the Re determined that the mean coefficient of variation among laboratories (i.e., reproducibility) increased as powers of two as the analyte level decreased as a power of 10. In other words, the Re doubled for every decrease of two orders of magnitude in the concentration of the analyte (expressed as mass fraction). Such a pattern should be independent from either the nature of the analyte or of the analytical technique that was used to make the measurement (Horwitz, 1982; Horwitz and Albert, 2006).

A direct evaluation of the observed R is obtained by calculating HorRat. Acceptable values of this ratio should be between 0.5 and 2.0 (Horwitz, 1982). For the proposed method, HorRat varied from 2.94 to 4.10 across feeds (Table 4). Generally, this would indicate that proposed method is unacceptable concerning precision (i.e., reproducibility). However, it must be understood that the aforementioned limits for HorRat are not absolute, because transgressions are occasionally permitted in both directions (Horwitz and Albert, 2006).

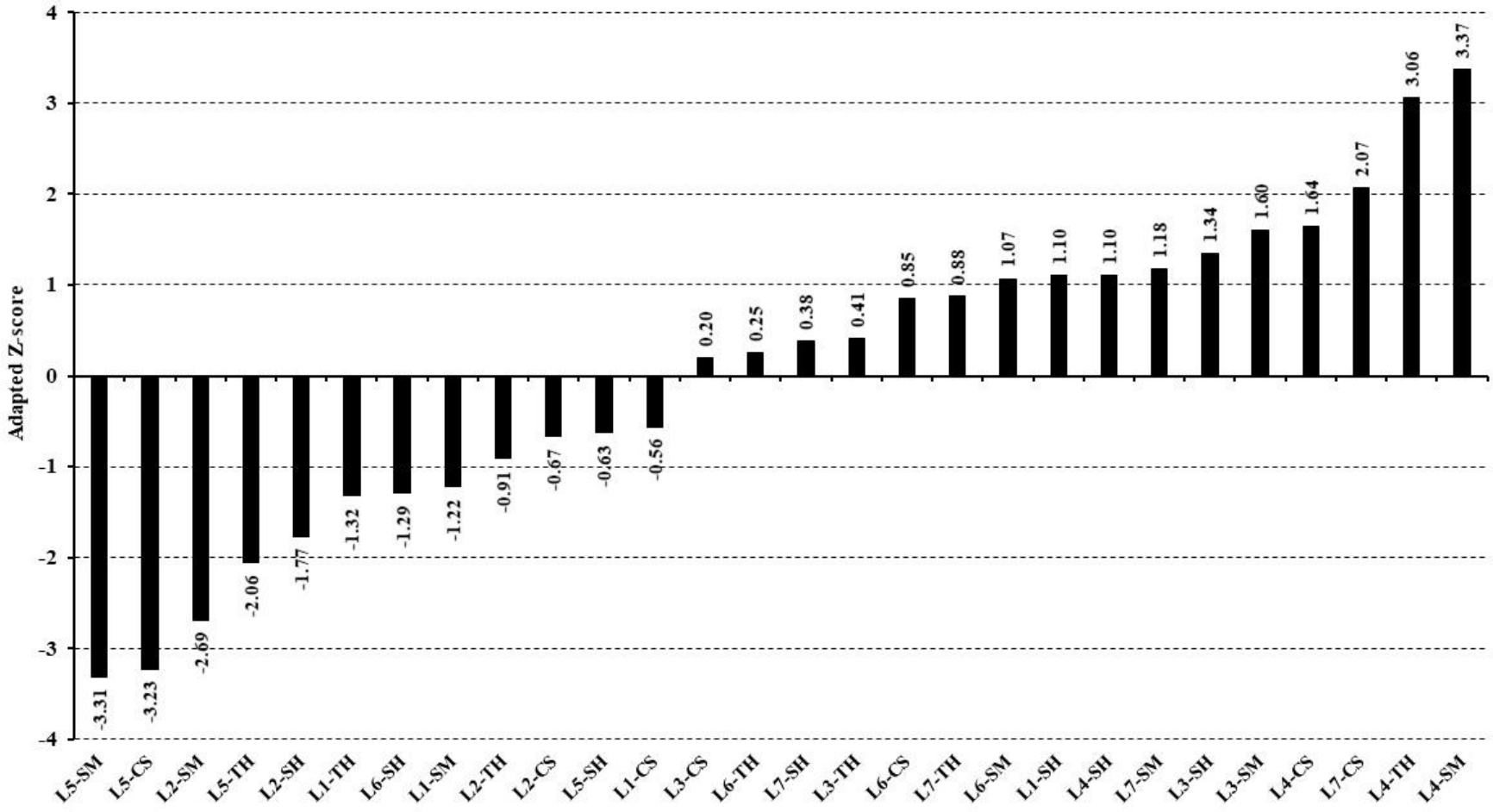

Figure 4. Adapted Z-scores for *in vitro* dry matter digestibility expressed according to different laboratories (L1-L7) and feeds (TH, Tifton hay; CS, corn silage; SM, soybean meal; and SH, soybean hulls). For details, see Equation (11).

To understand the pattern of observed R and HorRat, a broader evaluation of technical indicators must be performed. First, despite of Re decreases as analyte concentration increases, the reproducibility expressed as absolute variation (i.e., as a standard deviation) must present a positive relationship with concentration (Horwitz, 1982). In fact, both s_R and s_{Re} present a very similar pattern according to estimates of IVDMD (Figure 5), including very similar slopes (0.022 versus 0.018, respectively). Despite of s_R be, on average, 3.3 percentage units higher than s_{Re}, their similar sensibility to analyte concentration variation indicates a functional agreement with the theoretical pattern of reproducibility parameters.

Table 4. Estimates of variance components and technical indicators of the proposed method for *in vitro* dry matter digestibility according to the evaluated feed

		Feed			
	Overall	Tifton 85 hay	Corn silage	Soybean meal	Soybean hulls
Variance components [(%)^2]					
Laboratories	11.07	9.43	15.63	20.00	8.74
Error	15.63	8.19	10.41	9.28	16.57
Technical indicators[a]					
aIVDMD (%)	68.3	49.4	57.1	91.2	75.4
s_r	3.95	2.86	3.23	3.04	4.07
r (%)	5.79	5.79	5.65	3.34	5.40
s_R	5.17	4.20	5.10	5.41	5.03
R (%)	7.57	8.50	8.94	5.93	6.67
r/R	0.76	0.68	0.63	0.56	0.80
ser	1.45	1.10	1.24	1.85	1.57
Re (%)	2.12	2.22	2.18	2.02	2.09
HorRat	3.57	3.83	4.10	2.94	3.19
RL	-	11.8	14.3	15.1	14.1
Δ_{max}[b]	-	8.0 (68)	10.5 (73)	12.0 (79)	7.5 (53)

[a] aIVDMD, average *in vitro* dry matter digestibility; s_r, standard deviation within laboratories; r, repeatability; s_R, standard deviation among laboratories; R, reproducibility; Re, expected reproducibility; HorRat, Horwitz ratio; RL, reproducibility limit (RL = 2.8 × s_R); Δ_{max}, maximum difference among the eBLUPs for IVDMD. b Values among parentheses expressed Δ_{max} as % of RL.

Second, r should ordinarily be one-half to two-thirds of R (Horwitz, 1982). This pattern was observed for three feeds here evaluated, excepting soybean hulls (Table 4), which directly implied on a high r/R for the overall dataset. At first glance, the r/R of 0.80 for soybean hulls could indicate that intra-laboratorial replications are so poor that they swamp out the between-laboratory variation. However, a closer evaluation of the soybean IVDMD variability shows that the high r/R was not caused by a high s_r (Table 4), and this pattern seems simply reflect any particularity of this feed which may affect that ratio without bring the variances for levels above ones considered as normal standard across feeds. Thus, despite the particular pattern of soybean hulls, the r-to-R ratios once more indicated that the proposed method presents an adequate reproducibility.

However, the main aspect to be highlighted when interpreting both R and HorRat is the nature of the proposed method. The IVDMD is an analytical entity defined by the method itself (i.e., Type I method; Codex Alimentarius Commission, 2018). HorRat higher than 2 are commonly observed for this type of analytical entity, such as crude fat (Thiex et al., 2003) and fiber (Horwitz et al., 1990). This pattern is attributed to the fact that Horwitz model does not apply to empirical analytes (i.e., those that are method-dependent), whose composition is ill defined and whose concentration estimate depends on the specific details of the method (Horwitz and Albert, 2006). In those cases, the fact that HorRat are >2.0 does not invalidate the method (Thiex et al., 2003).

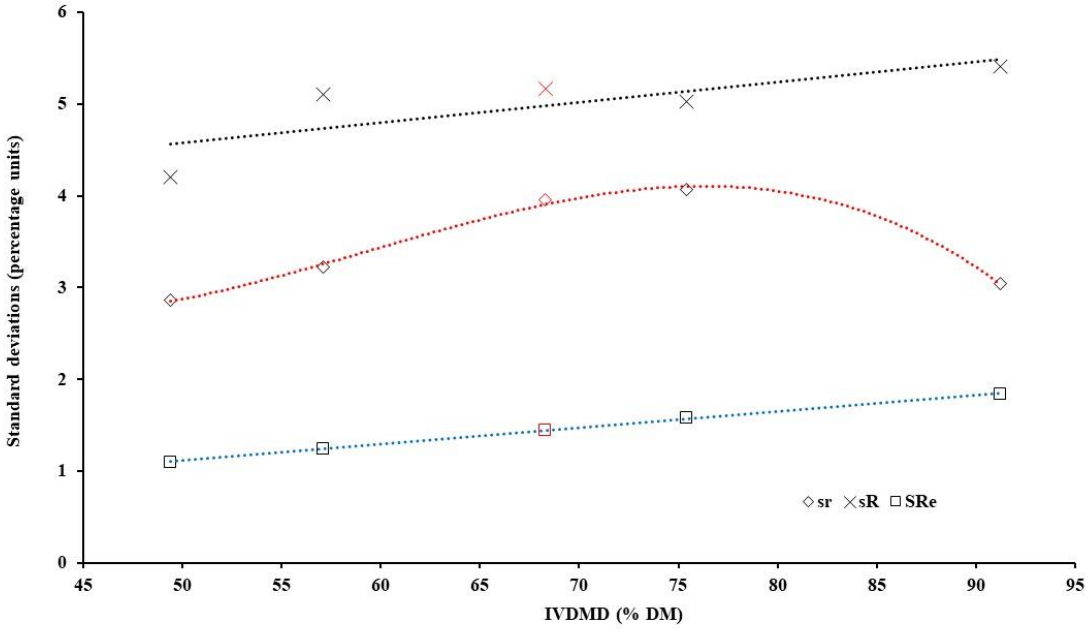

Figure 5. Descriptive pattern of the standard deviations of repeatability (s_r), reproducibility (s_R), and expected reproducibility (s_{Re}) according to average values of *in vitro* dry matter digestibility (the red point corresponds to the mean digestibility for overall dataset).

Due to differences in the digestibility of cell wall and cell contents, the apparent undigested residue (Equation 1) is mainly formed by fibrous compounds. According to Horwitz et al. (1990), fiber-related analytes are not defined chemically. In the presence of such identity problem, the methods are necessarily empirical and accompanied by problems in methodology and internal quality control that are reflected in high R values. Total gas production at fixed

incubation times is strongly correlated with extent of substrate digestion (Pell and Schofield, 1993). Some collaborative studies have found R values for gas production of 26.3% at 24 hours, 15.4% at 48 hours (Cornou et al., 2013), and of 8.2-9.4% at 72 hours of incubation (Van Laar et al., 2006). From this, the observed values of R for IVDMD found in our study (3.34-5.79%, Table 4) can be considered low and corroborate the reproducibility of the proposed method.

Moreover, the reproducibility limit represents the maximum acceptable difference between two single tests on identical test material with the same method in different laboratories with different operators using different equipment (Chui et al., 2009; AOCS, 2017). For all feeds, the maximum difference between laboratories did not exceed reproducibility limit (Table 4), varying from 53 to 79%. This pattern adds to our previous arguments about the adequate reproducibility of the proposed method.

CONCLUSIONS

A standardized method for evaluating the *in vitro* digestibility of dry matter for ruminant feeds and diets was proposed and evaluated through a collaborative study including seven Brazilian laboratories. The results highlighted that, if the method is followed exactly, its results are precise and present adequate levels of repeatability and reproducibility.

ACKNOWLEDGEMENTS

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), and Instituto Nacional de Ciência e Tecnologia de Ciência Animal (INCT-CA), for financial support. To the professors R. Mezzomo (UFRA), E.D. Batista (UFMT/UFLA), S.A. Santos (UFBA), I.A.M.A. Teixeira (FCAV-UNESP), E.O.S. Saliba (UFMG), and M.A.C. Danés (UFLA), for their support as coordinators in the collaborator laboratories.

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Appendix

Method G-00X/1 – *In vitro* digestibility of dry matter using artificial fermenters (models Daisy^{II} Ankom, TE-150 Tecnal, or similar)

Apparatus

- Analytical balance (0.0001 g)
- Forced air-circulation oven
- Oven without forced-air circulation
- Non-woven textile (100 g/m2; 4 × 4.5 cm) or F57 (Ankom®) filter bags
- Sealer
- Desiccator
- Artificial fermenter
- CO_2 cylinder
- Cheesecloth
- Erlenmeyer flasks
- Volumetric flasks
- Blender
- Thermos flasks
- Digital Potentiometer
- Inoculum donor animal
- Washing machine
- Aluminum pan
- Aluminum trays
- Stove

Reagents

- Sodium bicarbonate ($NaHCO_3$) P.A.
- Dibasic sodium phosphate (Na_2HPO_4) P.A. anhydrous, dihydrate or heptahydrate
- Potassium chloride (KCl) P.A.
- Sodium chloride (NaCl) P.A.
- Magnesium sulphate ($MgSO_4$) heptahydrate P.A.
- Calcium chloride ($CaCl_2$) dihydrate P.A.
- Urea P.A.
- Neutral household detergent

Solutions

Buffer solution

- In a 1 L (10 L) volumetric flask containing 0.1 L (1 L) of distilled water, add 9.8 g (98 g) of $NaHCO_3$, 3.71 g (37.1 g) of anhydrous Na_2HPO_4 or 4.65 g (46.5 g) of Na_2HPO_4 dihydrate or 7.00 g (70.0 g) of Na_2HPO_4 heptahydrate, 0.57 g (5.7 g) of KCl; 0.47 g (4.7 g) of NaCl; 0.12 g (1.2 g) of $MgSO_4$ heptahydrate and 0.05 g (0.5 g) of $CaCl_2$ dihydrate. Shake for partial dissolution. Add 0.8 L (8 L) of distilled water and stir until complete dissolution. Make up the volume of the balloon to 1 L (10 L). This procedure should preferably be carried out 24 hours before incubation.

Urea solution
- Add 5.5 g of urea in a 100 mL volumetric flask. Add, approximately, 70 mL of distilled water and mix until completely dissolved. After the temperature has stabilized with the environment, complete the volume. The solution must be stored under refrigeration until utilization.

Neutral detergent solution
- In a 4 L Erlenmeyer, add 1 L of distilled water and 80 mL of neutral household detergent. Shake until completely homogenized and make up to volume with distilled water.

Procedures

In all procedures, use an analytical balance calibrated by INMETRO, with an accuracy of 0.0001 g, on a special laboratory bench, and in a climate-controlled environment (20-25ºC or according to the manufacturer's specifications). Switch on the balance and wait 30 minutes for its stabilization.

Preparation of filter bags

1. Identify the filter bags with a permanent marker
2. Boil the filter bags in neutral detergent solution for 15 minutes
3. Wash the filter bags under running water until the detergent is completely removed
4. Dry the filter bags in trays (thin layer without overlapping) sequentially in a forced air-circulation oven at 55°C for 24 hours and then in a non-ventilated oven at 105°C for 2 hours
5. Place the filter bags in a desiccator (maximum 20 units per procedure) and wait for the equilibrium with room temperature
6. Weigh the filter bags and record the weight. This will be the tare weight
7. Weigh approximately 500 mg of air-dried sample processed to pass through a 1-mm screen sieve, record the weight, and place inside the filter bag
8. Seal the filter bags using a sealer and set them aside for further incubation
9. For each incubation jar it is necessary to use at least two sealed blank filter bags (i.e., without sample)

Preparation of buffer solution

1. Add McDougall's buffer solution in an Erlenmeyer flask in sufficient volume to carry out the incubation procedure. It is important that buffer solution stirring overnight for better dilution of reagents
2. Add urea solution to McDougall's solution at a rate of 5 mL/300 mL. **This addition is considered to be optional, since the use of urea can decrease the discrimination power among samples (see details in Camacho et al., 2019). Therefore, using or not urea is the only step here let to the analyst's discretion.**
3. Attach a silicone hose to the CO_2 cylinder
4. Insert the free end of the hose into the buffer solution and release the CO_2 providing bubbling
5. Using the digital potentiometer, monitor the pH of the solution. The bubbling with CO_2 is ended when the pH is adjusted down to 6.8. This procedure can take a long time. Thus, it is recommended to perform the pH adjustment before collecting ruminal inoculum
6. Using an oven or climate-controlled room, acclimatize the buffer solution to a temperature of 39°C
7. This procedure must be performed prior to the collection of rumen inoculum

Inoculum collection and processing

1. Turn on the fermenter heating (39°C) with empty jars
2. The inoculum can be collected from a rumen canulated bovine; however, the production of inoculum from a pool of ruminal digesta obtained from three or more animals is recommended
3. The donor animal(s) must be adapted to the basal diet for at least 14 days before inoculum collection
4. The basal diet must consist of forage and concentrate in the proportion of 80:20 with 12% of crude protein, both on dry matter basis. Mineral supplementation and water must be available *ad libitum.* The practice of fasting animals prior the collection should not be performed
5. Open the rumen cannula and collect portions of the solid and liquid parts of the digesta at different points in the rumen mat and store them in pre-heated thermos bottles (39°C)
6. Take the material immediately to the laboratory
7. Using a blender, mix the digesta for approximately 30 seconds
8. In an Erlenmeyer flask, filter the homogenized digesta through four layers of cheesecloth. Discard the material retained in the cheesecloth
9. Keep the filtrate (i.e., inoculum) acclimatized (39°C) with the use of thermos bottles, oven, or climate-controlled room
10. This procedure must be performed immediately before starting the incubation

Incubation procedure

1. Remove the pre-heated jars from the fermenter and accommodate filter bags inside them. It is recommended that each jar operates with 20 to 25 filter bags containing samples and two extra blank filter bags
2. Add 400 mL of inoculum and 1600 mL of buffer solution to each jar
3. Quickly flush the headspace of each jar with CO_2 to replace atmospheric air with carbon dioxide. Cap the jars immediately
4. Place the jars in the fermenter, start the rotation mechanism and close the door. Make sure the heating is properly turned on and set at 39°C
5. Keep jars under standard temperature and rotation conditions for 48 hours. Check them regularly during whole fermentation process
6. After 48 hours, open the fermenter and remove the filter bags
7. Wash them superficially with clean water and press them gently to remove gases
8. Place the filter bags in a washing machine, fill it with clean tap water, adjust it for delicate setting, and activate the washer for 1 minute. Drain water after washing. This procedure must be performed at least three times
9. After the washing cycles, gently press the filter bags to remove excess of water (which must be clear)
10. Dry the filter bags in trays (thin layer without overlapping) sequentially in an oven forced air-circulation oven at 55°C for 24 hours and then in a non-ventilated oven at 105°C for 16 hours
11. Place the filter bags in a desiccator (maximum 20 units per procedure) and wait for equilibrium with room temperature
12. Weigh the filter bags and record the weight

Calculation of in vitro dry matter digestibility

Calculation of the residue obtained with blank filter bags

$$B = \boldsymbol{F_B} - \boldsymbol{T}$$

where: B = contamination from the incubation procedure (g of dry matter); F_B = blank filter bag weight after incubation (g); and T = tare or pre-incubation filter bag weight (g).

The "blank" value to be used in the other calculations must be obtained by averaging the contamination (B) obtained in the blank filter bags evaluated within the incubation run. It is important to emphasize that contamination is inherent to each incubation procedure. Therefore, blanks are required for each procedure, and it is not possible to use "average values" obtained from previous procedures.

Calculation of apparently undigested residue:

$$U = \boldsymbol{F_A - T}$$

where: U = apparently undigested residue (g of dry matter); FA = weight of the filter bag containing the sample after incubation (g); and T = tare or pre-incubation filter bag weight (g).

Calculation of *in vitro* dry matter digestibility:

$$\%\boldsymbol{U_{ADS}} = \left(\frac{\boldsymbol{U - B}}{\boldsymbol{ADS}}\right) \times \mathbf{100}$$

$$\%\boldsymbol{IVDMU} = \frac{\%\boldsymbol{U_{ADS}}}{\%\boldsymbol{DM_{105}}} \times \mathbf{100}$$

$$\%\boldsymbol{IVDMD} = \mathbf{100} - \%\boldsymbol{IVDMU}$$

where: $\%U_{ADS}$ = undigestibility based on the air-dried sample (%); ADS = air-dried sample mass (g); %IVDMU = *in vitro* dry matter undigestibility (%); and $\%DM_{105}$ = percentage of dry matter of the sample evaluated at 105°C; and %IVDMD = *in vitro* dry matter digestibility (%). The other terms were previously defined.

When exposing the results, it is mandatory to report the following information: 1. brand and model of the artificial fermenter, 2. type of filter bag used, and 3. addition or not of urea to the buffer solution. These three factors will affect digestibility estimates and their knowledge is crucial for interpretation and comparative evaluation of results from different assays.