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# *In situ* techniques to predict *in vivo* digestibility and to evaluate the impact of flint maize processing methods on degradation parameters

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## Abstract

This study aimed to (1) evaluate the effects of flint maize processing methods on the estimation of the readily soluble fraction (*a*), the potentially degradable fraction (*b*) and the rate of degradation of *b* (*c*) for dry matter (DM), organic matter (OM) and starch in the rumen; and (2) verify whether two different applications of *in situ* technique can be used to estimate *in vivo* DM, OM and starch digestibilities. Five ruminally cannulated Nellore bulls (265 ± 18.2 kg; 8 ± 1.0 mo) were distributed in a 5 × 5 Latin square. Three experimental diets were composed of 0.30 whole-plant maize silage, 0.10 supplement and 0.60 of one of the following processing methods: dry ground maize grain (DMG); high-moisture maize (HMM); reconstituted maize grain silage (RMG). Two additional diets were composed of 0.10 supplement, 0.80 snaplage and 0.10 stalklage (SNAP-80); or 0.10 supplement and 0.90 snaplage (SNAP-90). Digestibilities were estimated using *in vivo* procedure or predicted from *in situ* technique using a single 24 h incubation point or an equation proposed in previous literature. Diets based on ensiled grains presented greater ($P < 0.05$) fraction *a* and *c* and lower ($P < 0.05$) fraction *b* of DM, OM and starch compared to DMG. Both alternative use of *in situ* technique accurately estimated ($P > 0.05$) *in vivo* DM, OM and starch digestibilities. The results suggest that ensilage process may increase the availability of nutrients. The two different applications of *in situ* technique showed precision and accuracy to estimate *in vivo* digestibility.

## Introduction

Flint maize is the most common cereal grain used to feed cattle, whereas fine grinding is the main grain processing method adopted by nutritionists in Brazilian feedlots (Oliveira and Millen, 2014; Pinto and Millen, 2018). However, the inclusion of ensiled grains in feedlot diets, such as high moisture maize (HMM), reconstituted maize grain silage (RMG) and snaplage (SNAP; grain, cob, husk and shank) has increased from 0 to 36% (Millen *et al*., 2009; Bernardes and Castro, 2019). The process of ensiling cereal grain can cause a breakdown of the starch-protein matrix (Hoffman *et al*., 2011; Junges *et al*., 2017; Silva *et al*., 2020), reduce the insoluble nitrogen (N) fraction (Valadares *et al*., 2018) and increase starch availability (Hoffman *et al*., 2012; Silva *et al*., 2020). Nevertheless, there is a lack of information regarding differences in availability and digestibility of nutrients between diets based on HMM, RMG and SNAP.

The *in vivo* procedure is the standard method to evaluate the availability of nutrients (Nocek, 1988). Products of digestion and energetic efficiency may change depending on the site of digestion. The products may be volatile fatty acids for starch fermented in the rumen or glucose for starch digested in the small intestine (Harmon *et al*., 2004; Owens *et al*., 2016). Regarding the efficiencies of energy use, it can be 80, 97 and 62% for starch digested in the rumen, small intestine and large intestine, respectively (Huntington *et al*., 2006). Therefore, according to Owens *et al*. (2016), diet formulation should consider the site and extent of digestion but even more the effects of grain processing methods on total tract digestibility of starch.

*In vivo* procedure is a costly, laborious and lengthy process, which requires a large number of animals to ensure repeatability (Benedeti *et al*., 2019). Also, ethics committees have recommended stricter protocols for animal use in experiments (Silva *et al*., 2020). Recent studies (Benedeti *et al*., 2019; Silva *et al*., 2020) have suggested the use of *in situ* technique to estimate the *in vivo* digestibility of diets. A multi-study analysis of beef cattle diets was performed by



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Benedeti *et al.* (2019) to develop an equation that predicts *in vivo* ruminal organic matter (OM) digestibility from *in situ* methods. Also, Silva *et al.* (2020) performed simultaneously *in situ* incubations and *in vivo* digestibility studies to evaluate the appropriate *in situ* incubation time that best estimates *in vivo* digestibility of dry matter (DM), OM and starch. According to these authors, more precise and accurate estimates of *in situ* digestibility might be obtained by evaluating complete diets instead of individual ingredients. However, in both studies, the authors suggested that diets based on different feedstuffs, roughage:concentrate ratio and processing methods should be evaluated.

*In situ* technique is quick and requires the use of fewer animals since it is possible to incubate several bags with different diets or ingredients in the same animal (Benedeti *et al.*, 2019). Thus, it may help to reduce costs and labour. Also, measurements of the digestion rate of feed fractions using the *in situ* technique can provide relevant information regarding relative differences among feeds (Allen, 2015). According to Silva *et al.* (2020), the processing methods may alter the degradation parameters in the rumen, such as the readily soluble fraction (*a*), the potentially degradable fraction (*b*) and the rate of degradation of *b* (*c*). Therefore, the *in situ* technique has the potential to optimize diet formulation, which could reduce the environmental impact of beef cattle production and increases producers' economic return.

We hypothesized that (1) diets based on HMM, RMG and SNAP will reduce fraction *b* and increase fraction *a* and the *c* of DM, OM and starch; and (2) the different applications of *in situ* technique can accurately and precisely estimate the *in vivo* digestibilities of DM, OM and starch. Thus, we aimed to (1) evaluate the effects of flint maize processing methods on fraction *a*, *b* and on the *c* of DM, OM and starch; and (2) verify whether two different applications of *in situ* technique previously reported in the literature can be used to estimate DM, OM and starch *in vivo* digestibilities of diets based on flint maize with different processing methods.

## Material and methods

### Maize processing methods

The maize fields were located at Animal Science Department of Universidade Federal de Viçosa (20°46′ 00″ S and 42°51′28″ W, 649 m above of sea level) in Viçosa, Minas Gerais, Brazil. From October to December 2017, the growing season of maize was characterized as spring with minimum temperatures between 13.8 and 21.5°C, maximum temperatures varying from 22.6 to 34.6°C and precipitations between 0.2 and 87.2 mm.

The maize hybrid, LG 6030 PRO 2 from LG Sementes (Curitiba, SP, Brazil) was planted in sufficient quantity to produce the dry ground maize grain (DMG), RMG, HMM, SNAP, stalklage and whole-plant maize silage for the entire experiment. A dose of 300 kg/ha of N-P-K fertilizer (the fertilizer composed of 8% N, 28% phosphorus and 16% potassium) was applied during the sowing. Maize seeds were planted with a row spacing of 90 cm and on-row plant spacing of approximately 18 cm. Sowing was performed with four-row pneumatic sowing machine. A dose of 100 kg/ha of N (approximately 222 kg/ha of urea) was applied after sowing in two equal aliquots: the first one was provided when the plants had four true leaves and the second aliquot when the plants had eight true leaves.

The DMG and RMG were harvested at the moisture content of 19% and was dried until it reached 13% of moisture. Approximately 6000 kg of dry maize grain was ground in a hammer mill (DMP-2, Nogueiras, São João da Boa Vista, São Paulo, Brazil) using a 3-mm sieve and the DM content was measured (method 934.01; AOAC, 2012). Subsequently, 3000 kg of the ground grain was reconstituted with water to reach a moisture content of 35%, using the following equation below:

$$\text{Amount of water (L/kg of grain as fed)} = \frac{\text{grain DM (\%)} - \text{target DM (\%)}}{\text{target DM (\%)}}$$

The grain was soaked, mixed in a cement mixer (Rental Mixer 400L, Menegotti Indústrias Metalúrgicas Ltda., Santa Catarina, Brazil) for 5 min and ensiled with a mean density of 1000 kg of fresh material/$m^3$ to obtain RMG. The remaining 3000 kg of DMG was stored dry in a grain bin.

The HMM and SNAP were harvested when the maize grain contained approximately 40% moisture. Immediately after the harvest, approximately 3000 kg of maize grain was ground in a hammer mill (DMP-2, Nogueiras, São João da Boa Vista, São Paulo, Brazil). Grains were broken into four to six pieces to achieve less than 5% whole kernels and 20% of fines (Hicks and Lake, 2006; Lardy and Anderson, 2016*b*; Salvo *et al.*, 2020) and ensiled with a mean density of 1000 kg of fresh feed/$m^3$. Moreover, about 6000 kg of whole ear maize (containing grain, cobs and husks) were chopped in a hammer and knife mill (DPM-JÚNIOR, Nogueiras, São João da Boa Vista, São Paulo, Brazil) and ensiled with a mean density of 600 kg of fresh feed/$m^3$ to obtain the SNAP. The grinder was set to break grains and cobs into small pieces and reduce the husk particle size (Mahanna, 2008; Akins and Shaver, 2014; Lardy and Anderson, 2016*a*).

Stalklage was produced with the remaining residue after harvest of SNAP. The material for stalklage and whole-plant maize silage production was harvested with approximately 45 and 30% of DM, respectively. A forage harvester (JF 1600 AT, JF Máquinas Agrícolas, São Paulo, Brazil) set at 22.3 mm theoretical cut length was used (Kononoff *et al.*, 2003; Cook *et al.*, 2016). Stalklage and whole-plant maize silage were ensiled with a mean density of 400 kg of fresh feed/$m^3$ and 500 kg of fresh feed/$m^3$, respectively.

Penn State Particle Separator (19-, 8-, 4-, and 1.18-mm sieves plus bottom pan; Heinrichs, 2013) was used to verify the particle size distribution of all maize corn processing methods. Thus, the grinder or forage harvester was adjusted when needed. The RMG, HMM, SNAP, stalklage and whole-plant maize silage were ensiled in round reinforced concrete pipe silos (1.0 m inside diameter × 1.0 m long × 8.0 cm wall thickness) at the same time, approximately 90 days before the beginning of the experiment.

### Animals, facilities and experimental design

Five ruminally cannulated Nellore bulls (age = 8 ± 1.0 mo; initial BW = 265 ± 18.2 kg) were distributed in a 5 × 5 Latin square design. Initially, the animals were individually identified with ear tags, treated for the elimination of internal and external parasites and, housed in a concrete floor tie-stall barn that was equipped with water and feed troughs. The experimental periods were divided into two subperiods of 12 days (Richards *et al.*, 2002; Machado *et al.*, 2016; Petzel *et al.*, 2019) for dietary adaptation and 5 days for *in vivo* digestibility and *in situ* degradability technique.

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### Experimental diets

Five experimental diets were used in the experiment where three of them were composed of 0.30 whole-plant maize silage, 0.10 mineral and protein supplement, and 0.60 (DM basis) of one of the following processing methods: DMG; high-moisture maize (HMM); RMG. The other two diets were composed of 0.10 mineral and protein supplement, 0.80 SNAP and 0.10 stalklage (DM basis; SNAP-80); or 0.10 mineral and protein supplement and 0.90 SNAP (DM basis; SNAP-90). The proportions of SNAP used in this experiment were approximately 0.78 grain, 0.13 cob and 0.09 husk.

The diets were formulated according to BR-CORTE recommendations (Valadares Filho *et al.*, 2016) to provide, approximately, 125 g CP/kg on a diet DM basis and to support an average daily gain of 1.2 kg/day. Ingredient and chemical composition of the experimental diets are presented in Table 1.

### Evaluation of in vivo digestibility

The diet ingredients were weighed separately, then mixed at the time of feeding, such that a total mixed ration was provided twice per day (08.00 h and 16.00 h). Feed bunks were evaluated each day to quantify refusals and to adjust daily feed allowance to a maximum of 5% of refusals. Feed intake was recorded during the data collection period (from day 13 to 17). Also, diet ingredients and refusals were sampled daily during the collection period and stored at −20°C.

Each diet ingredient and refusals samples were partially dried in a forced-air oven at 55°C for 72 h, ground in a knife mill (Tecnal, Piracicaba, São Paulo, Brazil) using a 1-mm sieve and packed in plastic bags for further laboratory analyses. Each diet ingredient sample was grouped for each period and refusals samples were grouped per animal for each period. These ingredients were analysed individually and used to calculate dietary composition.

From day 15 to 17 of the experimental period, 24 h faecal output was determined for all bulls. Faeces were collected from the concrete floor and placed in 30 l buckets. At the end of each collection day (24 h), the buckets containing the samples were weighed, homogenized and a subsample was collected, dried in a forced-air oven at 55°C for 72 h and ground in a knife mill (Tecnal, Piracicaba, São Paulo, Brazil) with a 1-mm sieve. Furthermore, faecal samples from the 3 day of the collection were combined proportionately for each animal per experimental period according to the dry weight of each collection day and stored in plastic bags for further laboratory analyses.

### Evaluation of in situ degradation

From day 13 to 17 of each experimental period, the *in situ* degradabilities of DMG, HMM, RMG, SNAP-80 and SNAP-90 diets were evaluated. All dietary ingredients were sampled from day 1 to 7 of each experimental period and partially dried in a forced-air oven at 55°C for 72 h. A composite sample from 7 day of the collection was made for each ingredient per period and ground in a knife mill (Tecnal, Piracicaba, São Paulo, Brazil) with a 2-mm sieve.

Approximately 5 g of dried total mixed ration sample was individually weighed into nylon bags (Sefar Nitex; Sefar, Thal, Switzerland; porosity of 50 µm, $8 \times 15$ cm$^2$) and incubated in each animal (Benedeti *et al.*, 2019; Menezes *et al.*, 2019; Silva *et al.*, 2020). To compose the total mixed ration, whole-plant maize silage, DMG, HMM, RMG, SNAP, stalklage, and mineral and protein supplement were weighed separately, maintaining diets composition (DM basis). The incubation for each diet was carried out in the same animal that was receiving the corresponding treatment in the *in vivo* procedure. Incubation was performed to allow the following ruminal degradation times: 0, 2, 4, 6, 12, 24, 48, 72 and 96 h. The number of bags varied as a function of the incubation time to guarantee enough residual samples after incubation (i.e. more bags per sample were incubated for the longer incubation times relative to the shorter incubation times).

The step by step of rumen *in situ* incubation procedure is presented in Supplementary Material. The bags containing previously weighed diet samples were attached to a steel chain ($90 \times 2$ cm$^2$; Menezes *et al.*, 2019) with a weight (300 grams) at the end to allow for complete immersion within ruminal contents. The steel chain was placed into the rumen and attached to the cannula cap using a plastic rope (15 cm) fixed to a PVC hose pipe (3 cm). The cannula was closed with the PVC hose pipe outside. The bags were placed into the rumen in reverse order so that all bags were removed at the same time. After the incubation period, the bags were washed in running water until the rinse water was clear (Silva *et al.*, 2020). The 0 h bags were not incubated in the rumen but were rinsed using the same procedure as incubated bags. The nylon bags with samples were oven-dried at 55°C for 72 h. In sequence, bags were placed in an oven at 105°C for 2 h and weighed. The residues of each diet were removed from the nylon bags, ground in a knife mill (Tecnal, Piracicaba, São Paulo, Brazil) with a 1-mm sieve, stored in a labelled plastic bag for further chemical composition.

### Chemical analyses

Diet ingredients samples were analysed for DM, OM, N and ether extract (EE), according to AOAC (2012) method numbers 934.01, 930.05 and 981.10 and AOAC (2006) method number 945.16, respectively. The neutral detergent fibre (NDF) analysis was performed according to techniques described by Mertens (2002), without the addition of sodium sulfite, but with the addition of thermostable alpha-amylase to the neutral detergent. The NDF content was corrected for residual ash and protein (apNDF). Estimations of neutral detergent insoluble nitrogen (NDIN) followed the technique described by Licitra *et al.* (1996). The starch analysis was performed following the recommendations of Silva *et al.* (2019). Refusals, faeces and *in situ* residues were analysed for DM, OM and starch contents, according to previously described methods.

### Degradation models and partition of starch digestion

For the *in situ* evaluation, the DM, OM and starch degradation profiles were estimated using the Ørskov and Mcdonald (1979) asymptotic function:

$$Y_t = a + b \times (1 - e^{(-ct)})$$

where $Y_t$ = degraded fraction of DM, OM or starch at time '$t$', g/kg; $a$ = readily soluble fraction, g/kg; $b$ = potentially degradable fraction in the rumen, g/kg; $c$ = rate constant for degradation of $b$, per hour; $t$ = time, hour.

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**Table 1.** Feedstuffs and chemical composition of experimental diets

| Item | Diets | | | | |
|---|---|---|---|---|---|
| | DMG | HMM | RMG | SNAP-80 | SNAP-90 |
| Feed, g/kg of dry matter | | | | | |
| Whole-plant maize silage | 300 | 300 | 300 | – | – |
| Dry ground maize grain | 600 | – | – | – | – |
| High-moisture maize | – | 600 | – | – | – |
| Reconstituted maize grain silage | – | – | 600 | – | – |
| Snaplage | – | – | – | 800 | 900 |
| Stalklage | – | – | – | 100 | – |
| Mineral and protein supplement | | | | | |
| Soybean meal | 60.5 | 60.5 | 60.5 | 59.4 | 60.5 |
| Mineral premix[a] | 30.7 | 30.7 | 30.7 | 30.7 | 30.7 |
| Urea + AS[b] | 8.80 | 8.80 | 8.80 | 9.90 | 8.80 |
| Dry matter, g/kg as fed | 559 | 459 | 479 | 440 | 496 |
| ME (MJ/kg)[c] | 12.2 | 13.4 | 13.1 | 12.1 | 12.7 |
| Chemical composition g/kg of dry matter | | | | | |
| Organic matter | 940 | 939 | 938 | 942 | 952 |
| Crude protein | 125 | 125 | 125 | 126 | 127 |
| Ether extract | 33.2 | 33.1 | 33.0 | 28.1 | 30.2 |
| Neutral detergent fibre[d] | 213 | 202 | 204 | 283 | 234 |
| Starch | 527 | 503 | 494 | 481 | 538 |

[a]Premix guarantees (per kg of dry matter): 200–220 g of Ca, 10 mg of Co (Min), 500 mg of Cu (Min), 22 g of S (Min), 333 mg of Fe (Min), 178.41 mg of F (Max), 10 g of P (Min), 25 mg of I (Min), 17 g of Mg (Min), 1500 mg of Mn (Min), 1100 mg of monensin, 100 × 109 CFU of Saccharomyces cerevisiae (Min), 6.6 mg of Se (Min), 50 g of Na (Min), 100 000 IU of vitamin A (Min), 13 000 IU of vitamin D3 (Min), 150 IU of vitamin E (Min) and 2000 mg of Zn (Min).
[b]Urea + ammonium sulfate in a 9 : 1 ratio.
[c]Total digestible nutrients (TDN) was estimated using concentrations of crude protein, ether extract, ash, neutral detergent fibre, lignin, acid and neutral insoluble crude protein as described by BR-CORTE (Valadares Filho *et al*., 2016) and then metabolizable energy (ME) was calculated using the following the equations: DE = TDN × 4.4; and ME = 0.9455 × ED – 0.3032 multiplied by a conversion factor of 4.184 to convert Mcal/kg to MJ/kg.
[d]Corrected for residual ash and nitrogen compounds.

The starch effective degradability (ED) for each diet was calculated according to the equation of Ørskov and McDonald (1979) and corrected for 6% of starch washing out of the bag that would escape rumen degradation (Offner and Sauvant, 2004):

$$\mathrm{ED}_{\mathrm{measured}} = (a + (b \times c)/(c + kp))$$
$$\mathrm{ED}_{\mathrm{corr}} = \mathrm{ED}_{\mathrm{measured}} - (0.06 \times a)$$

where ED = starch effective degradability, g/kg; $a$ = readily soluble fraction, g/kg; $b$ = potentially degradable fraction in the rumen, g/kg; $c$ = rate constant for degradation of $b$, per hour; kp = passage rate of starch. The kp for each diet was obtained by *in vivo* ruminal emptying procedure and it can be found in Godoi *et al.* (2021).

The ruminal digestibility of starch (Rd) was predicted according to the equation proposed by Offner and Sauvant (2004):

$$\mathrm{Rd} = 0.302 + 0.59\mathrm{ED}$$

The intestinal digestibility of starch (Id) was obtained by the difference between the total digestibility observed from *in vivo* procedure and the prediction of ruminal digestibility as described above.

$$\mathrm{Id} = \mathrm{Total\ tract} - \mathrm{Rd}$$

### *Use of* in situ *techniques to predict* in vivo *digestibility*

Silva *et al.* (2020) performed simultaneous *in situ* and *in vivo* trials and suggested that the optimal *in situ* incubation time required to accurately estimate *in vivo* digestibility of DM, OM and starch was 24 h. Moreover, a multi-study analysis was performed by Benedeti *et al.* (2019) to develop and validate an equation to estimate *in vivo* OM digestibility from *in situ* methods. According to these authors, stepwise regression results showed that $c$ contributed significantly to predictions of *in vivo* digestibility. Thus, the equation below was suggested to estimate the *in vivo* digestibility coefficient of OM using the *in situ* technique:

$$Y = 0.5695204 + 2.8597612 \times c$$

where $Y$ = *in vivo* digestibility coefficient of OM; $c$ = rate constant for degradation of $b$ estimated using different *in situ* incubation points, per hour; $t$ = time, hour.

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The *in vivo* digestibilities coefficients of DM, OM and starch estimated for each animal were compared to the *in situ* estimations using a single 24 h *in situ* incubation point suggested by Silva *et al.* (2020) and the equation proposed by Benedeti *et al.* (2019). It is worth mentioning that the present study adopted a similar *in vivo* and *in situ* procedures to the studies mentioned above.

### Statistical analysis

Statistical analysis for *in situ* degradation parameters and partition of starch digestion was performed using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Data were analysed using the following model:

$$Y_{ijk} = \mu + D_i + a_j + p_k + e_{ijk}$$

where $Y_{ijk}$ = response variable; $\mu$ = overall mean; $D_i$ = fixed-effect of $i$th dietary treatment (5 levels); $a_j$ = random effect of the $j$th animal (5 levels); $p_k$ = random effect of the $k$th period (5 levels) and $e_{ijk}$ = residual error, assuming $e_{ijk} \sim N\ (0,\ s^2)$.

Least-squares means were separated using Fisher's least significant difference test. Results were deemed significant when $P \leq 0.05$.

The estimated DM, OM and starch digestibility ($n = 25$) using a single 24 h *in situ* incubation point suggested by Silva *et al.* (2020) and the OM digestibility equation proposed by Benedeti *et al.* (2019) were compared to the values observed in the *in vivo* trial ($n = 25$) using the following regression model:

$$Y = \beta_0 + \beta_1 \times X,$$

where $X$ = predicted *in situ* digestibility; $Y$ = observed values using the *in vivo* trial; $\beta_0$ = intercept of equation; and $\beta_1$ = slope of equation. Regression was evaluated according to the following statistical hypotheses (Mayer et al., 1994):

$$H_0{:}\beta_0 = 0 \text{ and } \beta_1 = 1, \text{ and } H_a{:}\text{not } H_0$$

If the null hypothesis was not rejected, it could be concluded that the equations and the 24 h of *in situ* incubation time accurately estimate the apparent digestibility of DM, OM and starch. Slope and intercept were separately evaluated to observe where equations have possible errors. Estimates were evaluated using the estimated value of mean square error of prediction (MSEP) and its components (Bibby and Toutenburg, 1977):

$$\text{MSEP} = \text{SB} + \text{MaF} + \text{MoF} = 1/n \sum_i =_1 (X_i - Y_i)^2,$$

$$\text{SB} = (X - Y)^2,$$

$$\text{MaF} = (s_X - s_Y)^2,$$

$$\text{MoF} = 2 s_X s_Y (1 - R),$$

where $X$ = predicted values; $Y$ = observed values; MSEP = mean squared error of prediction; SB = squared bias; MaF = component relative to the magnitude of random fluctuation; MoF = component relative to the model of random fluctuation; $s_X$ and $s_Y$ = standard deviations of predicted and observed values, respectively; and $R$ = Pearson linear correlation between predicted and observed values.

For all variance and covariance calculations, the total number of observations was used as a divisor since it was a prediction error estimate (Kobayashi and Salam, 2000). Prediction of efficiency was determined by estimating the correlation and concordance coefficient (CCC) or reproducibility index described by Tedeschi (2006). Validation analyses were performed with the Model Evaluation System [MES; version 3.1.16 (Tedeschi, 2006)] and significance was established at $\alpha = 0.05$.

## Results

### In situ *degradation parameters and partition of starch digestion*

The data used to estimate the *in situ* degradation parameters of DM, OM and starch are presented in Table 2. Also, Fig. 1 presents the *in situ* degradation curves of diets based on flint maize with different processing methods. Diets based on HMM, RMG, SNAP-80 and SNAP-90 showed a greater ($P < 0.05$) fraction *a* of DM, OM and starch compared to the DMG diet (Table 3). Regarding fraction *a* of starch, it was greater ($P < 0.05$) in HMM compared to remaining diets and there was no difference ($P > 0.05$) in faction *a* of starch between SNAP-80 and SNAP-90.

DMG diet presented greater ($P < 0.01$) fraction *b* of DM, OM and starch compared to diets based on HMM, RMG, SNAP-80 and SNAP-90. The fraction *b* of STA was lower ($P < 0.05$) in HMM compared to the other diets and there was no difference ($P > 0.05$) in fraction *b* of starch between SNAP-80 and SNAP-90.

Diets based on HMM and RMG showed greater ($P < 0.05$) *c* of DM and OM compared to diets based on DMG and SNAP-80. Diets based on SNAP-90 had an intermediate *c* for DM and OM that did not differ from the other diets. The *c* of starch was lower ($P < 0.05$) for the DMG diet compared to other diets and there was no difference ($P > 0.05$) in the *c* of starch between HMM, RMG, SNAP-80 and SNAP-90.

Ruminal, intestinal and total tract digestibility of starch were lower ($P < 0.05$) for the diet based on DMG and did not differ ($P > 0.05$) among the other diets (Table 4).

### *Use of* in situ *techniques to predict* in vivo *digestibility*

Data used to evaluate the two different methods using *in situ* procedures to estimate *in vivo* DM, OM and starch digestibilities are presented in Table 5 and the comparisons between observed and predicted DM, OM and starch digestibilities values are presented in Fig. 2. The OM digestibility equation proposed by Benedeti *et al.* (2019) and the single 24 h time-point method suggested by Silva *et al.* (2020) for DM, OM and starch digestibilities both accurately estimated ($P > 0.05$) the *in vivo* digestibility (Table 6). The null hypothesis of intercept and slope equal to 0 and 1, respectively, was not rejected in both models.

The equation proposed by Benedeti *et al.* (2019) showed lower CCC (0.74 *v.* 0.93) and R (0.53 *v.* 0.87); and higher MSEP (0.0006 *v.* 0.0001) compared to the time incubation suggested by Silva *et al.* (2020). The 24 h *in situ* incubation suggested by Silva *et al.* (2020) for DM, OM and starch digestibilities presented values of R and CCC close to 1.

## Discussion

### In situ *degradation parameters and partition of starch digestion*

In maize, the hydrophobic starch-protein matrix promotes a physiochemical impediment to starch digestion in ruminants

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**Table 2.** Descriptive statistics of the data used to estimate the *in situ* disappearance parameters of dry matter (DM), organic matter (OM) and starch

| | Diets[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | DMG | | HMM | | RMG | | SNAP-80 | | SNAP-90 | |
| Item | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. |
| DM disappearance at time, g/kg | | | | | | | | | | |
| 0 | 268 | 44.8 | 572 | 10.0 | 523 | 10.8 | 527 | 83.2 | 519 | 77.8 |
| 2 | 347 | 48.0 | 625 | 27.0 | 588 | 16.0 | 587 | 65.0 | 604 | 78.7 |
| 4 | 382 | 70.9 | 679 | 16.0 | 660 | 15.8 | 611 | 70.0 | 627 | 56.7 |
| 6 | 430 | 68.6 | 724 | 11.3 | 709 | 27.4 | 636 | 36.1 | 671 | 42.5 |
| 12 | 601 | 84.1 | 757 | 21.5 | 745 | 23.0 | 690 | 38.9 | 729 | 21.0 |
| 24 | 747 | 27.8 | 803 | 21.6 | 809 | 37.0 | 794 | 16.6 | 815 | 23.9 |
| 48 | 845 | 14.7 | 830 | 13.1 | 834 | 23.0 | 819 | 17.0 | 843 | 36.6 |
| 72 | 867 | 8.8 | 854 | 10.2 | 854 | 12.3 | 828 | 18.6 | 850 | 33.3 |
| 96 | 874 | 9.9 | 870 | 9.7 | 865 | 20.4 | 849 | 11.8 | 857 | 24.2 |
| OM disappearance at time, g/kg | | | | | | | | | | |
| 0 | 255 | 47.8 | 583 | 10.3 | 528 | 12.3 | 529 | 77.5 | 520 | 77.1 |
| 2 | 332 | 46.9 | 638 | 26.7 | 593 | 11.2 | 592 | 67.2 | 608 | 77.7 |
| 4 | 378 | 68.6 | 689 | 18.4 | 668 | 17.7 | 613 | 73.1 | 630 | 59.5 |
| 6 | 425 | 70.4 | 730 | 20.6 | 715 | 27.0 | 648 | 36.4 | 664 | 42.7 |
| 12 | 608 | 87.4 | 769 | 25.5 | 764 | 19.2 | 701 | 36.0 | 731 | 21.1 |
| 24 | 767 | 22.2 | 837 | 14.1 | 842 | 18.1 | 832 | 15.0 | 846 | 34.2 |
| 48 | 864 | 13.3 | 844 | 18.1 | 865 | 24.9 | 837 | 20.8 | 858 | 37.3 |
| 72 | 877 | 12.6 | 867 | 10.6 | 871 | 11.2 | 846 | 18.8 | 865 | 36.4 |
| 96 | 883 | 11.2 | 883 | 5.8 | 892 | 15.1 | 855 | 7.5 | 870 | 26.3 |
| Starch disappearance at time, g/kg | | | | | | | | | | |
| 0 | 227 | 43.5 | 841 | 46.5 | 573 | 43.8 | 650 | 37.7 | 711 | 97.6 |
| 2 | 351 | 76.8 | 888 | 47.5 | 760 | 46.4 | 720 | 41.3 | 783 | 95.9 |
| 4 | 461 | 56.8 | 926 | 39.8 | 859 | 60.7 | 841 | 36.2 | 865 | 78.1 |
| 6 | 529 | 68.5 | 954 | 36.9 | 940 | 30.0 | 915 | 79.3 | 923 | 67.0 |
| 12 | 743 | 75.6 | 975 | 24.3 | 977 | 9.1 | 971 | 31.9 | 981 | 14.6 |
| 24 | 848 | 33.8 | 993 | 2.0 | 993 | 3.4 | 992 | 3.7 | 992 | 5.0 |
| 48 | 929 | 29.3 | 996 | 3.4 | 994 | 4.3 | 996 | 0.6 | 994 | 3.0 |
| 72 | 973 | 18.2 | 994 | 3.7 | 996 | 2.1 | 995 | 2.5 | 998 | 1.2 |
| 96 | 993 | 1.4 | 995 | 2.5 | 996 | 2.3 | 997 | 1.0 | 996 | 1.7 |

Notes: S.D. = standard deviation.
[a]Three experimental diets were composed of 0.30 whole-plant maize silage, 0.10 mineral and protein supplement and 0.60 (DM basis) of one of the following processing methods: dry ground maize grain (DMG); high-moisture maize (HMM); reconstituted maize grain silage (RMG). Two other diets were composed of 0.10 mineral and protein supplement, 0.80 snaplage and 0.10 stalklage (DM basis; SNAP-80); or 0.10 mineral and protein supplement and 0.90 snaplage (DM basis; SNAP-90).

(Owens *et al.*, 1986). Thus, factors including processing, conservation method (dry or ensiled), ration composition and animal characteristics influence the starch digestibility (Zinn *et al.*, 2011; Allen, 2015). Therefore, evaluation of maize processing methods may help to verify methods that improve the availability of nutrients and consequently, reduce the environmental impact of beef cattle production and increase economic returns for producers.

According to several studies (Hoffman *et al.*, 2011; Valadares *et al.*, 2018; Silva *et al.*, 2020), zein protein subunits that crosslink starch granules undergo proteolysis during grain ensiling process, which might explain the increases in starch availability in grain silages compared with DMG. Measurement of the digestion rate of feed fractions *in situ* can provide relevant information regarding relative differences among feeds (Allen, 2015).

We hypothesized that HMM, RMG and SNAP would promote changes in the fractions *a* and *b* of DM, OM and starch. Corroborating with our hypotheses, diets based on HMM, RMG, SNAP-80 and SNAP-90 presented a greater fraction *a*, and lower fraction *b* of DM, OM and starch compared to the DMG-based diet.

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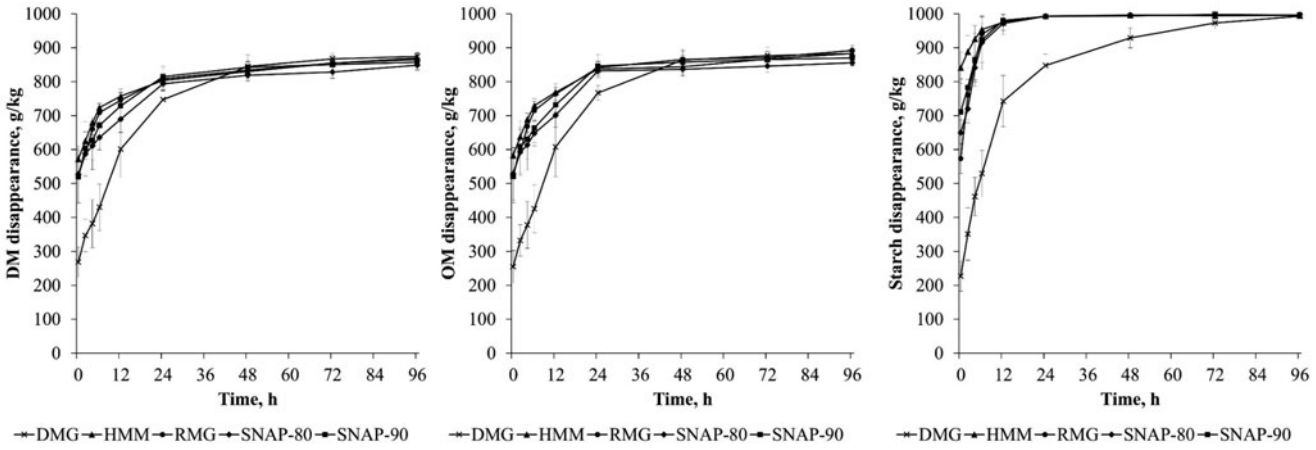


**Fig. 1.** Ruminal disappearance curves of diets based on flint maize with different processing methods estimated from *in situ* incubations. Vertical bars represent standard deviations of the means. Notes: A = dry matter; B = organic matter; C = starch. Three experimental diets were composed of 0.30 whole-plant maize silage, 0.10 mineral and protein supplement, and 0.60 (DM basis) of one of the following processing methods: dry ground maize grain (DMG); high-moisture maize (HMM); reconstituted maize grain silage (RMG). Two other diets were composed of 0.10 mineral and protein supplement, 0.80 snaplage and 0.10 stalklage (DM basis; SNAP-80); or 0.10 mineral and protein supplement and 0.90 snaplage (DM basis; SNAP-90).

**Table 3.** Ruminal degradation parameters of dry matter (DM), organic matter (OM) and starch of diets based on flint maize with different processing methods estimated from *in situ* incubations

| | Diets[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| Item | DMG | HMM | RMG | SNAP-80 | SNAP-90 | SEM | *P* value |
| DM, g/kg | | | | | | | |
| *a* | 261[b] | 578[a] | 538[a] | 531[a] | 553[a] | 24.9 | < 0.01 |
| *b* | 628[a] | 267[b] | 301[b] | 312[b] | 291[b] | 21.1 | < 0.01 |
| *c* | 0.07[b] | 0.10[a] | 0.10[a] | 0.07[b] | 0.09[ab] | 0.006 | < 0.01 |
| OM, g/kg | | | | | | | |
| *a* | 245[b] | 589[a] | 533[a] | 536[a] | 565[a] | 25.0 | < 0.01 |
| *b* | 663[a] | 270[b] | 315[b] | 322[b] | 297[b] | 22.3 | < 0.01 |
| *c* | 0.08[b] | 0.10[a] | 0.010[a] | 0.08[b] | 0.09[ab] | 0.005 | < 0.01 |
| Starch, g/kg | | | | | | | |
| *a* | 214[d] | 840[a] | 570[c] | 634[bc] | 700[b] | 25.7 | < 0.01 |
| *b* | 784[a] | 156[d] | 427[b] | 365[bc] | 299[c] | 26.2 | < 0.01 |
| *c* | 0.10[b] | 0.22[a] | 0.25[a] | 0.21[a] | 0.21[a] | 0.022 | < 0.01 |

Notes: *a* = readily soluble fraction (g/kg); *b* = potentially degradable fraction in the rumen (g/kg); *c* = rate constant for degradation of b (per hour).
[a]Three experimental diets were composed of 0.30 whole-plant maize silage, 0.10 mineral and protein supplement and 0.60 (DM basis) of one of the following processing methods: dry ground maize grain (DMG); high-moisture maize (HMM); reconstituted maize grain silage (RMG). Two other diets were composed of 0.10 mineral and protein supplement, 0.80 snaplage and 0.10 stalklage (DM basis; SNAP-80); or 0.10 mineral and protein supplement and 0.90 snaplage (DM basis; SNAP-90).
[a,b,c]Within row, means without a common superscript significantly differ ($P < 0.05$).

DMG and SNAP-80-based diets showed a lower *c* of DM and OM. In the DMG-based diet, it may be due to the higher resistance of zein protein subunits to degradation by bacteria, which probably affected the availability of some components. On the other hand, in the SNAP-80-based diet, the inclusion of stalklage may have reduced the quality of the DM components. Stalks have greater amounts of lignin (10.8% DM basis) than husks (6.1% DM basis) and leaves (4.5% DM basis), thus, the fibre in stalks is less accessible to ruminal fermentation (Petzel *et al.*, 2019).

Diets based on HMM, RMG, SNAP-80 and SNAP-90 showed similar *c* of starch, but fractions *a* and *b* of starch presented some alterations among these diets. The fraction *a* of starch for RMG diet was lower compared to the HMM diet. Although storage time and moisture were similar between HMM and RMG, the harvest of HMM was earlier than RMG. At this earlier stage, the starch-protein matrix of the maize is not totally consolidated in the endosperm (Caetano *et al.*, 2015), making it more soluble. As zein proteins develop and distend with advancing maturity, $\beta$- and $\gamma$-zeins cross-link and $\alpha$- and $\delta$-zeins penetrate their network, thereby encapsulating starch into a hydrophobic starch-protein matrix (Mu-Forster and Wasserman, 1998).

According to Kung *et al.* (2018), factors such as buffering capacity, sugar content, predominant organism types and pack density may alter fermentation profile among ensiled crops and

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**Table 4.** Partition of starch digestion using ruminal degradation parameters estimated from *in situ* incubations and *in vivo* procedure data

| | Diets[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| Item | DMG | HMM | RMG | SNAP-80 | SNAP-90 | SEM | *P* value |
| ED[b], kg/kg | 761[b] | 952[a] | 897[a] | 907[a] | 906[a] | 20.8 | < 0.01 |
| EDcorr[c], kg/kg | 748[b] | 902[a] | 862[a] | 869[a] | 864[a] | 19.9 | < 0.01 |
| Partition of starch digestion, kg/kg | | | | | | | |
| Rumen[d] | 0.75[b] | 0.83[a] | 0.81[a] | 0.81[a] | 0.81[a] | 0.012 | < 0.01 |
| Intestines[e] | 0.10[b] | 0.16[a] | 0.18[a] | 0.18[a] | 0.18[a] | 0.017 | 0.01 |
| Total | 0.85[b] | 0.99[a] | 0.99[a] | 0.99[a] | 0.99[a] | 0.009 | <0.01 |

[a]Three experimental diets were composed of 0.30 whole-plant maize silage, 0.10 mineral and protein supplement, and 0.60 (DM basis) of one of the following processing methods: dry ground maize grain (DMG); high-moisture maize (HMM); reconstituted maize grain silage (RMG). Two other diets were composed of 0.10 mineral and protein supplement, 0.80 snaplage and 0.10 stalklage (DM basis; SNAP-80); or 0.10 mineral and protein supplement and 0.90 snaplage (DM basis; SNAP-90).
[b]Starch effective degradability calculated according to Ørskov and McDonald (1979).
[c]Starch effective degradability corrected according to Offner and Sauvant (2004).
[d]Ruminal digestibility of starch (Rd) was predicted according to the equation proposed by Offner and Sauvant (2004): Rd = 0.302 + 0.59ED.
[e]The intestinal digestibility of starch (Id) was obtained by the difference between the total digestibility observed from *in vivo* procedure and the prediction of ruminal digestibility: Id = Total tract – Rd.

**Table 5.** Descriptive statistics of the data used to evaluate the prediction of *in vivo* digestibility of dry matter (DM), organic matter (OM) and starch after 24 h of *in situ* ruminal incubation or an alternative equation

| | Diets[a] | | | | |
|---|---|---|---|---|---|
| Item | DMG | HMM | RMG | SNAP-80 | SNAP-90 |
| *N* | 5 | 5 | 5 | 5 | 5 |
| OM | | | | | |
| *In vivo* digestibility coefficient | 0.77 ± 0.022 | 0.85 ± 0.011 | 0.84 ± 0.026 | 0.83 ± 0.013 | 0.85 ± 0.028 |
| Predicted values | | | | | |
| At 24 h[b] | 0.77 ± 0.022 | 0.84 ± 0.014 | 0.84 ± 0.018 | 0.83 ± 0.015 | 0.85 ± 0.034 |
| Equation B20[c] | 0.79 ± 0.029 | 0.83 ± 0.021 | 0.86 ± 0.025 | 0.84 ± 0.023 | 0.84 ± 0.024 |
| DM | | | | | |
| *In vivo* digestibility coefficient | 0.75 ± 0.023 | 0.81 ± 0.012 | 0.81 ± 0.031 | 0.80 ± 0.014 | 0.82 ± 0.025 |
| Predicted values | | | | | |
| At 24 h | 0.75 ± 0.028 | 0.80 ± 0.022 | 0.81 ± 0.037 | 0.79 ± 0.017 | 0.81 ± 0.024 |
| Starch | | | | | |
| *In vivo* digestibility coefficient | 0.85 ± 0.043 | 0.99 ± 0.001 | 0.99 ± 0.001 | 0.99 ± 0.002 | 0.99 ± 0.002 |
| Predicted values | | | | | |
| At 24 h | 0.85 ± 0.034 | 0.99 ± 0.002 | 0.99 ± 0.003 | 0.99 ± 0.005 | 0.99 ± 0.005 |

[a]Three experimental diets were composed of 0.30 whole-plant maize silage, 0.10 mineral and protein supplement and 0.60 (DM basis) of one of the following processing methods: dry ground maize grain (DMG); high-moisture maize (HMM); reconstituted maize grain silage (RMG). Two other diets were composed of 0.10 mineral and protein supplement, 0.80 snaplage and 0.10 stalklage (DM basis; SNAP-80); or 0.10 mineral and protein supplement and 0.90 snaplage (DM basis; SNAP-90).
[b]Values obtained after 24 h of *in situ* ruminal incubation as suggested by Silva *et al.* (2020) to predict *in vivo* digestibility coefficient of dry matter, organic matter and starch using *in situ* procedure.
[c]Values obtained with equation suggested by Benedeti *et al.* (2019) to predict the *in vivo* digestibility coefficient of organic matter using *in situ* procedure. $Y = 0.5695204 + 2.8597612 \times c$, where $Y$ = *in vivo* digestibility coefficient of organic matter and $c$ = degradation rate of fraction *b*, per hour.

consequently, influence the main mechanisms (solubilization and proteolysis) that are responsible for the disruption of zein-proteins cross-linking to starch granules. Although HMM, SNAP-80 and SNAP-90, were harvested at the same time, the cobs and husk in the SNAP may have influenced the fermentation profile, promoting lower fraction *a* for SNAP-80 and SNAP-90 compared to HMM.

Regarding the partition of starch digestion, the results showed that a higher amount of starch escapes ruminal fermentation on the DMG diet compared to HMM, RMG, SNAP-80 and SNAP-90-based diets. Studies (Owens *et al.*, 1986; Harmon and Swanson, 2020) have suggested that the small intestine of ruminants has a limited capacity for starch digestion due to inadequate access and insufficient time of starch granules exposure to enzymes. Therefore, the grain processing method may influence the amount of starch escapes ruminal fermentation and, consequently, the total tract *in vivo* digestibility.

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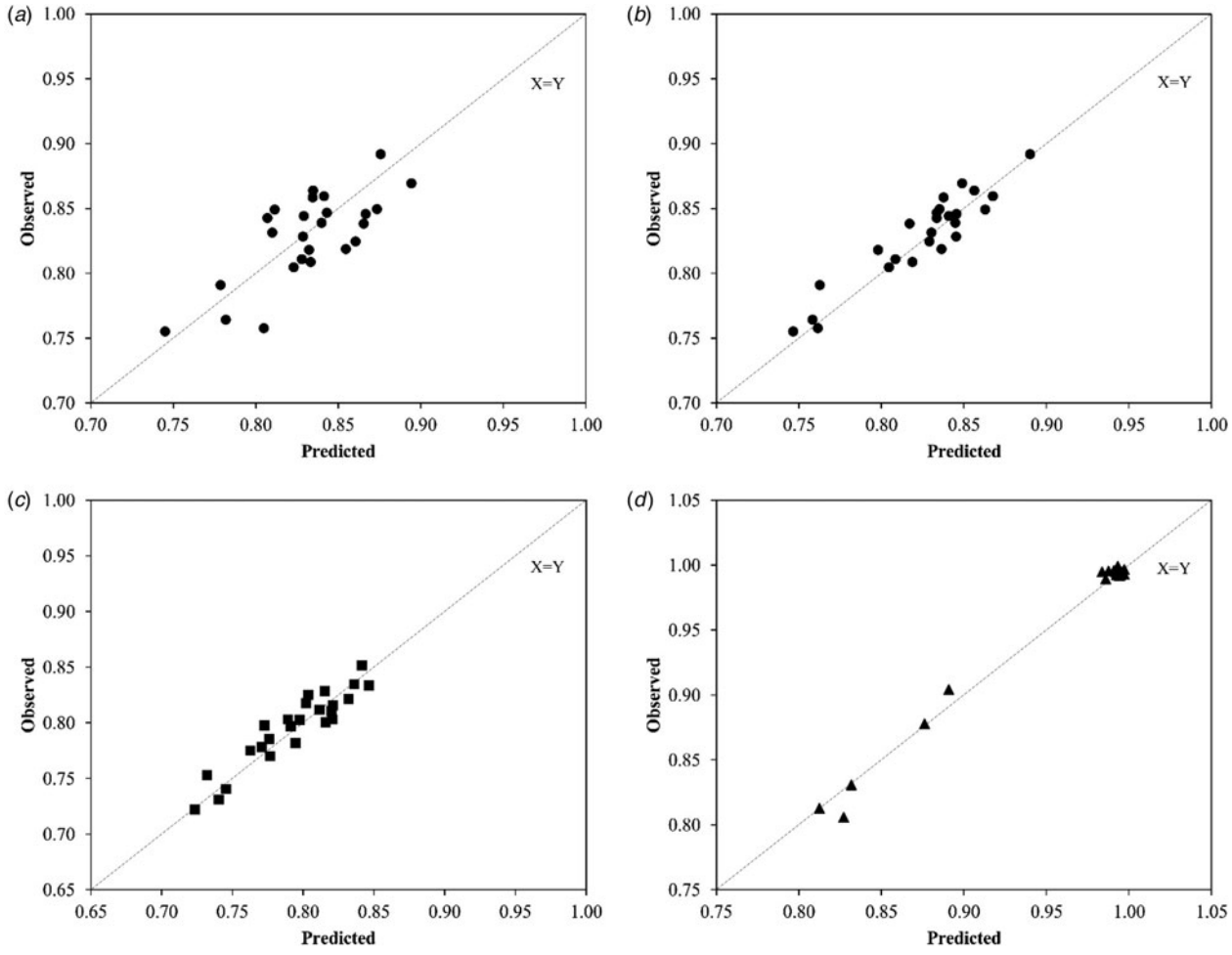


**Fig. 2.** Relationship among observed (*in vivo*) and predicted digestibilities values of dry matter, organic matter and starch at 24 h of *in situ* ruminal incubation as suggested by Silva *et al*. (2020) and equation proposed by Benedeti *et al*. (2019) using *in situ* technique. Notes: A = organic matter [Benedeti *et al*. (2019)], B = organic matter [Silva *et al*. (2020)], C = dry matter [Silva *et al*. (2020)] and D = starch [Silva *et al*. (2020)].

**Table 6.** Mean and descriptive statistic of the relationship among the observed (*in vivo*) digestibility coefficient and predicted values of dry matter, organic matter and starch using a single point of 24 h of *in situ* ruminal incubation and predicted values of organic matter using an alternative equation

| | Dry matter | | Organic Matter | | | Starch | |
|---|---|---|---|---|---|---|---|
| Item | Observed | At 24 h[a] | Observed | At 24 h | Equation B20[b] | Observed | At 24 h |
| *Validation analysis* | | | | | | | |
| Mean | 0.79 | 0.79 | 0.83 | 0.82 | 0.83 | 0.96 | 0.96 |
| Standard deviation | 0.033 | 0.034 | 0.034 | 0.036 | 0.033 | 0.061 | 0.063 |
| Maximum | 0.85 | 0.84 | 0.89 | 0.89 | 0.89 | 0.99 | 0.99 |
| Minimum | 0.72 | 0.72 | 0.75 | 0.74 | 0.74 | 0.81 | 0.80 |
| *R* | – | 0.86 | – | 0.87 | 0.53 | – | 0.99 |
| CCC | – | 0.93 | – | 0.93 | 0.74 | – | 0.99 |
| *P* value ($H_0$: $\beta_0 = 0$, $\beta_1 = 1$) | – | 0.29 | – | 0.14 | 0.21 | – | 0.18 |
| MSEP | – | 0.0002 | – | 0.0001 | 0.0006 | – | 0.0001 |
| SB | – | 2.63 | – | 7.84 | 2.45 | – | 3.67 |
| MaF | – | 7.56 | – | 8.23 | 10.19 | – | 9.78 |
| MoF | – | 89.81 | – | 83.93 | 87.36 | – | 86.55 |

Notes: *R* = determination coefficient; CCC = correlation and concordance coefficient; MSEP = mean square error of prediction; SB = squared bias (% of the MSEP); MaF = magnitude of random fluctuation (% of the MSEP); MoF = model of random fluctuation (% of the MSEP).
[a]Values estimated after 24 h of *in situ* ruminal incubation as suggested by Silva *et al*. (2020) to predict *in vivo* digestibility of dry matter, organic matter and starch using *in situ* technique.
[b]Values obtained with equation suggested by Benedeti *et al*. (2019) to predict the *in vivo* digestibility coefficient of organic matter using *in situ* procedure. $Y = 0.5695204 + 2.8597612 \times c$, where $Y$ = *in vivo* digestibility coefficient of organic matter and $c$ = degradation rate of fraction *b*, per hour.

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### *Use of* in situ *techniques to predict* in vivo *digestibility*

The development of accurate and precise procedures to use as an alternative to *in vivo* method may contribute to improving diet formulation and animal performance (Benedeti *et al.*, 2019), since these procedures allow for obtaining faster results, with lower costs, labour and animal usage (Nocek, 1988). *In situ* techniques have been studied for several years, but the lack of standardization makes difficult comparisons between studies (Silva *et al.*, 2020), as well as the utilization of that procedure.

The composition of diets used in the present study was close to those used by Silva *et al.* (2020), whereas diets used by Benedeti *et al.* (2019) had a greater variation in diet composition. That high variation may have been the reason for the lower R and CCC, and greater MSEP in the current study for the equation method proposed by Benedeti *et al.* (2019) compared to the time method described by Silva *et al.* (2020) for OM digestibility. According to Tedeschi (2006), CCC and MSEP are parameters that indicate the model's efficiency and reproducibility. However, R needs to be analysed together with other variables in a statistical model to indicate the correctness of the regression model (Benedeti *et al.*, 2019). Approximately 87% of MSEP of the equation proposed by Benedeti *et al.* (2019) were mostly associated with random errors (MoF) and not with problems associated with linear regression or systematic bias (MaF) and/or central tendency or bias (SB).

Therefore, the equation proposed by Benedeti *et al.* (2019) for OM digestibility and the 24 h of *in situ* incubation time described by Silva *et al.* (2020) for DM, OM and starch digestibilities were accurate and precise, being appropriate to predict *in vivo* digestibilities. Futures studies are recommended to test the efficacy of these methods on the DM, OM and starch digestibility estimation of diets with higher fibre content.

In conclusion, diets based on HMM, RMG, SNAP-80 and SNAP-90 present a lower faction *b* and greater fraction *a* and the *c* for DM, OM and starch compared to DMG. The results suggest that HMM, RMG, SNAP-80 and SNAP-90 diets increase the breakdown of the grains protein matrix, enhancing starch availability. The two different applications of *in situ* technique evaluated in this study showed precision and accuracy to estimate the *in vivo* digestibility of DM, OM and starch.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S0021859621000034.

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**Conflict of interest.** The authors declare that they have no competing interests.

**Ethical standards.** The experiment was conducted following the approval of the animal handling and procedures described herein by the Ethics Committee for Animal Use (protocol CEUAP/DZO/UFV 27/2017).

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