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Can Associative Effects Affect In Vitro Digestibility Estimates Using Artificial Fermenters?

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Abstract: We aimed to test the associative effects among forages, and between forage and concentrates on the in vitro digestibility of dry matter and neutral detergent fibre using an artificial ruminal fermentation system. The study consisted of two assays, in which associative effects were evaluated among three forages, sugarcane, maize silage, and Tifton 85 hay under two incubation conditions (single feed or all feeds together in a jar), and the associative effects between sugarcane and soybean meal and/or ground maize. For the first assay, sugarcane digestibility increased ($p < 0.02$), whereas the maize silage digestibility decreased ($p < 0.01$) when forages were incubated together in the same jar. Tifton hay digestibility was not altered ($p \geq 0.57$) by the incubation condition. In the second assay, the sugarcane digestibility was depressed ($p < 0.05$) when the forage was incubated along with maize grain. For both assays, the pattern of repeatability for digestibility estimates presented an influence of the incubation condition. We concluded that the incubation of different feeds together in the same jar using artificial fermenters causes associative effects among them. These effects can influence the estimates of in vitro dry matter and fibre digestibility and alter their repeatability.

Keywords: concentrates; feed analysis; forages; in vitro systems; ruminal digestion

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1. Introduction

The method for evaluating in vitro digestibility of ruminants' feeds suggested by Ti-lley and Terry [1] was based on the incubation of forage samples in individual tubes containing ruminal inoculum and buffer solution. However, the utilisation of artificial fermenters has increased in recent decades, as laboratorial analyses are considered to be simpler, faster, less expensive, and have greater operational capacity [2–4].

The first artificial fermenter model was developed by a North American company (DaisyII, Ankom Technology Corporation Fairport, New York, NY, USA) and was introduced to the public in 1994 [3]. However, nowadays, different commercial brands of this equipment can be found on the market. Overall, the equipment possesses four jars, where samples are kept in contact with the inoculum under controlled temperature and rotation. Particularly, the greater operational capacity of artificial fermenters occurs because several samples can be evaluated simultaneously [5] and the utilisation of filter bags could minimize errors caused by material transfer during filtration procedures [6]. On the other hand, the simultaneous evaluation of feeds with different chemical characteristics could cause associative effects among samples incubated in the same jar, influencing microbial populations and activity, and, consequently, altering feed digestibility estimates [7–9].

In vitro digestibility estimates of feeds can be used according to two main objectives: prediction of in vivo digestibility [10] and comparative evaluation among feeds [5,11]. In this sense, the occurrence of associative effects among feeds incubated together in a jar could affect both objectives by biasing the estimates, and by altering the ranking of feeds in relation to digestibility characteristics. However, studies performed to evaluate the

occurrence of associative effects amongst feeds using in vitro artificial fermenters are still scarce regarding tropical feeds.

Therefore, our objective was to study the occurrence of associative effects among forages, and between forage and concentrates on in vitro digestibility of dry matter (IVDMD) and neutral detergent fibre (IVNDFD) using an artificial ruminal fermentation system.

2. Materials and Methods

The experiment was carried out at the Animal Science Department of the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. The Ethics Committee on the Use of Production Animals of the Universidade Federal de Viçosa (protocol number 029/2019) approved all animal care and handling procedures applied in this work.

2.1. Incubations and Measurements

The experiment was performed in two assays. In the first, the occurrence of associative effects among forages with different chemical characteristics (sugarcane, maize silage, and Tifton 85 hay) was evaluated. In the second assay, the occurrence of associative effects between one forage (sugarcane) and energy and/or protein concentrate feeds (maize grain and, or soybean meal) was evaluated.

Primary samples of fresh sugarcane (*Saccharum officinarum* L.), maize silage (*Zea mays* L.), Tifton 85 hay (*Cynodon* sp. Rich.), maize grain, and soybean meal were obtained in Viçosa, Minas Gerais, Brazil. The high-moisture forages were oven-dried (55 °C/72 h). After that, all materials were processed in a knife mill (model 3; Arthur H. Thomas, Philadelphia, PA, USA) to pass through a 1 mm screen sieve and were analysed with regard to the contents of dry matter (DM, dried 105 °C/16 h; method G-003/1), nitrogen (N, Kjeldahl procedure; method N-001/2), and neutral detergent fibre (NDF, method F-002/1) according to the standard analytical procedures of the Instituto Nacional de Ciência e Tecnologia de Ciência Animal ([12], Table 1). The NDF contents were evaluated using a heat-stable α-amylase (Termamyl 2X; Novozymes, Araucária, Paraná, Brazil), omitting sodium sulphite, and were expressed including residual ash and protein. The crude protein (CP) content was expressed as N × 6.25.

Table 1. Average contents of crude protein (CP) and neutral detergent fibre (NDF) in the different study materials.

	CP	**NDF**
Feeds	**g/kg Dry Matter**	
Sugarcane	30.2	586
Maize silage	79.3	562
Tifton 85 hay	75.9	797
Soybean meal	515	233
Maize grain	84.0	198

In the first assay, the IVDMD and IVNDFD were evaluated with the forages incubated either in different jars or all of them simultaneously in the same jar. In the second assay, the digestibility of fresh sugarcane was evaluated by incubating only the forage in a jar or the forage along with maize grain, soybean meal, or maize grain plus soybean meal.

The digestibility assays were performed in a TE-150 artificial fermenter (Tecnal Equipamentos Científicos, Piracicaba, São Paulo, Brazil) and filter bags which were made of non-woven textile (100 g/m^2, [13]). The filter bags were 4 × 4.5 cm and were previously washed, dried, and weighed as described by Camacho et al. [4] to obtain the tare weight.

The incubations procedures lasted eight days, with each assay lasting four consecutive days, with two consecutive 48 h incubation runs [1]. In each assay, each incubation run included the evaluation of four fermentation jars containing 30 filter bags with approximately 500 mg test portions per filter bag (in a DM basis). All filter bags were heat-sealed.

In the first assay, the filter bags were distributed to the fermentation jars in each run as follows: one jar for each individual forage (n = 30 for each jar), and one jar for all forages simultaneously (n = 10 for each forage). In the second assay, the filter bags were distributed as follows: one jar for sugarcane (n = 30 for sugarcane), one jar for sugarcane and maize grain (n = 15 for each feed), one jar for sugarcane and soybean meal (n = 15 for each feed), and one jar for sugarcane, maize grain, and soybean meal (n = 10 for each feed). Each jar contained two blank filter bags. It must be noticed that the artificial fermenter possessed four jars (3200 mL).

McDougall's buffer solution [14] was used for all incubations ($NaHCO_3$, 9.80 g/L; anhydrous Na_2HPO_4, 3.71 g/L; KCl, 0.57 g/L; NaCl, 0.47 g/L; $MgSO_4.5H_2O$, 0.12; $CaCl_2.2H_2O$, 0.05 g/L). Urea was not added to the buffer solution in order to avoid favouring the microbial utilisation of the low-quality feeds [11,15], and to allow a clearer visualization of associative effects. The solution was prepared 24 h prior to each incubation run and kept in an acclimatized room (39 °C). Prior to each incubation, the pH of the buffer solution was adjusted to 6.8 by bubbling CO_2 into the solution, as described by Camacho et al. [4].

The ruminal inoculum was obtained from a rumen-fistulated Nelore heifer weighing 350 kg. The basal diet of the animal consisted of fresh sugarcane and a commercial concentrate containing 220 g CP/kg as fed. The commercial concentrate was composed of maize grain, soybean meal, wheat bran, urea, and minerals. The forage-to-concentrate ratio of the diet was 80:20 on a DM basis. The animal was fed ad libitum twice daily at 0800 and 1600 h, allowing approximately 100 g/kg in orts (as feed). The animal also had ad libitum access to fresh water and a complete mineral mixture, and was adapted to the basal diet for 14 days prior to the inoculum collections [16].

Ruminal inoculum (liquid and solid digesta) was collected at several points in the cranial and caudal rumen mat shortly before the beginning of incubation. Ruminal inoculum was stored in preheated (39 °C) thermal bottles and then mixed for a few seconds in a blender to homogenize liquid and solid phases [4]. The fluid was then filtered through four layers of cheesecloth. The steps from rumen inoculum collection to incubation onset were conducted within 20 min in an acclimatized room (39 °C). In each jar, 400 mL of ruminal inoculum and 1600 mL of McDougall's buffer solution were added (1:4 vol/vol). Carbon dioxide was flushed into the headspace of each jar, which was closed and placed into the preheated (39 °C) artificial fermenter.

After 48 h of incubation, the filter bags were washed with hot distilled water (90 °C) until the water became clear, and bags were gently pressed to remove gases. Then, to estimate the apparently undigested DM residue, filter bags were oven-dried (55 °C/24 h and 105 °C/16 h, sequentially), placed in a desiccator, and weighed.

For the IVNDFD evaluations, filter bags containing the incubation residues were placed into polypropylene screw-capped flasks (120 mL; autoclavable universal collection vial, Bioplast 2605, Porto Alegre, Rio Grande do Sul, Brazil) with 80 mL of neutral detergent solution and 500 µL of a heat-stable α-amylase (Termamyl 2X, Novozymes, Araucária, Paraná, Brazil). Flasks containing the filter bags were closed and autoclaved for 1 h at 105 °C [17]. After that, the filter bags were washed with hot distilled water (90 °C) and acetone. The drying and weighing procedures were performed as previously described.

The IVDMD and IVNDFD were calculated as

$$D = \frac{M - (R - B)}{M} \times 1000 \quad (1)$$

where D is the IVDMD or IVNDFD (g/kg); M is the incubated mass of DM or NDF (g); R is the undigested residue of DM or NDF (g); and B is the DM or NDF residue in blank filter bags (g).

To evaluate the pH and concentration of ammonia nitrogen (NH_3-N) in the medium (i.e., the mixture of rumen inoculum and buffer solution), aliquots of the fluid were taken from each jar within each incubation run, filtered through a triple layer of cheesecloth, and transferred to 50 mL conical polypropylene tubes with screw caps. The pH was immediately measured using a digital potentiometer (TEC-3P-MP; Tecnal, Piracicaba, São

Paulo, Brazil). Then, 1 mL of a H_2SO_4 solution (9 mol/L) was added to each tube, and all tubes were kept at 4 °C until NH_3-N analysis. Ammonia nitrogen was estimated by indophenol-catalysed colorimetric reactions (method N-006/1, [12]).

2.2. Statistical Analysis

For the first assay, the evaluation of the associative effects among forages was analysed according to the model:

$$Y_{ijkl} = \mu + F_i + C_j + FC_{ij} + R_k + \varepsilon_{ijkl} \quad (2)$$

where Y_{ijkl} is the IVDMD or IVNDFD measured on test portion l of forage i under incubation condition j in run k; μ is the general constant; F_i is the fixed effect of forage i; C_j is the fixed effect of the incubation condition j (i.e., single feed or all feeds together in a jar); FC_{ij} is the fixed effect of interaction between forage i and incubation condition j; R_k is the random effect of incubation run k; and ε_{ijkl} is the random error assumed to be NIID (0, $\sigma^2{}_\varepsilon$).

For the second assay, the evaluation of associative effects among forage and concentrate feeds followed the model:

$$Y_{ijk} = \mu + C_i + R_j + \varepsilon_{ijk} \quad (3)$$

where Y_{ijk} is the IVDMD or IVNDFD measured on test portion k under incubation condition i in run j; μ is the general constant; C_i is the fixed effect of the incubation condition i; R_j is the random effect of incubation run j; and ε_{ijk} is the random error assumed to be NIID (0, $\sigma^2{}_\varepsilon$).

All statistical evaluations were performed using the MIXED procedure of SAS 9.4. The components of variance were estimated according to the restricted maximum likelihood method. Significant results were declared at $p < 0.05$.

Initially, we performed an outlier evaluation on the overall dataset. An outlier was identified when its restricted likelihood distance was greater than 1.0. After the outlier elimination, analyses of variance were performed again to interpret the significance of the effects. For the model (2), when a significant interaction was found, we studied the effect of incubation condition nested within each forage using the SLICE statement of the MIXED procedure. When a significant effect of incubation condition was found in model (3), comparisons were performed using the Bonferroni's test.

After performing the analyses based on the full models (Equations (2) and (3)), a second set of analyses of variance was carried out. In this case, we evaluated the data obtained for each forage/incubation condition to quantify their specific residual variances. The following model was used:

$$Y_{ij} = \mu + R_i + \varepsilon_{ij} \quad (4)$$

where Y_{ij} is the IVDMD or IVNDFD measured on test portion j in incubation run i; μ is the general constant; R_i is the random effect of incubation run i; and ε_{ij} is the random error assumed to be NIID (0, $\sigma^2{}_\varepsilon$).

From the results obtained in model (4), the repeatability was calculated as

$$r = \frac{\sqrt{\hat{\sigma}^2_\varepsilon}}{\bar{Y}} \times 100 \quad (5)$$

where r is the repeatability (%); $\hat{\sigma}^2_\varepsilon$ is the residual variance [(g/kg)^2]; and $\bar{Y}$ is the average IVDMD or IVNDFD (g/kg).

The initial and final values of pH and NH_3-N concentration were evaluated following what was previously described, but considering each jar as a subject. Evaluation times (i.e., initial and final) were considered as repeated measures. Due to the restricted number of replicates ($n = 2$ for each assay), the (co)variance matrix structure was modelled according to a variance component structure (VC), and the degrees of freedom were estimated using the Satterthwaite's approximation. Comparison among incubation conditions followed the previously stated methods.

3. Results

We did not detect variation between incubation runs ($p > 0.05$) for the response variables evaluated here. For the first assay, there was an interaction between forage and incubation condition ($p < 0.01$) for both IVDMD and IVNDFD. Sugarcane digestibility increased ($p < 0.02$), whereas maize silage digestibility decreased ($p < 0.01$) when the forages were incubated together in a same jar. Tifton hay digestibility was not altered ($p \geq 0.57$) by the incubation condition (Table 2).

Table 2. Average and standard error of the mean of in vitro digestibility of dry matter (IVDMD) and neutral detergent fibre (IVNDFD) in several forages according to incubation conditions.

	Incubation Condition [1]		
Forage	**Single Feed**	**All Feeds**	***p*-Value**
	IVDMD (g/kg)		
Sugarcane	574 ± 20.2 (60)	600 ± 21.3 (20)	0.007
Maize silage	666 ± 20.2 (59)	625 ± 21.3 (20)	<0.001
Tifton 85 hay	552 ± 20.3 (55)	557 ± 21.3 (20)	0.599
	IVNDFD (g/kg)		
Sugarcane	347 ± 21.4 (58)	377 ± 23.1 (20)	0.019
Maize silage	503 ± 21.3 (60)	442 ± 23.1 (20)	<0.001
Tifton 85 hay	508 ± 21.4 (55)	501 ± 23.1 (20)	0.577

[1] Single feed, each feed located in different bags from a separated jar; All feeds, all the feeds located in different bags from the same jar. The number in parentheses represents the number of replicates.

In the second assay, the sugarcane IVDMD and IVNDFD did not change ($p > 0.05$) when the forage was incubated together with soybean meal or soybean meal plus maize (Table 3). However, its digestibility was depressed ($p < 0.05$) when the forage was incubated along with maize grain.

Table 3. Average and standard error of the mean of in vitro digestibility of dry matter (IVDMD, g/kg) and neutral detergent fibre (IVNDFD, g/kg) in sugarcane incubated alone or along with different concentrate feeds.

Incubation Condition	**IVDMD** [1]	**IVNDFD** [1]
Sugarcane + maize + soybean meal	660 ± 21.6 [a] (16)	422 ± 31.5 [a] (18)
Sugarcane + soybean meal	651 ± 21.2 [a] (29)	418 ± 31.0 [a] (29)
Sugarcane + maize	615 ± 21.2 [b] (29)	370 ± 31.1 [b] (29)
Sugarcane	641 ± 21.0 [a] (56)	407 ± 30.6 [a] (57)
p-value	<0.001	<0.001

[1] Means in column followed by different letters differ at $p < 0.05$. The number in parentheses represents the number of replicates for the forage feed.

The evaluation of associative effects among forages showed that the repeatabilities for digestibility of sugarcane and maize silage were improved when the incubation was performed with all feeds in the same jar (Figure 1). Though, the opposite pattern was verified for Tifton 85 hay, whose best repeatability occurred when this forage was incubated alone in the jar.

Regarding the associative effects between forage and concentrates, we did not observe variations in the repeatability for sugarcane IVDMD regardless of the incubation condition. However, repeatability for sugarcane IVNDFD was better when forage was incubated along with soybean meal (Figure 2). The incubation of forage alone or the addition of maize to the same incubation jar compromised the repeatability, which worsened when maize and soybean meal were added together to the incubation jar.

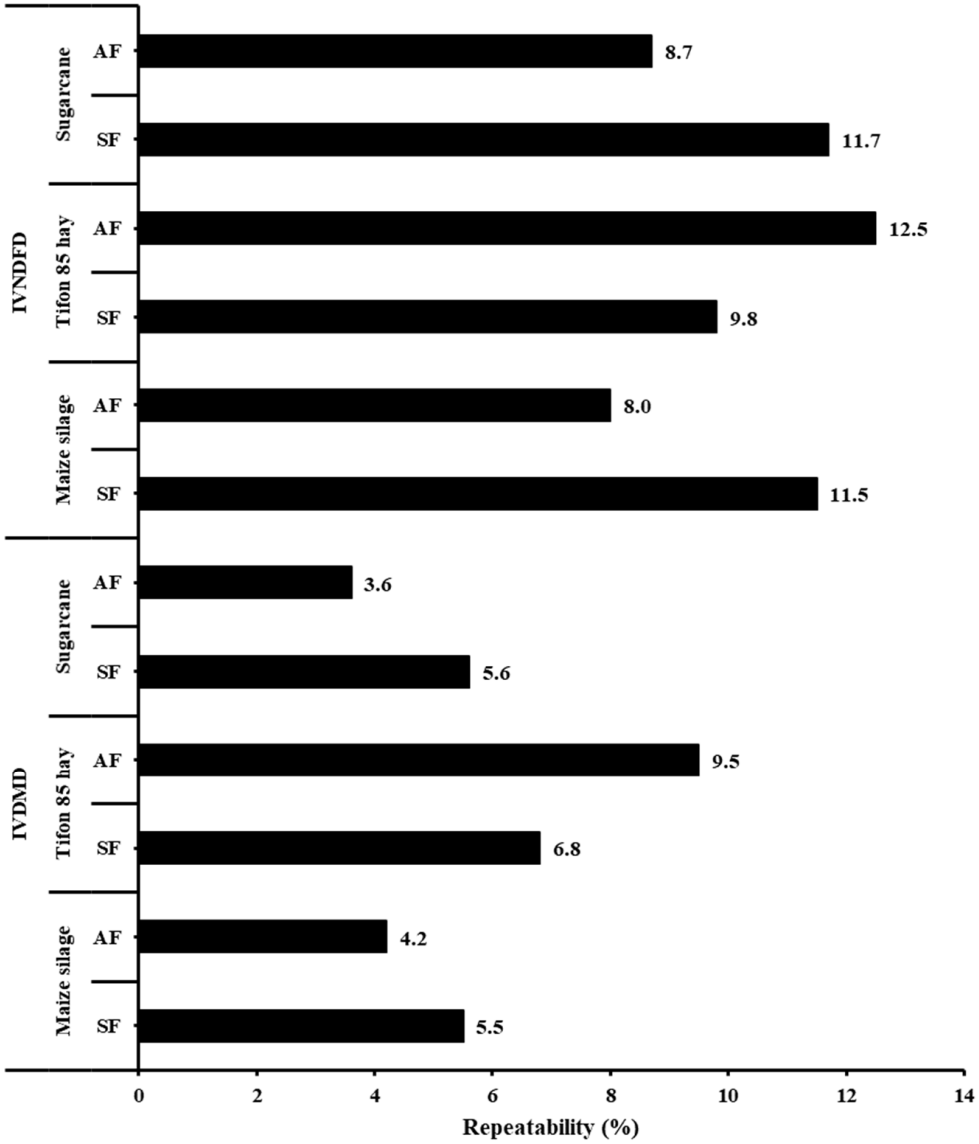

Figure 1. Repeatabilities for in vitro digestibility of dry matter (IVDMD) and neutral detergent fibre (IVNDFD) according to incubation conditions in assay 1 [SF, single feed (each feed in a separated jar); AF, all feeds (all the feeds in the same jar)] for the three forages under study.

In the first assay, pH and NH_3-N concentration in the incubation jars were not affected by either incubation condition ($p \geq 0.30$), or by the interaction between incubation condition and time ($p \geq 0.35$). However, both variables differ according to the time of evaluation ($p < 0.01$). On average, the pH decreased (6.99 vs. 6.73) and NH_3-N concentration increased (0.51 vs. 4.94 mg/dL) from the beginning to the end of incubation period (Table 4). Regarding the evaluation of associative effects between forage and concentrates, both pH and NH_3-N concentration showed an interaction between incubation condition and time ($p < 0.01$). Slicing of this effect showed no differences between incubations conditions for the initial pH ($p > 0.90$) and NH_3-N ($p > 0.98$), whose average values were 6.91 and 2.16 mg/dL, respectively. The final pH was higher ($p < 0.05$) for the incubation of sugarcane along with soybean meal, when compared to the incubation of forage alone or along with maize. Incubating all feeds together caused an intermediate final pH. The final NH_3-N concentration followed the same pattern observed for the final pH.

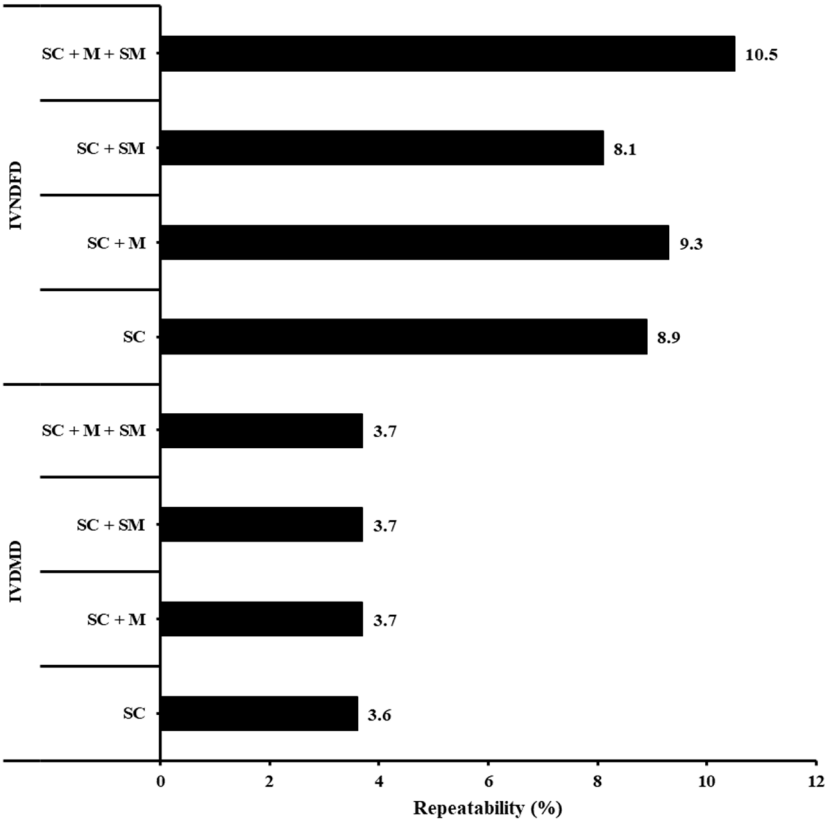

Figure 2. Repeatabilities for in vitro digestibility of dry matter (IVDMD) and neutral detergent fibre (IVNDFD) according to incubation conditions in assay 2 (SC, sugarcane; SC + M, sugarcane plus maize; SC + SM, sugarcane plus soybean meal; SC + M + SM, sugarcane plus maize plus soybean meal).

Table 4. Average descriptive values of pH and ammonia nitrogen (NH_3-N) concentration under different incubation conditions.

	Ph [1]		**NH_3-N (mg/dL)** [1]	
Incubation Condition	**Initial**	**Final**	**Initial**	**Final**
Forages (Assay 1)				
Sugarcane	7.01	6.76	0.47	5.73
Maize silage	6.99	6.77	0.62	6.27
Tifton 85 hay	7.00	6.72	0.29	4.03
All forages together	6.99	6.70	0.66	3.72
p-value				
Incubation condition (IC)	0.764		0.305	
Time (T)	<0.001		<0.001	
IC × T	0.809		0.350	
SEM	0.040		0.762	
Forage plus concentrates (Assay 2)				
Sugarcane + soybean meal + maize	6.91	6.69 ^{ab}	1.90	8.12 ^{ab}
Sugarcane + soybean meal	6.92	6.86 ^{a}	2.04	13.59 ^{a}
Sugarcane + maize	6.93	6.61 ^{b}	2.54	4.05 ^{b}
Sugarcane	6.90	6.63 ^{b}	2.17	3.69 ^{b}
p-value				
Incubation condition (IC)	0.009		0.039	
Time (T)	<0.001		0.001	
IC × T	0.012		0.028	
SEM	0.037		1.429	

[1] Means in column followed by different letters differ at $p < 0.05$.

4. Discussion

Associative effects occur when the digestibility of a mixture of feeds is different from the weighted sum of individual feed digestibilities [18]. In this sense, we understand that the results obtained in our work provide evidence for the occurrence of associative effects between forages, and also between forages and concentrates in terms of DM and fibre digestibility. In agreement with our results, several researchers have pointed out differences in digestibility characteristics when feeds are evaluated separately or together [5,7,19,20].

The effective ruminal digestibility of a feed is an integration between its potential digestibility (an inherent characteristic of the feed) and the digestion environment [21], and its expression depends on the intensity of microbial activity on the substrate [22]. The environment itself encompasses all major factors that can affect the activity of microbial enzyme systems on substrates, such as pH, minerals, nitrogen compounds (i.e., ammonia and peptides), branched-chain fatty acids, etc. [23]. Even though a part of the environment is defined by the own animal (e.g., buffer releasing and N recycling), the feed itself is a potential supplier of substrates for microbial growth, as well as influencing the rumen physicochemical characteristics. Consequently, the rumen environment conditions are interrelated with feed characteristics. When a mixture of feeds is fed into the rumen, most environmental conditions would be determined through the mutual interactions among the components of different feeds [21]. For the case of closed in vitro systems with a single donor animal, such as the one evaluated here, the environmental characteristics among the different incubation conditions would be almost exclusively affected by the feeds.

There are several causes for the occurrence of associative effects among feeds, which can manifest in either a positive or negative way [18]. Evidence of a positive associative effect was verified through sugarcane digestibility in the first assay, whose digestibility was increased by incubation along with the other forages. Overall, sugarcane is a forage with a low content of nitrogen compounds. Hence, under restriction of nitrogenous substrates (i.e., ammonia, amino acids, or small peptides), the microbial fibre degradation is compromised [24] and, consequently, digestibility decreases. When sugarcane was incubated along with forages with higher CP content, an improvement in the nitrogen availability for microbial growth possibly occurred, and its IVDMD and IVNDFD were increased. An improvement in ammonia concentration has not been detected in the first assay. However, it is likely that improvements in the availability of other forms of nitrogenous compounds (e.g., small peptides) were responsible for the better microbial degradation of sugarcane when incubated along with better quality forages. It is noteworthy that these forages had a higher CP content when compared to sugarcane.

On the other hand, the results from the first assay also showed that positive and negative associative effects can be simultaneously observed. Despite the improvement in sugarcane digestibility, the joint incubation of forages decreased the maize silage digestibility. Likely, the joint incubation increased the carbohydrate diversity in the medium. Under this condition, some microbial species (e.g., *Butyrivibrio fibrisolvens Bryant* and *Streptococcus gallolyticus* Orla-Jensen) may change their priority regarding the carbohydrate source used for energy metabolism and growth [25]. This priority shift could decrease the degradation of some carbohydrate sources that would previously be considered as a primary source for microbial growth. Moreover, the inclusion of fast-fermenting carbohydrates to the incubation medium might favour the growth of certain microbial groups with a greater competitive capacity for substrates [26]. Both statements could be associated with the high sucrose content in sugarcane, which seemed to negatively affect the microbial utilisation of maize silage. According to Huhtanen [18], the decrease in fibre digestibility tends to be more pronounced in higher quality forages.

Negative associative effects were also observed in the second assay, when sugarcane was incubated along with maize grain. This inhibited digestion of sugarcane emphasised the occurrence of the carbohydrate effect, which is characterised by a decrease in the cellulolytic activity when fast-fermenting carbohydrates (e.g., starch) are added to the medium. Its causes seem to involve an increased competition among microbial species for

essential nutrients and other compounds necessary for microbial growth. Species with a faster growth rate, such as those that degrade starch, outcompete the fibrolytic microorganisms, which have a slower growth rate, and imply a decreased fibre digestion [26–28]. The carbohydrate effect can be minimized or overcome when the availability of essential substrates in the medium increases, mainly in terms of amount and chemical profile of nitrogenous compounds [28]. This statement explains why the sugarcane digestibility was not compromised when soybean meal was incubated in the same jar, with or without the presence of maize grain, as soybean meal was able to improve the nitrogen availability in the medium.

On the other hand, the forages that showed an evident associative effect in the first assay (i.e., maize silage and sugarcane) also displayed an improved repeatability. Repeatability is a measure of the ability of the method to generate similar results for multiple preparations of the same sample considering the same intra-laboratorial conditions. This pattern highlights that despite the altered digestibility, there was greater homogeneity in microbial activity on the test portions when the feeds were incubated together in the same jar. However, even though Tifton hay has not shown evident associative effects on digestibility, its repeatability worsened when it was incubated together with the other forages. This differentiated pattern seems to indicate that associative effects may affect feed digestibility trough in different ways, which might include loss of precision in some cases. In a logical way, it can be understood that feed itself is an important factor that affects the incubation medium [21]. Thus, the associative effect may incur different changes in digestibility estimates depending on the combination of feeds incubated together in the same jar.

We should highlight that the associative effects evaluated herein are characteristic of artificial fermenters based on the Daisy incubator system, where several samples are incubated together in the same jar. Hence, these effects would not be observed in the classical method proposed by Tilley and Terry [1], in which each test portion is incubated in an individual test tube. According to Wilman and Adesogan [7], this particular associative effect in artificial fermenters could be associated with the escape of soluble material from the samples, which may influence the overall microbial population in the incubation medium. This statement agrees with the results obtained in our work.

In vitro digestibility estimates of feeds can be used according to two different objectives: prediction of in vivo digestibility and comparative evaluation among feeds [11]. For the first case, the occurrence of associative effects observed here warns us that the likelihood of the estimates obtained through artificial fermenters could be improved with the incubation of all feeds that compose the diet, following the proportion planned for in vivo use. In this case, it would be expected that the in vitro environment could present similar interactions among feeds compared to the in vivo ruminal digestion. However, simultaneous in vivo and in vitro evaluations are necessary to provide adequate support for this statement. Moreover, the mutual influence among the forages evaluated here shows that any evaluation aiming at discriminating feeds can be compromised if the incubation of those feeds is performed together in the same jar. However, more studies with a larger number of feeds are suggested to enhance our understanding about the influence of associative effects on a procedure to rank or discriminate feeds.

Additionally, researchers and technicians have used both forms of incubation worldwide (i.e., individually incubated feeds and collectively incubated feeds). Inadvertently, many of them have considered both techniques as similar. We showed that they are not similar. Consequently, most researchers and technicians may not be aware that interactive effects among feeds can affect the in vitro estimates of feed digestibility. Our results showed that they should be aware and should use this information in the interpretation of the laboratory test results and/or to adjust more adequate analysis protocols.

5. Conclusions

The incubation of different feeds together in the same jar using artificial fermenters causes associative effects among them. These effects can influence the estimates of in vitro dry matter and fibre digestibility, and change their repeatability pattern.

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Institutional Review Board Statement: The animal care and handling procedures were approved by the Ethics Committee on the Use of Production Animals of the Universidade Federal de Viçosa (protocol number 029/2019).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data generated during the current study are available from the corresponding authors on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Tilley, J.M.A.; Terry, R.A. A two-stage technique for the in vitro digestion of forage crops. *J. Br. Grassl. Soc.* **1963**, *18*, 104–111. [CrossRef]
2. Adesogan, A.T. What are feeds worth? A critical evaluation of selected nutritive values methods. In Proceedings of the 13th Annual Florida Ruminant Nutrition Symposium, Gainesville, University of Florida, Gainesville, FL, USA, 10–22 January 2002; pp. 33–47.
3. Tassone, S.; Fortina, R.; Peiretti, P.G. In vitro techniques using the Daisy^{II} incubator for the assessment of digestibility: A review. *Animals* **2020**, *10*, 775. [CrossRef] [PubMed]
4. Camacho, L.F.; Silva, T.E.; Rodrigues, J.P.P.; Franco, M.O.; Detmann, E. A standard procedure for in vitro digestion using rumen fermenters: A collaborative study. *Animals* **2022**, *12*, 2842. [CrossRef] [PubMed]
5. Lopes, F.C.F.; Dornelas, R.A.C.; Portugal, J.A.B.; Carneiro, J.C.; Verneque, R.S.; Oliveira, J.S.; Arcuri, P.B.; Duque, A.C.A. Dry matter digestibility of corn silages and concentrates determined by in vitro procedures. *Arq. Bras. Med. Vet. Zootec.* **2010**, *62*, 1167–1173. [CrossRef]
6. Holden, L.A. Comparison of methods of in vitro dry matter digestibility for ten feeds. *J. Dairy Sci.* **1999**, *82*, 1791–1794. [CrossRef]
7. Wilman, D.; Adesogan, A. A comparison of filter bags method with conventional tube methods of determining the in vitro digestibility of forages. *Anim. Feed Sci. Technol.* **2000**, *84*, 33–47. [CrossRef]
8. Adesogan, A.T. Effect of bag type on the apparent digestibility of feeds in Ankom Daisy^{II} incubators. *Anim. Feed Sci. Technol.* **2005**, *119*, 333–344. [CrossRef]
9. Mould, F.L.; Kliem, K.E.; Morgan, R.; Mauricio, R.M. In vitro microbial inoculums: A review of its function and properties. *Anim. Feed Sci. Technol.* **2005**, *123–124*, 31–50. [CrossRef]
10. Huhtanen, P.; Rinne, M.; Nousiainen, J. A meta-analysis of feed digestion in dairy cows. 2. The effects of feeding level and diet composition on digestibility. *J. Dairy Sci.* **2009**, *92*, 5031–5042. [CrossRef]
11. Silva, T.E.; Detmann, E.; Camacho, L.F.; Saliba, E.O.S.; Palma, M.N.N.; Valadares Filho, S.C. Comparison of in vitro methods to quantify the dry matter and neutral detergent fibre digestibility of forages and concentrates. *Arq. Bras. Med. Vet. Zootec.* **2017**, *69*, 1635–1644. [CrossRef]
12. Detmann, E.; Costa e Silva, L.F.; Rocha, G.C.; Palma, M.N.N.; Rodrigues, J.P.P. *Métodos Para Análise de Alimentos*, 2nd ed.; Suprema: Visconde do Rio Branco, Brazil, 2021; 350p.
13. Valente, T.N.P.; Detmann, E.; Valadares Filho, S.C.; Da Cunha, M.; Queiroz, A.C.; Sampaio, C.B. In situ estimation of indigestible compounds contents in cattle feed and feces using bags made from different textiles. *Rev. Bras. Zootecn.* **2011**, *40*, 666–675. [CrossRef]
14. McDougall, E.I. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Bioch. J.* **1948**, *43*, 99–109. [CrossRef]
15. Camacho, L.F.; Silva, T.E.; Palma, M.N.N.; Assunção, A.S.; Rodrigues, J.P.; Costa e Silva, L.F.; Detmann, E. Evaluation of buffer solutions and urea addition for estimating the in vitro digestibility of feeds. *J. Anim. Sci.* **2019**, *97*, 922–931. [CrossRef] [PubMed]

16. Machado, M.G.; Detmann, E.; Mantovani, H.C.; Valadares Filho, S.C.; Bento, C.B.P.; Marcondes, M.I.; Assunção, A.S. Evaluation of the length of adaptation period for changeover and crossover nutritional experiments with cattle fed tropical forage-based diets. *Anim. Feed Sci. Technol.* **2016**, *222*, 133–148. [CrossRef]
17. Barbosa, M.M.; Detmann, E.; Rocha, G.C.; Franco, M.O.; Valadares Filho, S.C. Evaluation of laboratory procedures to quantify the neutral detergent fiber content in forage, concentrate, and ruminant feces. *J. AOAC Internat.* **2015**, *98*, 883–889. [CrossRef] [PubMed]
18. Huhtanen, P. Associative effects of feeds in ruminants. *Norw. J. Agric. Sci.* **1991**, *5*, 37–57.
19. Moss, A.R.; Givens, D.I.; Phipps, R.H. Digestibility and energy value of combinations of forage mixture. *Anim. Feed Sci. Technol.* **1992**, *39*, 151–172. [CrossRef]
20. Ramin, M.; Vaga, M.; Cabezas-Garcia, E.H.; Detmann, E. Comparison of methane production from individual feeds and total diets—An in vitro evaluation. *Agraarteadus* **2016**, *27*, 42–47.
21. Detmann, E.; Paulino, M.F.; Valadares Filho, S.C. Avaliação nutricional de alimentos ou dietas? In *Uma Abordagem Conceitual, Proceedings of the 6th Simpósio de Produção de Gado de Corte, Viçosa, Brazil*; Universidade Federal de Viçosa: Viçosa, Brazil, 22–24 May 2008; pp. 21–52.
22. Michalet-Doreau, B.; Ould-Bah, M.Y. In vitro and in sacco methods for the estimation of dietary nitrogen degradability in the rumen: A review. *Anim. Feed Sci. Technol.* **1992**, *40*, 57–86. [CrossRef]
23. Leng, R.A. Factors affecting the utilization of 'poor-quality' forages by ruminants particularly under tropical conditions. *Nutr. Res. Rev.* **1990**, *3*, 277–303. [CrossRef]
24. Detmann, E.; Paulino, M.F.; Mantovani, H.C.; Valadares Filho, S.C.; Sampaio, C.B.; Souza, M.A.; Lazzarini, I.; Detmann, K.S.C. Parameterization of ruminal fibre degradation in low-quality tropical forage using Michaelis-Menten kinetics. *Livest. Sci.* **2009**, *126*, 136–146. [CrossRef]
25. Russell, J.B.; Baldwin, R.L. Substrate preferences in rumen bacteria: Evidence of catabolite regulatory mechanisms. *Appl. Environ. Microbiol.* **1978**, *36*, 319–329. [CrossRef] [PubMed]
26. Carvalho, I.P.C.; Detmann, E.; Mantovani, H.C.; Paulino, M.F.; Valadares Filho, S.C.; Costa, V.A.C.; Gomes, D.I. Growth and antimicrobial activity of lactic acid bacteria from rumen fluid according to energy or nitrogen source. *Rev. Bras. Zootecn.* **2011**, *40*, 1260–1265. [CrossRef]
27. Mould, F.L.; Ørskov, E.R.; Manns, O. Associative effects of mixed feeds. I. Effects of type and level of supplementation and the influence of the rumen pH on cellulolysis in vivo and dry matter digestion of various roughages. *Anim. Feed Sci. Technol.* **1983**, *10*, 15–30. [CrossRef]
28. Costa, V.A.C.; Detmann, E.; Valadares Filho, S.C.; Paulino, M.F.; Henriques, L.T.; Mantovani, H.C. In vitro degradation of neutral detergent fiber from high-quality tropical forage according to supplementation with protein and (or) carbohydrates. *Rev. Bras. Zootecn.* **2009**, *38*, 1803–1811. [CrossRef]