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Comparison of indigestible markers from *in situ* and *in vivo* incubation to predict apparent digestibility in hay- and corn-fed horses

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ABSTRACT. Four castrated crossbred horses were used in a randomized block design to study the use of indigestible internal markers iNDF and iADF obtained *in situ* (from bovines) or *in vivo* (from equines). Treatments consisted of determining digestibility by the direct method comprising total feces collection (TC) and by the indirect method comprising internal markers iNDF and iADF obtained by *in situ* incubation in bovine rumen or i*n vivo* by the mobile nylon bag (MNB) technique with horses. iNDF-IV and iADF-IV resulted in better marker recovery rate (RR) (91.50%), similar to TC. The *in situ* technique resulted in lower RR values for the two indigestible markers, averaging 86.50% ($p < 0.05$). Estimates of the nutrient coefficient of digestibility (CD) were adequately predicted by iADF-IV, for horses fed on hay exclusively, with rates 46.41, 48.16, 47.92 and 45.51% for dry matter (DM), organic matter (OM), FDN and gross energy, respectively. Results show that MNB may be used to obtain iADF in horses fed on coast-cross hay exclusively, whereas NDFi and ADFi were selected for horses fed on mixed diets to predict the coefficient of nutrient digestibility.

Keywords: equine, internal marker, indirect method, mobile nylon bag.

Comparação de indicadores indigestíveis obtidos por incubação *in situ* e *in vivo* para predizer a digestibilidade aparente em cavalos alimentados com feno e milho

RESUMO. Foram utilizados quatro cavalos castrados sem raça definida pelo delineamento em blocos casualizados. Objetivou-se viabilizar os indicadores internos indigestíveis, FDNi e FDAi, obtidas pelas técnicas *in situ* nos bovinos e *in vivo* nos equinos por meio do saco de náilon móvel (SNM). Os tratamentos consistiram de digestibilidade determinada por método direto com a coleta total de fezes (CT) e indireto pelo uso de FDNi e FDAi obtidos pelas técnicas *in situ* (IS) na cavidade ruminal de bovinos e *in vivo* (IV) nos equinos por meio do SNM. As FDNi-IV e FDAi-IV promoveram a melhor taxa de recuperação (TR) do indicador, igualando-se ao grupo CT, em 91,50%, enquanto a técnica *in situ* resultou nas piores taxas, na média 86,50%, para ambos os indicadores indigestíveis ($p < 0,05$). As estimativas dos CD dos nutrientes foram adequadamente preditos pela FDAi-IV, para equinos alimentados com feno, no qual os valores observados foram de 46,41; 48,16; 47,92 e 45,51% para MS, MO, FDN e EB, respectivamente. Concluiu-se que o SNM em equinos pode ser usado para obter a FDAi em equinos alimentados exclusivamente com feno de *coast-cross* e para cavalos consumindo dieta mista, as FDNi e FDAi foram eleitas para predizer CD de nutrientes.

Palavras-chave: equino, indicador interno, método indireto, saco de náilon móvel.

Introduction

Digestive food efficiency and the prediction of nutrition rates in horses are highly relevant for the growth of horse industry and for diet manufacturers. The development of exact methodologies applicable to horses, without any complex techniques and with the lowest manpower rates, are highly urgent due to their special digestive systems. The application of indirect methods of digestibility through internal markers is an alternative to solve these difficulties. Most internal markers may be employed to evaluate food, although Lippke et al. (1986) insist that indigestible fiber may be successfully used to predict food digestion when adequately assessed. The above issue has been the theme of many research works in experimentation with horses. The most important

markers require in-depth investigation: indigestible cellulose has a good capacity to predict the digestion of dry matter (DM), very similar to *in vivo* (ALVARENGA et al., 1997; OLIVEIRA et al., 1998, 2003; STEIN et al., 2006) and in neutral (FDNi) and acid (FDAi) detergent fibers (ALVARENGA et al., 1997; OLIVEIRA et al., 1998, 2003; STEIN et al., 2006) with contradictory results.

Indigestible components of the cell wall, which are present in food, have already been evaluated in ruminants by state-of-the-art methodologies. Indigestible markers may thus be determined through the incubation of nylon bags *in situ* within the rumen cavity (BERCHIELLI et al., 2005; FREITAS et al., 2002; HUHTANEN et al., 1994; ÍTAVO et al., 2002a and b; LIPPKE et al., 1986) or through *in vitro* incubation in the rumen liquid (COCHRAN et al., 1986; FREITAS et al., 2002; HUHTANEN et al., 1994; KRYSL et al., 1988; LIPPKE et al., 1986). However, methodology in the above-mentioned assays with horses for FDNi and FDAi has always been *in vitro,* with the rumen liquid as inoculum. Although *in vitro* methods are generally precise, they require highly complex techniques and equipments (MIRAGLIA et al., 1999), besides keeping bovines with a rumen cannula.

It should be emphasized that available techniques to obtain these markers have been prepared for ruminants, although important differences have been point out with regard to the digestion magnitude and process between horses and cattle. Discrepancies exist on the digestive capacity among the species which may be intensified with fibrous food (CYMBALUK, 1990; MARTIN-ROSSET; DULPHY, 1987). The above observations imply the need for developing methodologies using horse substrata or obtained from the same animal species so that results from tested food which accelerate their nutrition rate process could be better understood.

The above shows that the mobile nylon bag (MNB), with modifications, used in horses to evaluate mainly the digestibility of several types of food by the entire digestive tract (ARAÚJO et al., 1996, 2001; FOMBELLE et al., 2004; MACHEBOUEF et al., 1995) may provide *in vivo* indigestible markers. In fact, surgery to reach the gastrointestinal tract to fix the cannula to the caecum is not practical in the species. Therefore, modified MNB may be an important tool in the precise evaluation of economical issues from biological modifications since they minimize the limiting factors which are inherent to the use of less specific techniques in horses.

Current analysis studies the viability of the internal indigestible markers FDNi and FDAi obtained by *in situ* techniques in bovines and *in vivo* techniques in horses by MNG so that apparent nutrient digestibility in horses fed on hay and on hay+corn could be predicted and compared to the total feces collection method.

Material and methods

Two assays were undertaken in the Equine Metabolism Section of the Department of Animal Sciences of the Federal University of Lavras and in the Laboratory of Animal Nutrition of the Department of Animal Sciences, Esalq/USP, Brazil. Four crossbreed castrated horses, mean age and weight 6 years and 330 kg respectively, were used. Digestion assays were designed in randomized blocks, with four repetitions, in which each horse produced data on total feces collection and in vivo technique by MNB. Each animal was considered as a block, with four repetitions. The five treatments consisted of digestibility evaluation methodologies constituted by the direct method through total feces collection and by the indirect method through internal indigestible markers FDNi and FDAi obtained by in situ techniques in bovines and by in vivo techniques in horses with MNG. Whereas the first assay comprised horses exclusively fed on coast-cross hay, the second comprised animals fed on 70% of coast-cross hay and 30% corn grains, coupled to mineral salt *ad libitum*.

Food quantity to horses followed recommendations by the National Research Council (NRC, 1989) and attended to the nutritional requirements of the species. Daily DM ingestion amounted to 2.0% BW and feeds were provided daily at 8, 12 and 17h. Waste food was removed and weighed 15 min. before each meal. Table 1 shows the chemical composition of hay and corn in assays 1 and 2.

Table 1. Chemical composition of food[1].

Food	DM (%)	Nutrient (% DM)				
		OM	Starch	CP	FDN	FDA
Coast-cross hay	91.20	95.21	2.59	7.47	80.70	42.79
Corn in grain	88.25	98.45	63.59	8.99	20.25	5.34

[1]Data from the Lab. of Animal Nutrition of FMVZ/Unesp and from the Bromatology Lab of Esalq/USP.

Research in each digestion assay had a total duration period of 32 days, divided into two experimental phases and comprising 20 days for Phase I and 12 days for Phase II. Phase I comprised the determination of digestibility by the direct method in which the first 15 days were reserved for the adaptation of animals to bays,

diets and management conditions. The horses were placed in individual 2 x 3 m bays, with a bed-less cement floor, provided with troughs for food, salt and water. The horses were then placed in metabolism cages with plastic pails for water and mineral salt, food trough and feces collector with total collection during 5 days. Feces collection was undertaken four times a day (6, 12, 18 and 24h), weighed, homogenized and sampled in 10%. Samples were placed in identified plastic bags and stored at –15°C for further analysis. Prior to the start of the experiment, wide spectrum anthelmintics were given to the horses.

At the end of Phase II feces samples were thawed at room temperature and homogenized per treatment to obtain a composed sample for each animal. Further, 10% aliquots were then retrieved, weighed and pre-dried in a buffer at 60ºC for 72h. After drying, the samples were once more weighed and ground in a 1 mm mesh sieve. Bromatological analyses (DM, OM and CP) for hay, corn and feces were undertaken according to methodology by Silva (1989), and for cell wall components (FDN and FDA), according to Van Soest et al. (1991), at the Animal Nutrition Laboratory of the Faculty of Veterinary Medicine and Animal Sciences, Unesp, Campus of Botucatu, São Paulo State, Brazil. Starch rates were determined following Macrae and Armstrong (1968) at the Bromatology Lab of the Department of Animal Sciences of Esalq-USP, Brazil. CE rates of grains, hay and feces were determined by adiabatic calorimeter (Parr Instruments Co).

Phase II comprised the measurement of indirect methodologies during six days for each in situ and in vitro techniques. Nylon bags, 7 x 14 cm and diameter 60 mm, with 7 g of ground sample at 1 m to maintain density between 10 and 20 mg DM of the sample per cm^2 of the bag surface, were used to obtain indigestible markers of bovines by the in situ method (NOCEK, 1988). Hay samples with six repetitions and feces samples in quadruplets for each horse of the previous phase, originating from assay 1, coupled to the hay, corn and equine feces of assay 2, respectively with 6, 12 and 4 repetitions, were incubated in the rumen of a Dutch cow which was fistulated in the rumen for 144h and fed on coast-cross hay and mineral salt.

The determination of in vivo indigestible markers in horses was done by MNB and this experiment phase was undertaken as a continuation of the digestibility assay by TC. The same horses housed in brick bays were used, fed on coast-cross hay, as described previously. White polyester nylon bags, 3.5 x 6.5 cm and porosity of 60 micros, following Araújo et al. (1996), were used. Further, 1 g of ground sample for each 1 mm of hay, corn or feces from the direct method was placed in each nylon bag. The relationship 10 to 20 mg of DM of the sample per cm^2 of bag surface was maintained, following Nocek (1988).

Nylon bags were inserted in four horses by a nasal-gastric tube during 6 days alternately, or rather, intubation bags of assay 1 were inserted on days 1, 3 and 5; intubation bags of assay 2 were inserted on days 2,4 and 6; re-intubations occurred on days 3, 4, 5 and 6. Therefore, nylon bags recovered in the feces were twice re-intubated by the nasal-gastric tube up to a minimum of 144h incubation. Mean passage time of nylon bags through the horses' digestive tract was 48h. Four bags with coast-cross hay and 16 bags, respectively with hay, corn and equine feces (4 per horse), totaling 20 bags $horse^{-1}$ of assay 1 were inserted at 13h of the first day of nasal-gastric tube passage. On the second intubation day, 4, 5 and 16 bags, respectively with hay, corn and equine feces (4 for each horse), totaling 25 bags $horse^{-1}$ of assay 2, were inserted per horse. The number of repetitions in assay 1 amounted to 16 bags of hay and feces for each, whereas assay 2 contained the same number of repetitions for hay and feces, although the corn one had 20 bags. Nylon bag collection occurred 4 times a day, at 6, 12, 18 and 24h. They were identified on a chart and then immediately frozen at –15°C in a freezer, till the next re-intubation. Bags were thawed at the appropriate moment at room temperature and pre-dried in a buffer at 55°C for later intubation.

After in situ or in vivo incubations, the bags were washed in a washing-machine with running water till water was transparent (40 min.) and kept in a buffer for 72h at 60°C. Nylon bags with digestion residues of hay, corn and feces, per animal and per methodology, were opened and a composed sample was formed. Digestion residues underwent extraction by neutral and acid detergents, with the formation of FDNi and FDAi.

Coefficient of Digestibility (DC) of DM was estimated by the equation suggested by Church (1993):

$$DCDM\ (\%) = 100 - \left(100 \times \frac{\%\ \text{Marker in Diet}}{\%\ \text{Marker in Feces}}\right)$$

$$DCN\ (\%) = 100 - \left(100 \times \frac{\%\ \text{Marker in Diet}}{\%\ \text{Marker in Feces}} \times \frac{\%\ \text{Nutrient in Feces}}{\%\ \text{Nutrient in Diet}}\right)$$

Recuperation Rate (RR) of markers was estimated according to Krysl et al. (1988) by the equation below:

$$RR\ (\%) = \frac{\%\ \text{Marker in Feces x Observed Fecal Production (g)}}{\text{Ingested Marker (g)}} \times 100$$

Nutrients' digestibility coefficients from direct and indirect methods by FDNi and FDAi with *in situ* and *in vivo* techniques from digestion assays, were processed by variance analysis Statistical Analysis System (SAS, 2000), following the model: $Yij = \mu + B_i + Tj + Eij$; in which Yij = fecal production and apparent digestibility coefficient of nutrients of horse i fed on treatments j; μ = general constant; B_i = effect of horse i, in which i = 1,2,3,4; Tj = effect of treatment j, in which j = 1,2,3,4,5; Eij = randomized error associated with each observation Yij.

Comparison of means was undertaken by Tukey's test at 5% significance.

Results and discussion

Table 2 shows concentration, intake and recuperation rate of markers FDNi and FDAi obtained from *in situ* and *in vivo* coast-cross hay. Methodology's significant effect ($p < 0.05$) was verified, namely, in situ in bovine rumen cavity x in vivo in horses by MNB, employed to obtain non-indigestible markers in horses fed exclusively on hay in which in vivo determinations produced highest FDNi and FDAi concentrations, or rather, 32.26 and 19.45%, respectively, when compared to in situ technique. Berchielli et al. (2005) obtained the same response when they worked with in vitro and in situ methods to determine FDNi and FDAi in cattle feeding. The authors reported that a greater attention must be taken when comparing estimates from different methodologies. They concluded that there probably exists a marker which is adequate for each roughage. Greater standardization of these techniques in experiments with horses is highly required.

Markers' rates in current assay varied between 23 to 32% for FDNi, very similar to that reported in the literature for forage between 31 and 33% (BERCHIELLI et al., 2005; LIPPKE et al., 1986), whereas FDAi with rates between 14 and 19% was lower than that reported by Berchielli et al. (2005) for Tifton hay in bovines which varied between 24 and 39% for in situ and in vitro incubations. Once more this fact reveals the lack of homogeneity of analysis methods when the distinct techniques used become the main divergence factors in the results of the research.

One of the most important characteristics in an ideal marker is foregrounded in its resistance capacity to digestion during exposition by the gastro-intestinal tract (FAICHNEY, 1975 apud COCHRAN et al., 1986). Analysis of the fecal recuperation rate of markers (Table 2) showed MNB-caused low digestion for FDN-IV and FDA-IV. This is due to the fact that it triggered a better fecal recuperation and became equal to the TC group, respectively with rates 91.76 and 91.28%. Above rates are in conflict with those by Oliveira et al. (2003) working on the same animal species and food. Fecal recuperation of in vitro-obtained FDNi and FDAi were positive, respectively 137.87 and 153.78%. In spite of the different techniques used to determine indigestible markers, the literature suggests the occurrence of an increase in residue after in vitro incubation and thus the overestimation of marker amount in the sample as a consequence of particles that adhere to the wall and tube lid (FREITAS et al., 2002). Cochran et al. (1986) also state that these positive recuperations are due to links between free phenolic monomers and diet components of low molecular weight, with a consequent increase in the contribution of the lignin fraction in the feces. However, mean rates for the recuperation of FDNi (90-94%) and FDAi (87-90%) for forage in cattle feed were coherent to those in current research (COCHRAN et al., 1986; KRYSL et al., 1988; LIPPKE et al.,1986).

Lowest rates in fecal recuperation of indigestible markers occurred in the in situ technique, with average 86.5% (Table 2). This fact shows their significant disappearance during the gastro-intestinal transition. Incapacity to recuperate quantitatively FDNi-IS and FDAi-IS shows the fragility in predicting the apparent digestibility of coast-cross hay nutrients when provided exclusively in horse feed.

Table 2. Concentration (% DM), intake (g day^{-1}) and recuperation rate (%) of indigestible markers in different methodologies for horses fed on coast-cross hay.

Variable	Methodology					CV (%)
	TC[1]	*in situ*		*in vivo*		
		FDNi[2]	FDAi[3]	FDNi	FDAi	
Concentration	-	23.83^{b}	14.54^{d}	32.26^{a}	19.45^{c}	0.10
Intake	-	130.65^{b}	79.72^{d}	176.87^{a}	106.63^{c}	0.10
Rate Recuperation	100^{a}	88.03^{ab}	85.15^{ab}	91.76^{a}	91.28^{a}	3.51

[1]TC = Total collection of feces; [2]FDNi = indigestible neutral detergent fiber; [3]FDAi = indigestible acid detergent fiber; Means with different letters on the same row differ ($p < 0.05$) among themselves by Tukey's test.

Table 3 shows coast-cross hay coefficients of apparent digestibility in horses' diet obtained with indigestible markers by in situ and in vivo methodologies. Estimates of digestible coefficients

of DM, OM, NDF and CE were predicted successfully with FDAi-IV, equaling with group TC ($p > 0.05$), FDNi-IS/IV and FDA-IS significantly underestimated the nutrients' determinations for horses fed on coast-cross hay. FDAi superiority in predicting nutrient digestibility with regard to FDNi obtained in situ in bovine rumen cavity has been reported in assays developed with horses and ruminants fed exclusively on roughage (BERCHIELLI et al., 2005; ÍTAVO et al., 2002a; PENNING; JOHNSON, 1983). This fact was confirmed by current essay, although it disagrees with regard to the aspect that only FDAi caused by in vivo incubation by MNB in horses was equal to the conventional method. The above shows the need for techniques specific to horses to standardize methods for the evaluation of nutrient apparent digestibility by the indirect method.

Protein digestibility was not adequately estimated by any indigestible markers under analysis (Table 3). Investigating the efficiency of markers FDAi, FDNi, CELi and lignin in horses fed on diets composed of concentrate:roughage in the proportions 60:40, 40:60, 20:80 and 0:100, Oliveira et al. (2003) obtained similar results. The authors concluded that CELi was the only marker with the best capacity to estimate the coefficient of digestibility of crude protein in diets with up to 80% hay. However, further research has to be undertaken to investigate this issue. Conversely, the coefficient of digestibility of crude energy of horses fed on coast-cross hay in current assay may be efficiently determined by all indigestible markers, FDNi and FDAi, regardless of the methodology used to obtain them ($p > 0.05$).

Table 3. Coefficients of apparent digestibility (CD) of dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (FDN) and crude energy (CE) of coast-cross hay obtained by different methodologies in horse feed (% DM).

Variable	Methodology					CV (%)
	TC[1]	*in situ*		*in vivo*		
		FDNi[2]	FDAi[3]	FDNi	FDAi	
CDMS	46.41[a]	39.08[b]	36.09[b]	41.66[b]	41.76[a]	4.97
CDMO	48.16[a]	41.07[b]	38.97[b]	43.56[b]	43.66[a]	4.56
CDPB	46.90[a]	39.71[b]	37.58[b]	42.21[b]	42.28[b]	4.54
CDFDN	47.92[a]	40.77[b]	38.66[b]	43.29[b]	43.40[a]	4.12
CDEB	45.51[a]	48.29[a]	48.04[a]	40.62[a]	40.78[a]	7.46

[1] TC = Total collection of feces; [2]FDNi = indigestible neutral detergent fiber; [3]FDAi = indigestible acid detergent fiber; Means with different letters on the same row differ among themselves ($p < 0.05$) by Tukey's test.

The second assay also showed the significant effect of in situ x in vivo methodology to obtain indigestible markers, FDNi and FDAi, for horses fed on mixed diets composed of 70% coast-cross hay and 30% corn grains (Table 4). However, in current study all markers, regardless of acquiring technique, were recovered efficiently in the feces, and thus equaled to the TC group ($p > 0.05$) with mean rate 101.7%. The event of practically zero digestion of FDNi and FDAi during the gastro-intestine transition is an asset to these markers as having great capacity in predicting exactly nutrient digestibility in horses fed on a mixed diet.

Contradictory results are verified in experiments with horses with regard to the efficiency of recuperation of the markers. Oliveira et al. (2003) verified positive recuperation rates 118.37 and 122.88% for FDNi and FDAi, respectively, obtained by in vitro incubation with rumen liquid in horses fed on mixed diet, in the proportion 60:40 of roughage:concentrate. Conversely, Stein et al. (2006) evaluated FDAi, CELi and lignin as markers in the diet of mares fed on a mixed diet and obtained a 100% fecal recuperation ($p < 0.05$) for FDAi. This fact confirms rates in current research.

Data analysis from digestion assays in current experiment, related to the efficiency of indigestible markers under analysis, shows that, besides the recommendation of Berchielli et al. (2005) with regard to the possible need of having a specific marker for each type of roughage, the type of food for the animals must be decided upon. Diet type, exclusively with roughage or mixed diet, interferes in the fecal recuperation rate of the markers. In other words, they behave differently in each situation.

Table 4. Concentration (% DM), intake (g day^{-1}) and recuperation rate (%) of indigestible markers with different methodologies for horses fed on coast-cross hay and corn grains.

Variable	Methodology					CV (%)
	TC[1]	*in situ*		*in vivo*		
		FDNi[2]	FDAi[3]	FDNi	FDAi	
Concentration	-	17.35[b]	10.55[d]	23.54[a]	14.09[c]	1.28
Intake	-	102.22[b]	62.11[d]	138.82[a]	83.26[c]	2.75
Rate Recuperation	100[a]	100.38[a]	99.92[a]	102.86[a]	103.67[a]	3.46

[1]TC = total collection of feces; [2]FDNi = indigestible neutral detergent fiber; [3]FDAi = indigestible acid detergent fiber; Means with different letters in the same row differ among themselves ($p < 0.05$) by Tukey's test.

Table 5 shows the coefficients of apparent digestibility of coast-cross hay and corn gains in horse feed from indigestible markers by in situ and in vivo methodologies. The CD of DM, OM, CP, FDN and starch determined by TC were similar ($p > 0.05$) to those estimated by FDNi and FDAi in the two techniques, which is different from what occurred in assay 1. The efficiency of these indigestible markers to estimate CD of nutrients coincides with predictions in several studies on ruminants (COCHRAN et al., 1986; KRYSL et al., 1988) and horses (OLIVEIRA et al., 2003; STEIN et al., 2006) when a mixed diet in the animals' feed was used.

When decisions have to be taken on indigestible markers to estimate the digestibility of nutrients in assays with horses, based on assays of current research, the adequate markers are FDNi and FDAi in roughage and grains feed system. However, in a diet composed exclusively on hay, only FDAi may be used since it causes less variations in laboratory analyses and has no hemicellulose, a component present in large proportions in roughage. According to Ítavo et al. (2002a), hemicellulose is greatly accountable for variations found in several experiments on FDNi.

Table 5. Coefficients of apparent digestibility (CD) of dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (FDN) and starch from different methodologies for horses fed on coast-cross hay and corn grains (% in DM).

Variable	TC[1]	Methodology				CV (%)
		in situ		*in vivo*		
		FDNi[2]	FDAi[3]	FDNi	FDAi	
CDMS	52.63	52.62	52.42	53.74	54.18	3.60
CDMO	54.17	54.61	54.42	55.68	56.10	3.31
CDPB	64.90	64.90	64.76	65.73	66.05	2.15
CDFDN	43.73	43.82	43.56	45.13	45.66	5.13
CDAmido	98.28	98.30	98.30	98.34	98.36	0.07

[1]CT = Total collection of feces; [2]FDNi = indigestible neutral detergent fiber; [3]FDAi = indigestible acid detergent fiber; Means with different letters on the same row differ among themselves ($p < 0.05$) by Tukey's test.

Conclusion

The *in vivo* method in horses by MNG may be used to obtain indigestible markers and is a promising technique in the experimentation on this species.

FDAi *in vivo* has proved to be the best marker to estimate the coefficients of digestibility of DM, OM, CP, FDN, CP and starch for horses fed exclusively on coast-cross hay. On the other hand, the chosen markers in mixed diet were FDNi and FDAi for the two techniques.

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