In vitro Digestibility Measurement of Feedstuffs in Donkeys Using the DaisyII Incubator

Manuscript Details

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Abstract

Successful studies on in vitro digestibility measurement of feedstuffs with fecal inoculum have been reported for horses, while data on donkeys are currently lacking. In this study we evaluated the use of the DaisyII Incubator (Ankom Technology, Fairport, NY, USA) for in vitro digestibility measurement of feedstuffs using donkey feces as source of microbial inoculum. The method was tested using 7 feedstuffs commonly used in donkey diets (alfalfa, bromegrass, ryegrass and timothy hays; wheat bran and wheat straw; barley grains). Feces were obtained from 4 female donkeys and incubations were carried out at one-week intervals for 4 consecutive weeks. Two bags of each feedstuff were incubated in digestion vessels containing a buffer/feces solution (90:10). In vitro apparent dry matter digestibility (DMD), true dry matter digestibility (IVTD), and neutral detergent fiber digestibility (NDFD) were evaluated at 4 incubation times: 30, 48, 60, and 72 h. All digestibility parameters significantly increased from 30 to 72 h of incubation. At 72 h of incubation, the within-laboratory repeatability and reproducibility of the method were 2.7% and 5.0% for DMD and 1.6% and 3.9% for IVTD, respectively. The method was less repeatable and reproducible for NDFD (4.5% and 10.4%, respectively).

Keywords	Equus asinus L.; fecal inoculum; DaisyII Incubator; measurement error; incubation time.

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Suggested reviewers	Jennifer E. Earing, Mauro Spanghero, Barbara Padalino, Valentina Ragno, Eric Davis

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To Dr. Edward L. Squires

Editor-in-Chief

Journal of Equine Veterinary Science

Re: Revision Requested - JEVS_2018_283_R1 for Journal of Equine Veterinary Science

Dear Dr. Edward L. Squires,

I have read carefully your letter dated January 28th, 2019 and the comments from reviewer 1 regarding our manuscript. I prepared a revised version of the paper according to these comments. All changes made to the R1 version of the manuscript have been highlighted in red font. I hope this modified version will fully satisfy the reviewers and the Editor, and match the standards of the *Journal of Equine Veterinary Science*.

The comments of the reviewer have been addressed one by one, and are listed in the “Response to reviewers” file.

Yours sincerely, Dr. Manuela Renna.

RESPONSE TO REVIEWERS

Reviewer 1

This reviewer congratulates the authors on a well written and detailed manuscript. Thank you for thoroughly addressing all comments and for your effort in adding new sections and references. In this reviewer's opinion, the authors' suggestion to change the manuscript from short communication to original research article should be accommodated.
Please see below my minor comments to this version of the manuscript.

AU Response: Thank you for the positive evaluation of the revised version of our manuscript.

Line 109: doubled weighed

AU Response: Changed to: "weighed in duplicate".

Line 227: improve "when" extending

AU Response: Corrected.

Line 237: provides

AU Response: The sentence has been modified.

Lines 238-239: "the absolute difference… will differ" I suggest trying to rephrase and shorten this sentence for clarity

AU Response: We rewrote the sentence as follows: "The estimated repeatability and reproducibility coefficients (Table 2b) are precision measures providing additional information, as they represent the maximum absolute difference that can be expected between repeated measurements on 95% of occasions [17]."

Lines 244-248: since conclusions (i) and (iii) appear to overlap, could they be condensed into one?

AU Response: We rewrote the sentence as follows: "From the obtained results, it can be concluded that an incubation period of 72 h is required to obtain accurate DMD, IVTD, and NDFD estimates in donkeys and to improve the repeatability and reproducibility of the method. For NDFD, the repeatability and reproducibility of the method were comparable to those obtained with the Daisy^{II} Incubator in ruminants."

Reviewer 2

The revision of the manuscript is satisfactory.

AU Response: Thank you for the positive evaluation of the revised version of our manuscript.

Highlights

- Daisy^{II} Incubator can be successfully used for digestibility studies in donkeys
- NDFD had lower repeatability and reliability than DMD and IVTD
- Digestibility increased significantly from 30 to 72 h of incubation

In vitro Digestibility Measurement of Feedstuffs in Donkeys Using the Daisy^{II} Incubator

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Abstract

Successful studies on *in vitro* digestibility measurement of feedstuffs with fecal *inoculum* have been reported for horses, while data on donkeys are currently lacking. In this study we evaluated the use of the Daisy^{II} Incubator (Ankom Technology, Fairport, NY, USA) for *in vitro* digestibility measurement of feedstuffs using donkey feces as source of microbial *inoculum*. The method was tested using 7 feedstuffs commonly used in donkey diets (alfalfa, bromegrass, ryegrass and timothy hays; wheat bran and wheat straw; barley grains). Feces were obtained from 4 female donkeys and incubations were carried out at one-week intervals for 4 consecutive weeks. Two bags of each feedstuff were incubated in digestion vessels containing a buffer/feces solution (90:10). *In vitro* apparent dry matter digestibility (DMD), true dry matter digestibility (IVTD), and neutral detergent fiber digestibility (NDFD) were evaluated at 4 incubation times: 30, 48, 60, and 72 h. All digestibility parameters significantly increased from 30 to 72 h of incubation. At 72 h of incubation, the within-laboratory repeatability and reproducibility of the method were 2.7% and 5.0% for DMD and 1.6% and 3.9% for IVTD, respectively. The method was less repeatable and reproducible for NDFD (4.5% and 10.4%, respectively).

Key words: *Equus asinus* L.; fecal *inoculum*; Daisy^{II} Incubator; measurement error; incubation time.

1. Introduction

The use of *in vitro* techniques to study diet digestion and fermentative end products has been successfully applied in horse nutrition. Lowman et al. [1] was the first to demonstrate that equine feces can be used as source of microbial *inoculum* and that fecal microflora can remain viable for several hours after excretion. According to Abdouli et al. [2], a two-stage *in vitro* technique (combining enzymatic pre-digestion and fermentation) can estimate the organic matter digestibility of both forages and concentrates using horse feces as source of microbial *inoculum*. Ringler et al. [3] demonstrated that the use of equine fecal *inoculum* in a closed-system fermentation apparatus can yield valid *in vitro* estimates of dry matter (DM) and fiber digestibility. Lattimer et al. [4] reported that the Daisy^{II} Incubator could be used to predict valid estimates of DM digestibility of diets using equine feces as *in vitro inoculum* source. Earing et al. [5] confirmed that feces are a suitable source of microbial *inoculum* for *in vitro* digestibility studies in horses. More recently, Murray et al. [6] compared samples from different sites of the equine intestine, showing that feces are a suitable alternative to cecal fluid, thus highlighting the potential of this kind of *inoculum* for *in vitro* digestibility studies.

Very few studies are currently available on digestibility measurement of feedstuffs in donkeys, most of the data resulting from *in vivo* feeding trials [7-9]. *In vitro* digestibility in donkeys was studied using the neutral cellulase plus gamanase technique developed by Ankom Technology (Fairport, NY, USA) and via fecal-Near Infrared Spectroscopy [9-11], but with poor success. Since the breeding of lactating donkeys in Western Europe is increasing [12], it becomes needful to evaluate tools to study the *in vitro* digestibility of feedstuffs used in donkey nutrition. Therefore, the objectives of this study were (i) to analyse, at different incubation times, the *in vitro* digestibility measurement of feedstuffs in donkeys using the Daisy^{II} Incubator and donkey feces as source of microbial *inoculum,* and (ii) to test the within-laboratory repeatability and reproducibility of the method.

2. Materials and Methods

The protocol was designed according to the guidelines of the current European Directive (2010/63/EU) on the care and protection of animals used for scientific purposes.

Ethical approval for this study was obtained from the Animal Welfare Committee of the Department of Veterinary Science of the University of Torino (Italy).

2.1. Feedstuffs

Seven feedstuffs (alfalfa hay, bromegrass hay, ryegrass hay, timothy hay, wheat bran, wheat straw, and barley grains) were chosen for the trial. Hays and straws are commonly used as forages in donkey nutrition; wheat bran and flaked barley are fed to working and lactating donkeys to increase the palatability and/or energy density of diet [13,14].

Feedstuffs were analysed for their proximate constituents and fiber fractions following the procedures reported by Fortina et al. [15].

2.2. Animals and diet

The feces were collected from 4 healthy (Body Condition Score = 5) female Ragusana donkeys (live weight: 193 ± 24 kg) fed with a constant ration during the whole trial. The ration was based on first cut meadow hay given *ad libitum* [DM 909 g/kg; ash 79 g/kg DM; crude protein (CP) 68 g/kg DM; ether extract 9 g/kg DM; neutral detergent fiber (NDF) 550 g/kg DM; acid detergent fiber (ADF) 313 g/kg DM; lignin 38 g/kg DM]. The donkeys were individually housed in 3 × 4 m covered pens at the experimental facility of the Department of Veterinary Science, University of Torino. Wood chips were used for bedding and fresh water was available at all times. On a daily basis, the donkeys were turned out in pairs into a sandy paddock, allowing free movement and socialization.

2.3. In vitro digestibility

For feces collection and *in vitro* analyses, the procedures described by Earing et al. [5] were followed. Approximately 250 g of feces were collected directly from the rectum of each donkey on a weekly basis, for 4 consecutive weeks (i.e. 4 runs), always at the same hour in the morning. Immediately after collection, the feces were sealed in individual airtight bags expelling air as much as possible to maintain anaerobic conditions [4,5] and transported to the laboratory (approximately 3 min travel from the barn to the laboratory). During transport, the temperature of the feces was maintained at about 39°C using a warm water-containing cooler. At the laboratory, the feces of the 4 donkeys were pooled and a mixture of them was used for the analysis.

The in *vitro* digestibility measures were performed using the Ankom Daisy^{II} Incubator (Ankom Technology Corporation, Fairport, USA). Feed samples (approximately 0.50 g) were weighed in duplicate into Ankom F57 filter bags, which were then heat sealed and put into each of the four vessels with 1800 mL of a mixture (5:1 ratio) of two buffer solutions (A and B), prepared as described by Ankom Technology [16]. About 200 g of feces per vessel were weighed and transferred to a blender jar with 400 mL of the warmed buffer solution. The content was purged with CO_2 for 15 s and blended for 30 s using a high-speed blender (Osterizer cyclo trol eight, Oster, Moncalieri (TO), Italy). The blended content was transferred to the digestion jar and gently mixed. The pH of the mixture was adjusted to 7.0 using additional amounts of the two (unmixed) buffers (A or B) as suggested by Earing et al. [5].

Considering previous experiments on horses [3-5], 4 incubation times were assigned to the vessels: 30, 48, 60, and 72 h. The shortest incubation time (30 h) was included considering that the mean retention time of the gastrointestinal tract in horses ranges from 18-20 h [8] to 45-60 h [7]. As donkeys retain feeds for longer time than other equids [7], the 72 h incubation time was chosen trying to ensure complete nutrient digestion [5].

At the end of the assigned time, the bags were removed from each vessel, rinsed thoroughly with cold tap water and placed in a 50 °C forced-air oven to dry for 24 h. The bags were weighed and then analysed for their NDF content with the Ankom^{200} Fiber Analyzer (Ankom Technology).

The obtained data were used to calculate:

- *in vitro* apparent DM digestibility (DMD, g/kg DM) = $1000 \times (DM_{0h} - DM_{residue}) / DM_{0h}$

- *in vitro* true digestibility (IVTD, g/kg DM) = $1000 \times (DM_{0h} - NDF_{residue}) / DM_{0h}$

- *in vitro* NDF digestibility (NDFD, g/kg NDF) = $1000 \times (NDF_{0h} - NDF_{residue}) / NDF_{0h}$.

To test the within-laboratory repeatability of the method, two replicates were carried out for each sample. The within-laboratory reproducibility of the method tested instead the variation in the measurements under changing conditions (i.e. the 4 runs) [17].

2.4. Statistical analysis

Data were analysed using SAS [18].

A mixed model repeated measures analysis [18] was carried out, considering incubation time as repeated measure, the effects of run, incubation time and their interaction, and the random effect of feedstuff [19]. Different covariance structures were fitted and, considering the Schwarz Bayesian Information Criterion, the autoregressive structure was chosen [19] to account for intra-feedstuff correlation.

For each incubation time, the repeatability and reproducibility of the method were calculated as reported by Spanghero et al. [20]. For this purpose, a generalized linear model (GLM) was applied according to the following equation:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk},$$

where: μ = overall mean; α = effect of run (i = 1,4); β = effect of feedstuff (j = 1,7); ε = residual error.

The standard deviation (SD) of repeatability and reproducibility were calculated as:

SD_repeatability (S_r) = $\sqrt{\sigma_e^2}$

SD_reproducibility (S_R) = $\sqrt{\left\{\left(\frac{\sigma_\alpha^2 - \sigma_{\alpha\beta}^2}{7x2}\right) + \left(\frac{\sigma_{\alpha\beta}^2 - \sigma_e^2}{2}\right) + \sigma_e^2\right\}}$,

where σ_α^2, $\sigma_{\alpha\beta}^2$, and σ_e^2 are the variance components of run effect, interaction between run and feedstuff effects, and error, respectively. Repeatability and reproducibility were then expressed as coefficients of variation: SD / mean × 100 [20].

In addition, the repeatability and reproducibility coefficients were also estimated, as reported by Bartlett and Frost [17]:

- repeatability coefficient = $1.96 \times \sqrt{2} \times S_r$
- reproducibility coefficient = $1.96 \times \sqrt{2} \times S_R$.

A linear regression analysis for incubation time was used to obtain, for each feedstuff, the regression parameters (intercept and slope) of each *in vitro* digestion (4 runs × 7 feedstuffs). Regression parameters were analysed by Canonical Discriminant Analysis (CDA), which finds linear combinations (canonical functions) of the quantitative variables that provide maximal separation between classes or groups [17].

Significance was set at $P < 0.05$.

3. Results

3.1. Effect of incubation time on estimates of DMD, IVTD, and NDFD

The chosen feedstuffs were characterized by a wide range of CP (66-166 g/kg DM), NDF (213-797 g/kg DM), ADF (69-550 g/kg DM), and lignin (12-100 g/kg DM) contents (Table 1).

The mixed model repeated measure analysis showed that DMD, IVTD, and NDFD were not influenced by run ($P > 0.05$). Incubation time significantly affected DMD, IVTD and NDFD (Table 2a). All considered digestibility parameters ranked according to the following order: 72 h > 60 h > 48 h > 30 h. For each feedstuff, DMD and NDFD trends over incubation time are shown in Fig. 1.

DMD (Fig. 1a) and NDFD (Fig. 1b) of hays and straw were lower than that of barley grains. Straw showed the absolute lowest DMD values (Fig. 1a). Wheat bran and ryegrass hay showed the highest NDFD values at 72 h, while alfalfa hay and wheat straw had the lowest; bromegrass hay, timothy hay, and barley grains showed intermediate values at this incubation time (Fig. 1b).

3.2. Repeatability and reproducibility of the method

For each incubation time, the repeatability and reproducibility of the method are reported in Table 2b. Repeatability and reproducibility, expressed as coefficients of variation, ranged 2.7 - 3.8% and 5.0 - 10.3% for DMD, 1.6 - 1.8% and 2.8 - 5.4% for IVTD, and 4.5 - 11.6% and 10.4 - 18.5% for NDFD, respectively. Particularly for NDFD, both repeatability and reproducibility improved from 30 to 72 h of incubation.

The results of the multivariate statistical analysis are shown in Fig. 2. The regression parameters (intercept and slope) describe each *in vitro* digestion (4 runs × 7 feedstuffs) and Fig. 2, for IVTD as an example, shows the separation among the different feedstuffs by CDA.

4. Discussion

4.1. Effect of incubation time on estimates of DMD, IVTD, and NDFD

As expected, estimates of DMD, IVTD and NDFD increased while increasing the incubation time (Table 2a). The obtained results indicate that an incubation period of 72 h or higher may be required for accurate DMD, IVTD, and NDFD estimates in donkeys. Similar findings were recently obtained by Franzan et al. [21], studying DM, organic matter, and NDF degradation using horse feces as *inoculum* in an *in vitro* fermentation assay. Moreover, comparing *in vivo* digestibility estimates in horses with those obtained *in vitro* using the Daisy^{II} Incubator at 30, 48, and 72 h of incubation, Earing et al. [5] showed that, for high-fiber diets (44 - 51% NDF), a 72 h incubation time provided estimates closer to *in vivo* data. However, the use of a set incubation time for all kind of feedstuffs

may be questionable, because more fibrous feedstuffs may need longer incubation times to estimate accurately DMD and NDFD [4,5]. In the current trial, the effect of incubation time was not evaluated for each considered feedstuff, because only one sample (in duplicate) per feedstuff was incubated. Further targeted trials are needed to assess the optimum incubation time for different feedstuffs using the Daisy^{II} Incubator for the digestibility evaluation of feedstuffs in donkeys.

As we did not use multiple samples per each feedstuff, at each incubation time differences among feedstuffs were not statistically evaluated. Despite lack of targeted statistical evaluation, the single feedstuffs showed expected trends according to their chemical composition (Fig. 1). High-fiber feedstuffs showed in fact lower DMD (Fig. 1a) and NDFD (Fig. 1b) values when compared to high-starch samples. The lowest NDFD values obtained for alfalfa hay and wheat straw at 72 h of incubation can be the consequence of their high lignin content (Fig. 1b). It is known that the type of feed, stage of maturity and processing can affect the digestive process and the gastrointestinal retention time in equids [22-23]. In forages, the cell wall components are the main factors influencing digestibility. Cell wall components increase with plant maturity [24] and lignification in particular imposes a barrier to complete cell wall polysaccharide digestion [25-26].

The CDA applied to our data (Fig. 2) allowed a good separation of the feedstuffs; this is also a way to synthesize the method's action according to the chemical composition of the feedstuffs.

4.2. Repeatability and reproducibility of the method

Concerning the repeatability and reproducibility of the method, it has to be pointed out that values can vary among laboratories [27]. Despite the large use of the Daisy^{II} Incubator to measure digestibility (especially in ruminants), very few studies aimed at verifying the associated measurement errors [20]. To the best of our knowledge, no published literature is available on the repeatability and reproducibility of DMD and IVTD obtained with the Daisy^{II} Incubator in ruminants or equids. The results obtained in our study are in accordance with those commonly found by Ankom Technology using the Daisy^{II} Incubator [Brian Layton, Ankom Technology;

personal communication]. Regarding fiber digestibility, in ruminants Spanghero et al. [20] showed that the repeatability and reproducibility of NDFD and digestible NDF using the Daisy^{II} Incubator improve when extending the fermentation time from 30 to 48 h. Our data also show a general improvement of the repeatability and reproducibility of the method, particularly for NDFD, as a consequence of longer incubations (Table 2b). At the shorter incubation times (30, 48, and 60 h), low repeatability and reproducibility for NDFD may be the consequence of insufficient degradation of the fiber fraction of the diet, which instead improved at 72 h of incubation, also allowing a better precision degree of the method. At 72 h of incubation, the method tested in our study was less repeatable and reproducible for NDFD than for DMD and IVTD (Table 2b). The repeatability and reproducibility found by Spanghero et al. [20] for NDFD at 48 h of incubation were equal to 6.83% and 10.46%; such values are quite similar to those found for NDFD at 72 h of incubation in the current study (4.5% and 10.4%, respectively). The estimated repeatability and reproducibility coefficients (Table 2b) are precision measures providing additional information, as they represent the maximum absolute difference that can be expected between repeated measurements on 95% of occasions [17].

5. Conclusion

We evaluated the use of the Daisy^{II} Incubator (Ankom Technology) for determining DMD, IVTD, and NDFD of feedstuffs using donkey feces as source of microbial *inoculum*. From the obtained results, it can be concluded that an incubation period of 72 h is required to obtain accurate DMD, IVTD, and NDFD estimates in donkeys and to improve the repeatability and reproducibility of the method. For NDFD, the repeatability and reproducibility of the method were comparable to those obtained with the Daisy^{II} Incubator in ruminants. Overall, the trial shows that the method described by different authors [3-5] for *in vitro* digestibility studies in horses using the Daisy^{II} Incubator could be successfully extended to donkeys.

Further studies should evaluate the among-laboratory repeatability and reproducibility of the method. In addition, trials aimed at comparing results from *in vitro* and *in vivo* digestibility are needed to assess the most suitable incubation time of different feedstuffs for donkeys, and to propose equations that fit the shortest *in vitro* incubation time with *in vivo* data.

Acknowledgements

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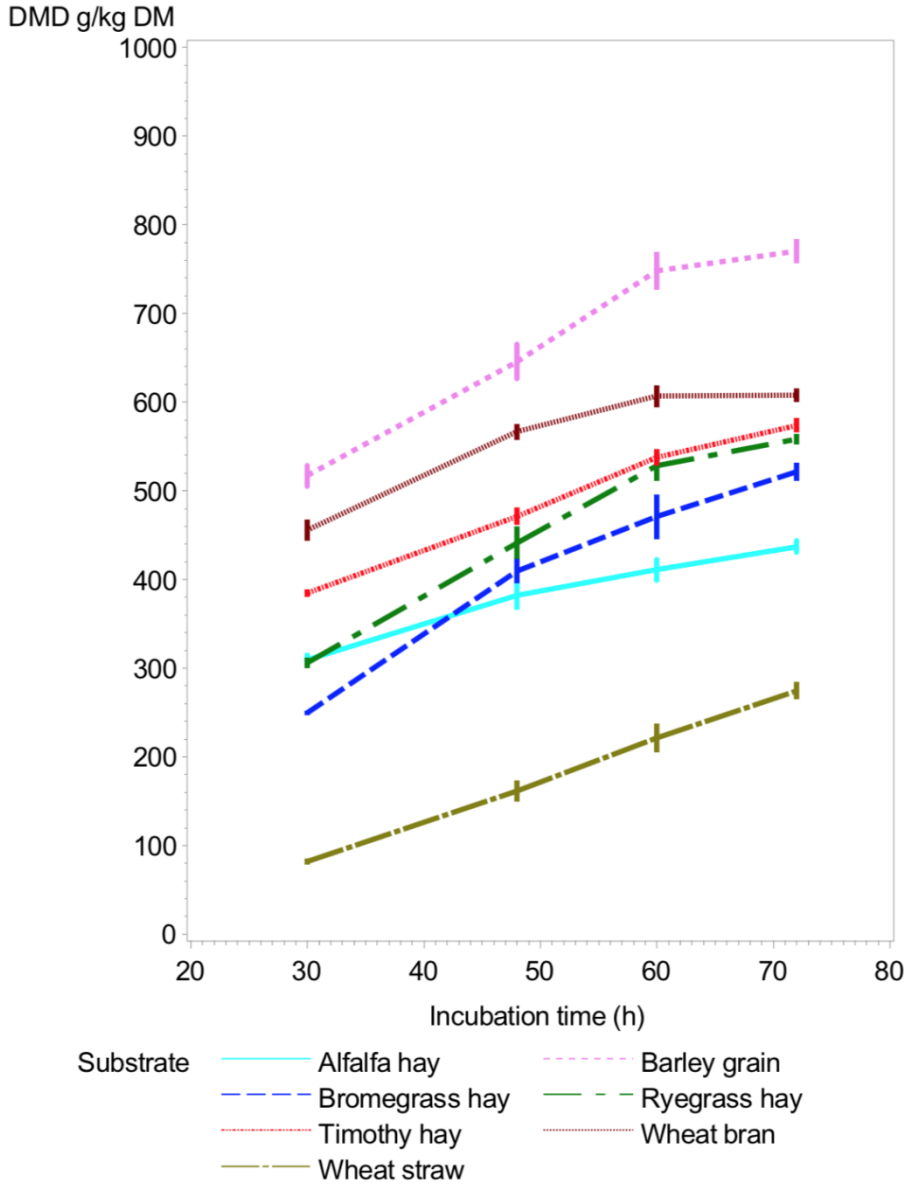
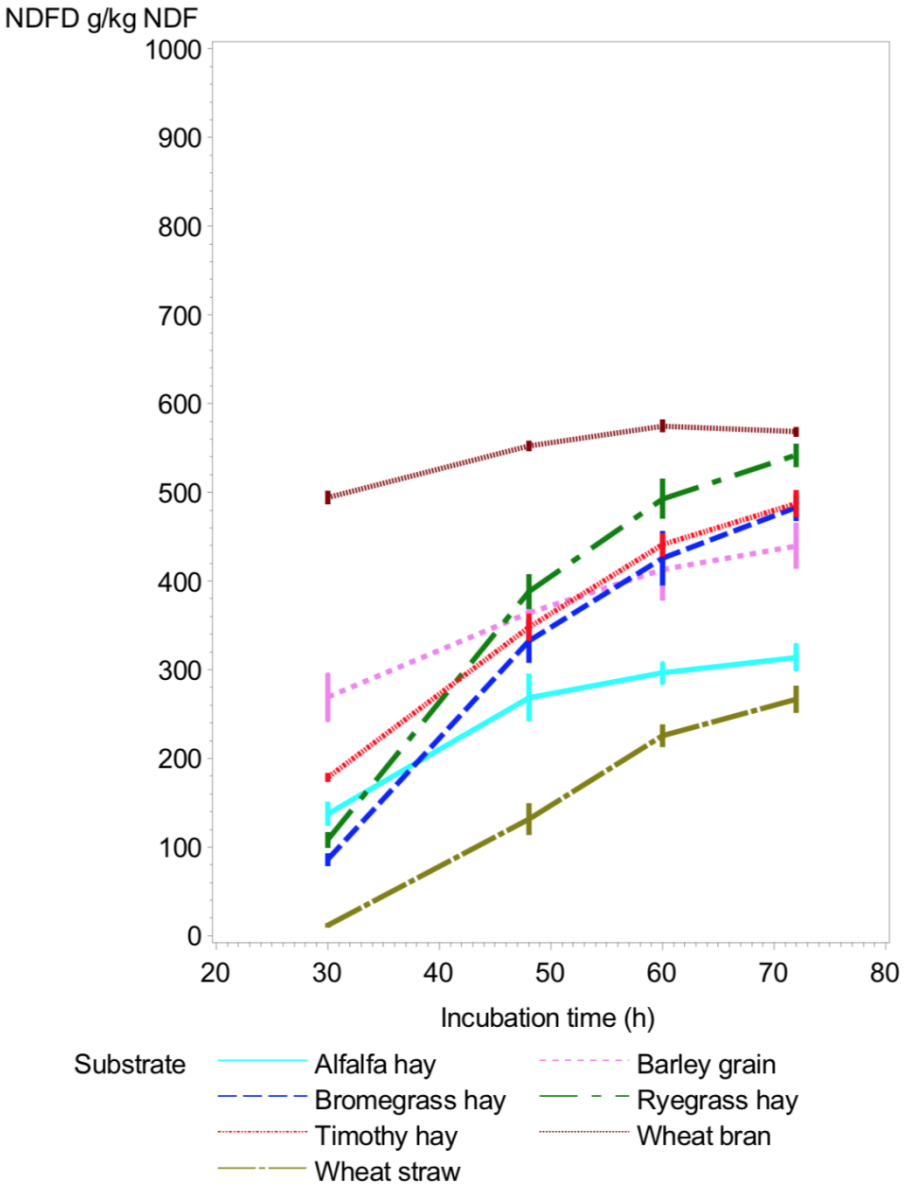
Figure 1.

(a)


DMD g/kg DM
1000
900
800
700
600
500
400
300
200
100
0
20
30
40
50
60
70
80
Incubation time (h)
Substrate
Alfalfa hay
Barley grain
Bromegrass hay
Ryegrass hay
Timothy hay
Wheat bran
Wheat straw

11 **(b)**


NDFD g/kg NDF
1000
900
800
700
600
500
400
300
200
100
0
20
30
40
50
60
70
80
Incubation time (h)
Substrate
Alfalfa hay
Barley grain
Bromegrass hay
Ryegrass hay
Timothy hay
Wheat bran
Wheat straw

12

13

Figure 2.

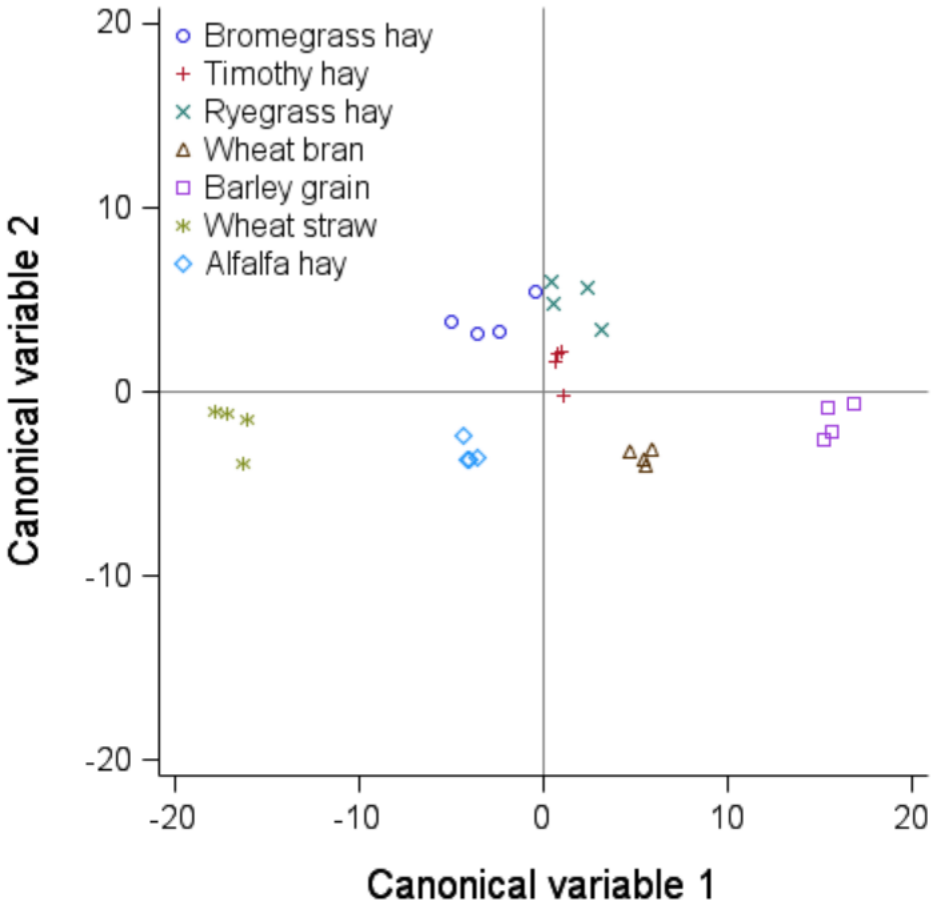

Bromegrass hay
Timothy hay
Ryegrass hay
Wheat bran
Barley grain
Wheat straw
Alfalfa hay
Canonical variable 2
Canonical variable 1
20
10
0
-10
-20
-20
-10
0
10
20

Figure captions

Figure 1. Trends for (a) dry matter digestibility (DMD, g/kg DM) and (b) neutral detergent fiber digestibility (NDFD, g/kg NDF) over incubation time for different feedstuffs. Error bars represent standard deviation.

Figure 2. Canonical Discriminant Analysis of regression parameters of *in vitro* true dry matter digestibility (IVTD) for different feedstuffs.

[Color should be used for all figures in print.]

Table 1. Chemical composition of feedstuffs (g/kg DM, unless otherwise stated).

	DM, g/kg	Ash	CP	EE	NDF	ADF	Lignin	NFC*
Alfalfa hay	911	91	156	10	616	480	100	127
Bromegrass hay	918	112	89	13	662	406	45	124
Ryegrass hay	870	90	156	32	588	332	25	135
Timothy hay	859	68	82	14	598	361	47	238
Wheat bran	899	59	166	48	675	145	39	51
Wheat straw	925	93	66	15	797	550	77	30
Barley grains	883	28	117	19	213	69	12	623

Abbreviations: DM, dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; NFC, non-fiber carbohydrates.

* Calculated as: NFC = 1000 – (ash + CP + EE + NDF).

Table 2. ***In vitro*** **apparent dry matter digestibility (DMD), true dry matter digestibility (IVTD) and neutral detergent fiber digestibility (NDFD) obtained using the Daisy^{II} Incubator and donkey feces as source of microbial** ***inoculum*** **(*n*=208). (a) Effect of incubation time on estimates of DMD, IVTD and NDFD. (b) Repeatability and reproducibility of the method.**

	DMD				IVTD				NDFD			
Incubation time (h)	30	48	60	72	30	48	60	72	30	48	60	72
(a)												
Mean*	330.9^{d}	432.6^{c}	497.1^{b}	525.2^{a}	510.7^{d}	587.4^{c}	639.6^{b}	658.3^{a}	193.2^{d}	338.4^{c}	412.5^{b}	443.1^{a}
(b)												
Variances												
Residual error	160.7	262.8	261.7	199.6	85.6	97.0	123.3	108.8	505.9	278.1	499.9	389.0
Runs	2185.5	6607.4	18963.6	3417.0	409.5	8495.9	7903.5	5562.9	968.8	22086.0	21907.9	14642.5
Run × Feedstuffs	529.4	1001.8	2642.9	776.0	292.6	859.0	1170.1	475.1	2220.3	1759.3	4389.6	2095.5
Precision parameters												
Repeatability (CV, %)	3.8	3.7	3.3	2.7	1.8	1.7	1.7	1.6	11.6	4.9	5.4	4.5
Reproducibility (CV, %)	6.5	7.4	10.3	5.0	2.8	5.4	5.3	3.9	18.5	14.7	14.7	10.4
Repeatability coefficient*	35.1	44.9	44.8	39.2	25.8	27.3	30.8	28.9	62.4	46.2	62.0	54.7
Reproducibility coefficient*	59.7	89.1	141.8	72.1	39.0	88.7	93.1	71.0	98.9	137.8	168.5	128.2

Abbreviations: CV, coefficient of variation.

For the effect of incubation time, different superscripts within row and digestibility parameter indicate significant differences (a, b, c, d: $P < 0.05$).

* Expressed as g/kg DM for DMD and IVTD; expressed as g/kg NDF for NDFD.

Conflict of interest statement

The Authors declare no conflict of interest.