

Manuscript Details

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Title	In vitro Digestibility Measurement of Feedstuffs in Donkeys Using the DaisyII Incubator
Article type	Research paper

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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UNIVERSITÀ DEGLI STUDI DI TORINO

DIPARTIMENTO DI SCIENZE VETERINARIE



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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.

Largo Braccini 2 – 10095 Grugliasco To

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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.

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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

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3 1 ***In vitro* Digestibility Measurement of Feedstuffs in Donkeys Using the Daisy^{II} Incubator**
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63 **21 Abstract**
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65 22 Successful studies on *in vitro* digestibility measurement of feedstuffs with fecal *inoculum* have been
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67 23 reported for horses, while data on donkeys are currently lacking. In this study we evaluated the use
68
69 24 of the Daisy^{II} Incubator (Ankom Technology, Fairport, NY, USA) for *in vitro* digestibility
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71 25 measurement of feedstuffs using donkey feces as source of microbial *inoculum*. The method was
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73 26 tested using 7 feedstuffs commonly used in donkey diets (alfalfa, bromegrass, ryegrass and timothy
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75 27 hays; wheat bran and wheat straw; barley grains). Feces were obtained from 4 female donkeys and
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77 28 incubations were carried out at one-week intervals for 4 consecutive weeks. Two bags of each
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79 29 feedstuff were incubated in digestion vessels containing a buffer/feces solution (90:10). *In vitro*
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81 30 apparent dry matter digestibility (DMD), true dry matter digestibility (IVTD), and neutral detergent
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83 31 fiber digestibility (NDFD) were evaluated at 4 incubation times: 30, 48, 60, and 72 h. All
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85 32 digestibility parameters significantly increased from 30 to 72 h of incubation. At 72 h of incubation,
86
87 33 the within-laboratory repeatability and reproducibility of the method were 2.7% and 5.0% for DMD
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89 34 and 1.6% and 3.9% for IVTD, respectively. The method was less repeatable and reproducible for
90
91 35 NDFD (4.5% and 10.4%, respectively).
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97 37 **Key words:** *Equus asinus* L.; fecal *inoculum*; Daisy^{II} Incubator; measurement error; incubation
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123 **47 1. Introduction**
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125 48 The use of *in vitro* techniques to study diet digestion and fermentative end products has been
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128 49 successfully applied in horse nutrition. Lowman et al. [1] was the first to demonstrate that equine
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130 50 feces can be used as source of microbial *inoculum* and that fecal microflora can remain viable for
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132 51 several hours after excretion. According to Abdouli et al. [2], a two-stage *in vitro* technique
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134 52 (combining enzymatic pre-digestion and fermentation) can estimate the organic matter digestibility
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136 53 of both forages and concentrates using horse feces as source of microbial *inoculum*. Ringler et al.
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138 54 [3] demonstrated that the use of equine fecal *inoculum* in a closed-system fermentation apparatus
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141 55 can yield valid *in vitro* estimates of dry matter (DM) and fiber digestibility. Lattimer et al. [4]
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143 56 reported that the Daisy^{II} Incubator could be used to predict valid estimates of DM digestibility of
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145 57 diets using equine feces as *in vitro inoculum* source. Earing et al. [5] confirmed that feces are a
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147 58 suitable source of microbial *inoculum* for *in vitro* digestibility studies in horses. More recently,
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149 59 Murray et al. [6] compared samples from different sites of the equine intestine, showing that feces
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152 60 are a suitable alternative to cecal fluid, thus highlighting the potential of this kind of *inoculum* for *in*
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154 61 *vitro* digestibility studies.

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156 62 Very few studies are currently available on digestibility measurement of feedstuffs in donkeys,
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158 63 most of the data resulting from *in vivo* feeding trials [7-9]. *In vitro* digestibility in donkeys was
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160 64 studied using the neutral cellulase plus gamanase technique developed by Ankom Technology
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162 65 (Fairport, NY, USA) and via fecal-Near Infrared Spectroscopy [9-11], but with poor success.
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165 66 Since the breeding of lactating donkeys in Western Europe is increasing [12], it becomes needful to
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167 67 evaluate tools to study the *in vitro* digestibility of feedstuffs used in donkey nutrition. Therefore, the
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169 68 objectives of this study were (i) to analyse, at different incubation times, the *in vitro* digestibility
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171 69 measurement of feedstuffs in donkeys using the Daisy^{II} Incubator and donkey feces as source of
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173 70 microbial *inoculum*, and (ii) to test the within-laboratory repeatability and reproducibility of the
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175 71 method.
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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.

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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 CO₂ 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].

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123 At the end of the assigned time, the bags were removed from each vessel, rinsed thoroughly with
124 cold tap water and placed in a 50 °C forced-air oven to dry for 24 h. The bags were weighed and
125 then analysed for their NDF content with the Ankom²⁰⁰ Fiber Analyzer (Ankom Technology).

126 The obtained data were used to calculate:

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

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

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

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

134 *2.4. Statistical analysis*

135 Data were analysed using SAS [18].

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

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

144 $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$,

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

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

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148 $SD_repeatability (S_r) = \sqrt{\sigma_e^2}$

149 $SD_reproducibility (S_R) = \sqrt{\left\{ \left(\frac{\sigma_\alpha^2 - \sigma_{\alpha\beta}^2}{7 \times 2} \right) + \left(\frac{\sigma_{\alpha\beta}^2 - \sigma_e^2}{2} \right) + \sigma_e^2 \right\}}$,

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

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

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

157 A linear regression analysis for incubation time was used to obtain, for each feedstuff, the
158 regression parameters (intercept and slope) of each *in vitro* digestion (4 runs \times 7 feedstuffs).

159 Regression parameters were analysed by Canonical Discriminant Analysis (CDA), which finds
160 linear combinations (canonical functions) of the quantitative variables that provide maximal
161 separation between classes or groups [17].

162 Significance was set at $P < 0.05$.

163 3. Results

166 3.1. Effect of incubation time on estimates of DMD, IVTD, and NDFD

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

169 The mixed model repeated measure analysis showed that DMD, IVTD, and NDFD were not
170 influenced by run ($P > 0.05$). Incubation time significantly affected DMD, IVTD and NDFD (Table
171 2a). All considered digestibility parameters ranked according to the following order: 72 h $>$ 60 h $>$
172 48 h $>$ 30 h. For each feedstuff, DMD and NDFD trends over incubation time are shown in Fig. 1.

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422
423 173 DMD (Fig. 1a) and NDFD (Fig. 1b) of hays and straw were lower than that of barley grains. Straw
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425 174 showed the absolute lowest DMD values (Fig. 1a). Wheat bran and ryegrass hay showed the highest
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427 NDFD values at 72 h, while alfalfa hay and wheat straw had the lowest; bromegrass hay, timothy
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429 hay, and barley grains showed intermediate values at this incubation time (Fig. 1b).
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431 432 177 433 434 178 *3.2. Repeatability and reproducibility of the method*

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436 179 For each incubation time, the repeatability and reproducibility of the method are reported in Table
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438 180 2b. Repeatability and reproducibility, expressed as coefficients of variation, ranged 2.7 - 3.8% and
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440 181 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
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442 182 NDFD, respectively. Particularly for NDFD, both repeatability and reproducibility improved from
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444 183 30 to 72 h of incubation.

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447 184 The results of the multivariate statistical analysis are shown in Fig. 2. The regression parameters
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449 185 (intercept and slope) describe each *in vitro* digestion (4 runs × 7 feedstuffs) and Fig. 2, for IVTD as
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451 186 an example, shows the separation among the different feedstuffs by CDA.

452 453 187 454 455 188 **4. Discussion**

456 457 189 458 459 190 *4.1. Effect of incubation time on estimates of DMD, IVTD, and NDFD*

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461 191 As expected, estimates of DMD, IVTD and NDFD increased while increasing the incubation time
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463 192 (Table 2a). The obtained results indicate that an incubation period of 72 h or higher may be required
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465 193 for accurate DMD, IVTD, and NDFD estimates in donkeys. Similar findings were recently obtained
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467 194 by Franzan et al. [21], studying DM, organic matter, and NDF degradation using horse feces as
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469 *inoculum* in an *in vitro* fermentation assay. Moreover, comparing *in vivo* digestibility estimates in
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471 horses with those obtained *in vitro* using the Daisy^{II} Incubator at 30, 48, and 72 h of incubation,
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473 Earing et al. [5] showed that, for high-fiber diets (44 - 51% NDF), a 72 h incubation time provided
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475 estimates closer to *in vivo* data. However, the use of a set incubation time for all kind of feedstuffs
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483 199 may be questionable, because more fibrous feedstuffs may need longer incubation times to estimate
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485 200 accurately DMD and NDFD [4,5]. In the current trial, the effect of incubation time was not
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487 201 evaluated for each considered feedstuff, because only one sample (in duplicate) per feedstuff was
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490 202 incubated. Further targeted trials are needed to assess the optimum incubation time for different
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492 203 feedstuffs using the Daisy^{II} Incubator for the digestibility evaluation of feedstuffs in donkeys.
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494 204 As we did not use multiple samples per each feedstuff, at each incubation time differences among
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496 205 feedstuffs were not statistically evaluated. Despite lack of targeted statistical evaluation, the single
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498 206 feedstuffs showed expected trends according to their chemical composition (Fig. 1). High-fiber
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500 207 feedstuffs showed in fact lower DMD (Fig. 1a) and NDFD (Fig. 1b) values when compared to high-
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502 208 starch samples. The lowest NDFD values obtained for alfalfa hay and wheat straw at 72 h of
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504 209 incubation can be the consequence of their high lignin content (Fig. 1b). It is known that the type of
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506 210 feed, stage of maturity and processing can affect the digestive process and the gastrointestinal
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508 211 retention time in equids [22-23]. In forages, the cell wall components are the main factors
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510 212 influencing digestibility. Cell wall components increase with plant maturity [24] and lignification in
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512 213 particular imposes a barrier to complete cell wall polysaccharide digestion [25-26].
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514 214 The CDA applied to our data (Fig. 2) allowed a good separation of the feedstuffs; this is also a way
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516 215 to synthesize the method's action according to the chemical composition of the feedstuffs.
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521 217 *4.2. Repeatability and reproducibility of the method*

523 218 Concerning the repeatability and reproducibility of the method, it has to be pointed out that values
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525 219 can vary among laboratories [27]. Despite the large use of the Daisy^{II} Incubator to measure
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527 220 digestibility (especially in ruminants), very few studies aimed at verifying the associated
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529 221 measurement errors [20]. To the best of our knowledge, no published literature is available on the
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531 222 repeatability and reproducibility of DMD and IVTD obtained with the Daisy^{II} Incubator in
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533 223 ruminants or equids. The results obtained in our study are in accordance with those commonly
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535 224 found by Ankom Technology using the Daisy^{II} Incubator [Brian Layton, Ankom Technology;
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543 225 personal communication]. Regarding fiber digestibility, in ruminants Spanghero et al. [20] showed
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545 226 that the repeatability and reproducibility of NDFD and digestible NDF using the Daisy^{II} Incubator
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548 227 improve **when** extending the fermentation time from 30 to 48 h. Our data also show a general
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550 228 improvement of the repeatability and reproducibility of the method, particularly for NDFD, as a
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552 229 consequence of longer incubations (Table 2b). At the shorter incubation times (30, 48, and 60 h),
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554 230 low repeatability and reproducibility for NDFD may be the consequence of insufficient degradation
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556 231 of the fiber fraction of the diet, which instead improved at 72 h of incubation, also allowing a better
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558 232 precision degree of the method. At 72 h of incubation, the method tested in our study was less
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560 233 repeatable and reproducible for NDFD than for DMD and IVTD (Table 2b). The repeatability and
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562 234 reproducibility found by Spanghero et al. [20] for NDFD at 48 h of incubation were equal to 6.83%
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564 235 and 10.46%; such values are quite similar to those found for NDFD at 72 h of incubation in the
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566 236 current study (4.5% and 10.4%, respectively). **The estimated repeatability and reproducibility**
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568 237 **coefficients (Table 2b) are precision measures providing additional information, as they represent**
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570 238 **the maximum absolute difference that can be expected between repeated measurements on 95% of**
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572 239 **occasions [17].**

573 240 574 575 241 **5. Conclusion**

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579 242 We evaluated the use of the Daisy^{II} Incubator (Ankom Technology) for determining DMD, IVTD,
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581 243 and NDFD of feedstuffs using donkey feces as source of microbial *inoculum*. **From the obtained**
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583 244 **results, it can be concluded that an incubation period of 72 h is required to obtain accurate DMD,**
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585 245 **IVTD, and NDFD estimates in donkeys and to improve the repeatability and reproducibility of the**
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587 246 **method. For NDFD, the repeatability and reproducibility of the method were comparable to those**
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589 247 **obtained with the Daisy^{II} Incubator in ruminants.** Overall, the trial shows that the method described
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591 248 by different authors [3-5] for *in vitro* digestibility studies in horses using the Daisy^{II} Incubator could
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593 249 be successfully extended to donkeys.
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603 250 Further studies should evaluate the among-laboratory repeatability and reproducibility of the
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605 251 method. In addition, trials aimed at comparing results from *in vitro* and *in vivo* digestibility are
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607 252 needed to assess the most suitable incubation time of different feedstuffs for donkeys, and to
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610 253 propose equations that fit the shortest *in vitro* incubation time with *in vivo* data.
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612 254

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630 631 263 **References**

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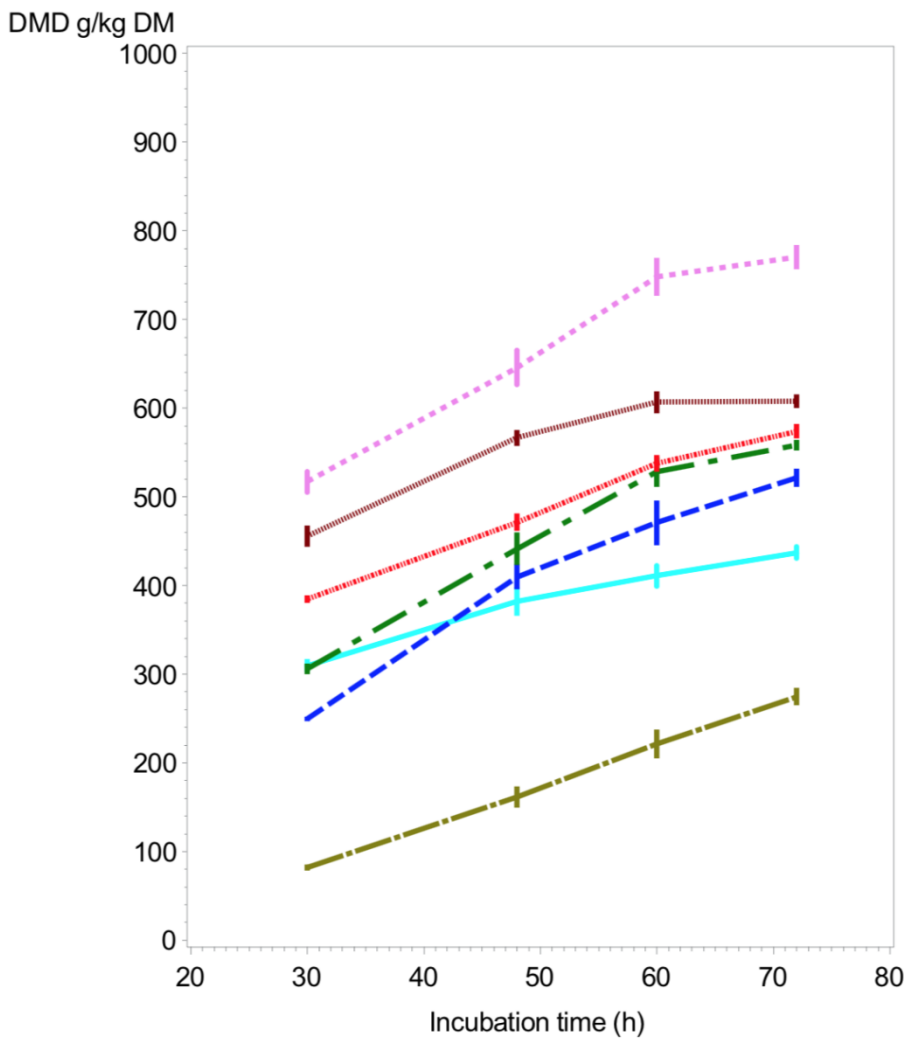
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1 **Figure 1.**

2 **(a)**



Substrate

- Alfalfa hay
- Bromegrass hay
- Timothy hay
- Wheat straw
- Barley grain
- Ryegrass hay
- Wheat bran

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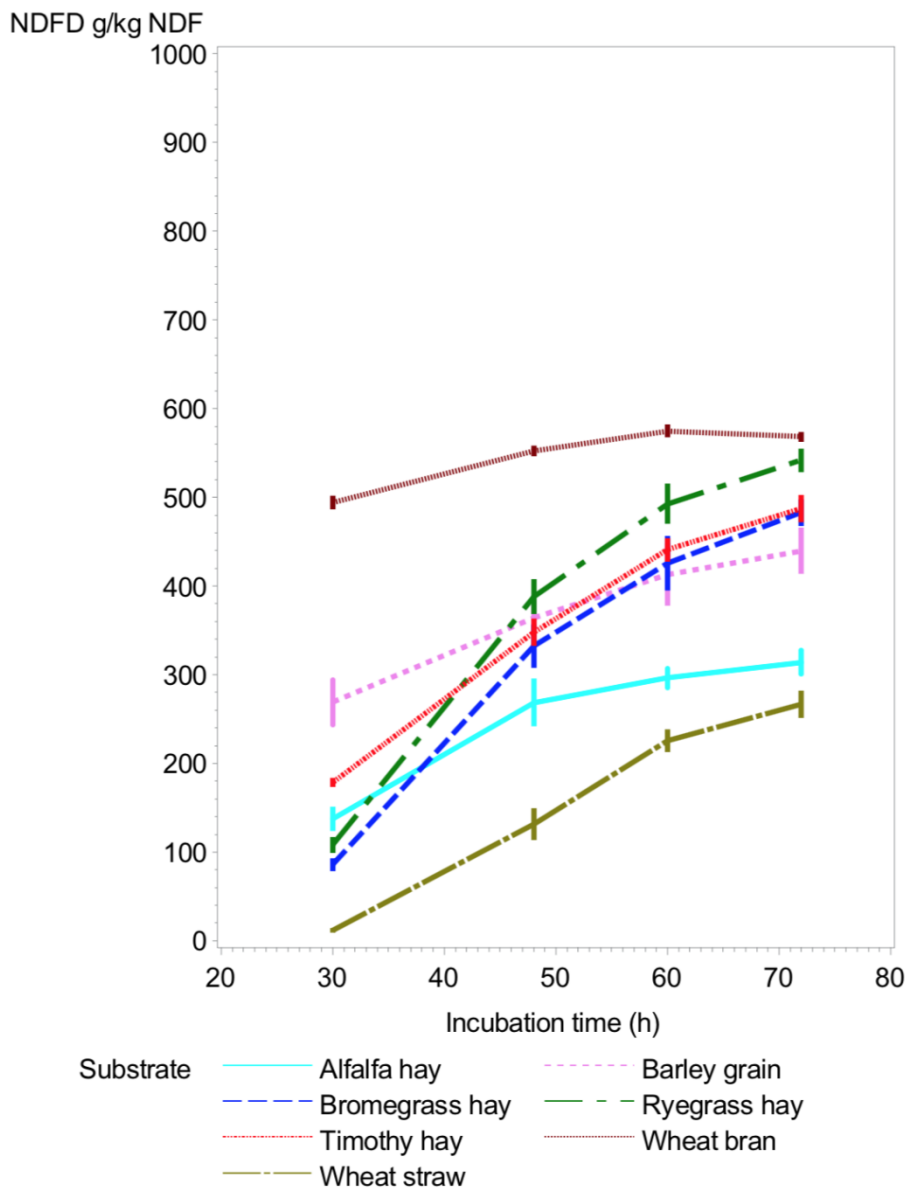
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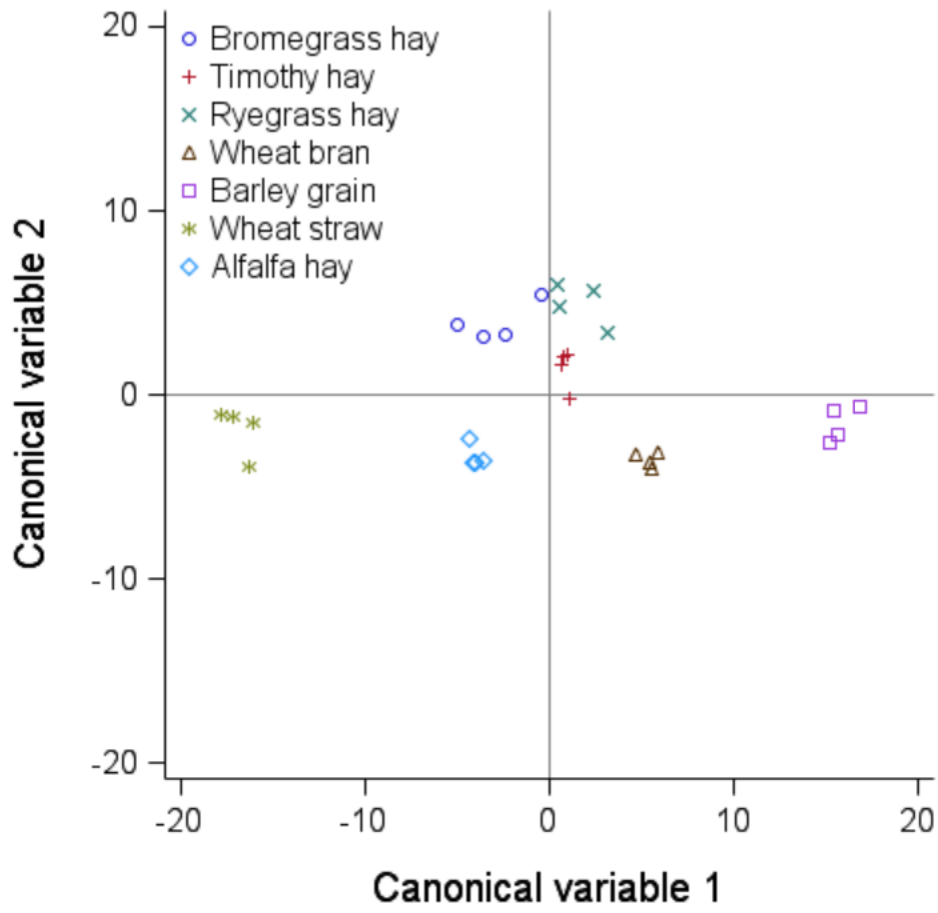


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1 **Figure 2.**

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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.]

1 **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

2 Abbreviations: DM, dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber;

3 ADF, acid detergent fiber; NFC, non-fiber carbohydrates.

4 * Calculated as: $NFC = 1000 - (\text{ash} + CP + EE + NDF)$.

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

Incubation time (h)	DMD				IVTD				NDFD			
	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

4 Abbreviations: CV, coefficient of variation.

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

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

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Conflict of interest statement

The Authors declare no conflict of interest.