WORD RABBIT SCIENCE 2000, VOL.8 (2), 93-97.

COMPARISON OF DIFFERENT *IN VITRO* DIGESTIBILITY METHODS FOR NUTRITIVE EVALUATION OF RABBIT DIETS.

PASCUAL J.J., CERVERA C., FERNÁNDEZ-CARMONA J.

Departamento de Ciencia Animal, Universidad Politécnica de Valencia, P.O. Box 22012, VALENCIA 46071, Spain.

Abstract : Seven simplified experimental diets for rabbits with a wide range of acid detergent fibre content (87 to 525 g kg^{-1} dry matter) were used to compare three different *in vitro* techniques for evaluating their dry matter and organic matter digestibilities, based on the use of multienzyme, caecal or faecal inocula. The multienzyme method always resulted in higher *in vitro* dry matter digestibility values (P<0.001) than those methods based on the use of digestive tract inocula. The prediction equations for dry matter digestibility obtained with the multienzyme and caecal *in vitro* techniques showed higher precision (r^2 = 0.95 and 0.88, respectively) and lower variability (se: 4.32 and 6.58, respectively) than that based on the chemical composition (r^2 = 0.80 and se = 8.34), while the method using faecal inocula showed lower precision and variability (r^2 = 0.68 and se = 10.64). Repeatability was good for the three techniques (0.38 to 1.05), although the multienzyme method was significantly better (P<0.05). However, the caecal and faecal *in vitro* methods showed clearly poorer results for reliability (P<0.001) than the multienzyme technique (2.61, 2.65 and 0.71 for dry matter digestibility prediction, respectively).

RESUME : Comparaison de différents méthodes de digestibilité *in vitro* pour l'évaluation de la valeur nutritive des aliments pour lapins.
Sept aliments expérimentaux contenant un large éventail de taux de lignocellulose (87 à 525 g ADF kg^{-1} de matière sèche) et dont la digestibilité *in vivo* avait été antérieurement déterminée, ont été utilisés pour comparer 3 techniques *in vitro* d'estimation de la digestibilité de la matière sèche (MS) et de la matière organique. La première, appelée "multienzymes", est basée sur l'utilisation en séquence de 3 cocktails enzymatiques, et les deux autres sur une incubation avec un inoculum caecal ou fécal. La technique multienzymes conduit systématiquement à des valeurs de digestibilité supérieures (P<0.001) à celles obtenues avec un inoculum provenant du tractus digestif, et très proches de celles observées *in vivo*. Les équations de prédiction de la digestibilité de la MS obtenues avec la méthode multienzymes ou l'inoculum caecal sont plus précises (R^2 = 0,95 et 0,88 respectivement) et moins variables (écart types de 4,32 et 6,58) que celle basée sur la composition chimique (en fait sur le taux d'ADF, R^2 = 0,80 et écart type de 8,34) ou surtout celle basée sur l'inoculum fécal (R^2 = 0,68 et écart type de 10,64). La répétabilité (détermination faite 3 fois un jour donné) est bonne pour les 3 méthodes (0,38 à 1,05) bien que la méthode multienzymes soit significativement meilleure (P<0,05). Par contre, les méthodes *in vitro* basées sur un inoculum caecal ou fécal ont une plus mauvaise fiabilité (3 séries de déterminations faites à un mois d'intervalle) que la méthode multienzymes (2,61 - 2,65 et 0,71 respectivement, lors de l'estimation de la digestibilité de la MS).

INTRODUCTION

Feed evaluation is frequently performed in time-consuming and costly experiments based on *in vivo* determinations, requiring animals and relatively large amounts of feed. Other methods have been developed to estimate the nutritive value of rabbit feeds using easy, quick and less costly techniques. In rabbits, the prediction of nutrient digestibility based on chemical composition (DE BLAS *et al.*, 1992; FERNÁNDEZ-CARMONA *et al.*, 1996; VILLAMIDE and FRAGA, 1998) has proved to be valuable, but *in vitro* techniques that simulate the digestive process (FERNÁNDEZ-CARMONA *et al.*, 1993; RAMOS and CARABAÑO, 1996), and more recently, the nutritive evaluation by mean of near-infrared reflectance spectra of feeds (XICCATO *et al.*, 1999), appear to be more precise techniques.

In vitro methods for the evaluation of feeds have been developed using either contents of the rabbit caecum or different parts of the digestive tract (ADERIBIGBE *et al.*, 1992; FERNÁNDEZ-CARMONA *et al.*, 1993), or blends of enzymes (RAMOS and CARABAÑO, 1996) as inocula for incubations. These methods have been shown to correlate relatively well with the *in vivo* apparent digestibilities of dry matter (DM) and organic matter (OM), but no attempts have previously been made to evaluate their repeatability and reliability over time. Thus, the aim of the present work was to provide some information about the precision, repeatability and reliability of three different *in vitro* methods based on enzymatic, caecal and faecal inocula for estimating digestibility in rabbits.

Contact author: J.J. Pascual
phone: +34 63 877432 ; fax: +34 63 877439
e.mail: jupascu@dca.upv.es

MATERIAL AND METHODS

Diets

Seven experimental and simplified pelleted diets for rabbits described by FERNÁNDEZ-CARMONA *et al.* (1996) were used to study the different *in vitro* digestibility techniques evaluated in the present work. Diets were selected in order to obtain a wide range of acid detergent fibre content (87 to 525 g ADF kg^{-1} DM), mainly responsible for low digestibility of rabbit diets. The ingredients, chemical composition and *in vivo* apparent digestibility for DM and OM are shown in Table 1.

Table 1 : Ingredients, chemical composition (% on DM basis), energy content (MJ kg^{-1} DM) and digestibility of diets (%)[a]

n°	Ingredients	OM	CP	EE	CF	NDF	ADF	GE	dDM	dOM
1	Olive oil cake	92.1	10.0	9.2	25.8	64.0	52.5	20.9	37.1	36.5
2	Grass hay	92.1	9.0	2.1	29.4	58.7	35.9	19.1	39.3	38.4
3	Alfalfa hay, 66.7% Paprika residue, 33.3%	88.0	17.2	3.0	24.8	48.6	31.3	18.6	54.2	52.4
4	Beet pulp	93.4	8.9	1.0	18.0	43.7	23.3	17.6	75.7	77.6
5	Barley, 66.7% Corn stover, 33.3%	93.6	10.3	2.1	15.4	38.8	17.5	18.6	61.6	62.8
6	Corn, 66.7% Alfalfa hay, 33.3%	95.5	12.5	3.4	11.7	28.3	14.2	18.8	72.7	73.4
7	Wheat shorts	94.4	14.4	3.4	6.1	30.9	8.7	18.6	78.3	79.3

[a] All diets were corrected by mineral-vitamin mixes (0.5-2.5%).

In vitro techniques

Three different *in vitro* techniques were evaluated in the present study: a multienzyme system described by RAMOS and CARABAÑO (1996); a caecal inoculum system described by FERNÁNDEZ-CARMONA *et al.* (1993) based on the methods developed by LINDGREN (1979) for ruminants and LÖWGREN *et al.* (1988) for pigs; and a faecal inoculum system following a similar procedure.

Multienzyme technique

As described by RAMOS and CARABAÑO (1996), 1 gram of 1 mm ground samples were carefully mixed in a flask with 25 ml of a phosphate buffer (0.1M, pH 6.0) and 10 ml of 0.2M HCl solution, and pH was adjusted to pH 2 with HCl or NaOH 1M solutions. Then 1ml of pepsin solution (25 mg of pepsine from porcine Merck n 7190 / ml 0.2M HCl) was added and, after gentlly stirring the flasks, were closed and incubated in an oven at 40°C for 1.5 h. After incubation, pH of each flask was increased up to 6.8 by the addition of 10 ml of a phosphate buffer (0.2M, pH 6.8) and 5 ml of 0.6M NaOH solution. After gentle stirring and a new pH adjustment, 1 ml of pancreatin solution (100 mg of pancreatin from porcine Sigma n 1750 /ml phosphate buffer pH 6.8) was added to each flask, and mixed. Flasks were closed and incubated in an oven at 40°C for 3.5 h. After the second incubation, pH was adjusted to 4.8 with acetic acid, and 0.5 ml of Viscozyme 120L (Novo Nordisk) was added. Flasks were again incubated in an oven at 40°C for 16 h after gentle stirring.

Caecal and faecal techniques

Caecal and faecal inocula, and the artificial saliva solution were prepared as described by FERNÁNDEZ-CARMONA *et al.* (1993). Twelve New Zealand White × Californian growing rabbits, given the same commercial diet and showing a normal weight gain and food intake, were randomly selected. Their faeces were collected daily for five d and then they were slaughtered. Two hundred grams of caecal or faecal content were diluted with 320 ml of artificial saliva solution (8 g of $NaHCO_3$, 4 g of K_2HPO_4, 0.5 g of $(NH_4)_2HPO_4$, 1.5 g of NaCl and 0.5 g of $MgSO_4 \cdot 7H_20$ per 1 of distilled water) under a stream of CO_2 gas. Caecal and faecal contents were filtered and macerated at 40°C under a constant stream of CO_2 gas, for 0.5 and 1 h. for caecal and faecal solutions, respectively. After maceration, caecal and faecal solutions were centrifuged at 3500 rpm for 5 min, and 1680 ml of artificial saliva solution were added to the supernatant, obtaining the caecal and faecal inocula, which were maintained at 40°C under a constant stream of CO_2. In each dried and pre-weighed filter crucible digestion glass (volume 100 ml and filter porosity n 2), 1 g of 1-mm ground sample was added to 50 ml of caecal or faecal inoculum. Digestion glasses were closed under a constant stream of CO_2, and incubated in an orbital bath at 40°C for 36 h under constant stirring at 40 fluctuations per min.

After incubation, undigested residue was collected by filtration, and washed with distilled water 5 times and with ethanol and acetone (50 ml) once. DM and OM of undigested residue were determined following the method of the Association of Official Analytical Chemists (1984). *In vitro* DM (dDM_i) and OM (dOM_i) digestibilities (%) were calculated as:

$$dDM_i = [((DR_s - G) - (DR_b - G))/W_s] \times 100$$
$$dOM_i = [((DR_s - IR_s) - (DR_b - IR_b))/W_s] \times 100$$

where, DR_s and DR_b are the weight of the glass and residue dried at 103°C for samples and blank samples, respectively; IR_s and IR_b are the weight of the glass and residue incinerated at 500°C for samples and blank samples, respectively; G is the weight of the glass; and W_s is the weight of sample on a DM basis.

Three replicates were carried out for each sample in order to determine the repeatability of methods, and this procedure was repeated three times (one a month) in order to calculate the reliability of methods.

Table 2 : Effect of method and time on the *in vitro* digestibility values of DM obtained for the evaluated diets (%)

n°	Ingredients	Method			Time[1]				Significance	
		Multi-enzyme	Caecal	Faecal	1	2	3	SE	Method	Time
1	Olive oil cake	36.43[b]	26.30[a]	25.77[a]	29.13	29.03	30.35	0.703	***	NS
2	Grass hay	44.76[b]	35.09[a]	35.42[a]	38.20	38.14	38.94	0.703	***	NS
3	Alfalfa hay, 66.7% Paprika residue, 33.3%	59.64[b]	39.98[a]	39.86[a]	46.58	46.50	46.40	0.703	***	NS
4	Beet pulp	80.19[c]	36.83[a]	41.83[b]	50.68[a]	52.59[a]	55.39[b]	0.718	***	**
5	Barley, 66.7% Corn stover, 33.3%	66.79[b]	56.95[a]	53.42[a]	60.12	58.37	58.67	0.703	***	NS
6	Corn, 66.7% Alfalfa hay, 33.3%	70.11[c]	46.87[b]	40.90[a]	55.48[b]	51.41[a]	50.99[a]	0.703	***	***
7	Wheat shorts	75.42[c]	69.76[b]	59.69[a]	71.80[b]	66.43[a]	66.49[a]	0.718	***	***

[1] Procedure was repeated three times (one a month) SE: standard error ; *** P<0.001
[a,b,c] Means within a row with different letters are different (P<0.05).

Repeatability is considered to be the standard deviation of laboratory consistency and was calculated as the mean of standard deviation for each determination. Moreover, reliability is considered to be the standard deviation through time and was calculated as the standard deviation of the means for each determination through time.

Statistical analysis

Data were analysed by variance analysis, using a mixed procedure (PROC MIXED) of SAS (STATISTICAL ANALYSIS SYSTEM INSTITUTE, 1996) and according to a repeated measures design that takes into account the variation between diets and covariation within them. To study the effect of method and time on the *in vitro* digestibility of diets, the model included as fixed effects the diets, the methods, the time and their interactions, adjusting (REPEATED statement) the data if correlated within the same diet. Repeatability was estimated using method and time as fixed effects, while for reliability only method was included as fixed effect. Covariance structures from the mixed procedures were objectively compared using the most severe criteria (Schwarz Bayesian criterion), as suggested by LITTELL *et al.* (1998). A linear regression method (PROC REG) was used to obtain the regression equations for digestibility prediction from each *in vitro* digestibility method evaluated.

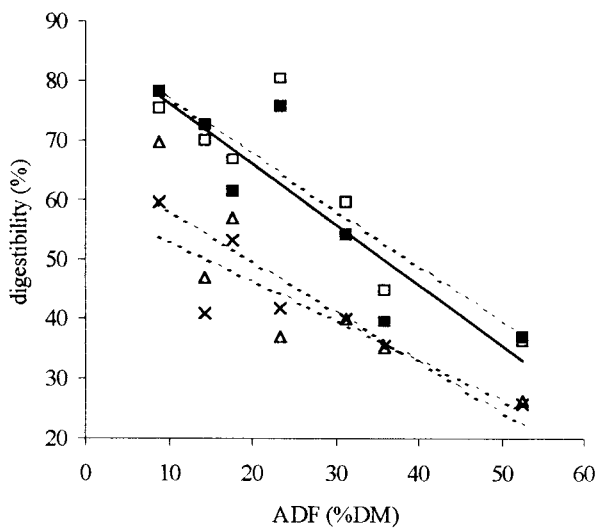

Figure 1 : Effect of ADF content on the *in vivo* (——) and *in vitro* (------) digestibility values of samples : ■ *in vivo*, □ multienzyme, Δ caecal and × faecal values.

RESULTS AND DISCUSSION

Data in Table 2 show the effect of the different *in vitro* digestibility methods and the time on the dDM_i values obtained for the different diets evaluated. The multienzyme method always showed significantly higher dDM_i values (P<0.001) and, as can be seen in Figure 1, nearer to *in vivo* values than caecal and faecal methods. Therefore , the multienzyme *in vitro* technique seemed to yield higher degradability of samples than caecal and faecal techniques.

However, the difference between the *in vivo* and caecal digestibility values decreased (Figure 1), as also showed by FERNÁNDEZ-CARMONA *et al.* (1993) for the caecal digestibility of barley grain (79.9 and 81.8% for *in vivo* and caecal digestibility, respectively). Caecal values for dDM_i were similar to those obtained by ADERIBIGBE *et al.* (1992) for similar diets and raw materials to those used in this experiment.

In general, time had no effect on mean dDM_i (50.3, 48.9 and 49.6% for the 1st, 2nd and 3th month, respectively), but some diets (4, 6 and 7) showed significantly differences in their dDM_i values at the different times. These differences were due to punctual variations on the caecal and faecal digestibility values,

Table 3 : Regression equations (a + bx), precision, repeatability and reliability of different *in vitro* digestibility methods evaluated.

	y	a	b	x	R^2	RSD	Repeatability	Reliability
				Dry matter (DM)				
Multienzyme method	DDM	-3.64	1.025	dDM_i	0.946	3.86	0.387^a	0.709^a
Caecal method		10.41	0.988		0.884	4.27	0.983^b	2.611^b
Faecal method		8.15	1.162		0.684	9.86	0.810^b	2.651^b
				Organic matter (OM)				
Multienzyme method	dOM	-3.12	1.041	dOM_i	0.949	3.98	0.377^a	0.797^a
Caecal method		8.51	1.049		0.888	4.86	1.045^b	3.838^b
Faecal method		7.22	1.184		0.678	10.47	0.874^b	2.698^b

dDM, dOM: *in vivo* DM and OM digestibilities (%). dDM_i, dOM_i: *in vitro* DM and OM digestibilities (%). R^2: coefficient of determination. RSD: residual standard deviation. Repeatability as standard deviation of laboratory consistency. Reliability as standard deviation through time. ^{a,b,c} Means within a column with different letters are different ($P<0.05$).

while the multienzyme technique did not show these fluctuations in their values across time.

With respect to dDM and dOM prediction, these can be estimated based on chemical composition of the diets, especially from their ADF and CF contents. The equations for the diets used in the present experiment are:

$dDM = 96.4 - 1.01\ ADF(\%DM)$ ($R^2 = 0.799$ $SE = 8.343$)
$dOM = 88.1 - 1.07\ ADF(\%DM)$ ($R^2 = 0.792$ $SE = 9.002$)

where the coefficient of determination and SE values are not very different from those deduced in other works (DE BLAS *et al.*, 1992; VILLAMIDE and FRAGA, 1998), and especially the work of FERNÁNDEZ-CARMONA *et al.* (1996) with all 23 diets.

The prediction equations obtained with the multienzyme and caecal *in vitro* techniques (Table 3) showed higher precision (R^2 = 0.95 and 0.88, respectively) and lower SE (4.32 and 6.58, respectively) than ADF based equations, but faecal technique gave poorer results (R^2 = 0.68 and SE= 10.64). The differences between them were mainly due to the inadequate prediction of beet pulp with the methods using digestive tract inocula, giving higher dDM and dOM for the beet pulp than those expected from their caecal and faecal *in vitro* digestibility (Figure 2). However, FERNÁNDEZ-CARMONA *et al.* (1996) suggested that the high *in vivo* digestibility values obtained for beet pulp samples could be due to low feed intake (43 g DM kg^{-1} day^{-1}).

Table 3 also shows the repeatability and reliability estimates of the different *in vitro* techniques evaluated. The multienzyme technique showed a better repeatability ($P<0.05$) than caecal and faecal techniques, but repeatability values obtained for all methods were good (S.D.: 0.38 to 1.05). However, as expected, the caecal and faecal *in vitro* methods showed clearly poorer reliability ($P<0.001$) than the multienzyme technique. These results seem to be related to the higher variability of the caecal and faecal inocula (from different animals and with a higher preparation variability) than multienzyme inoculum.

In conclusion, the multienzyme technique seems to show a higher degradability of samples than caecal and faecal techniques, especially for fibrous diets.

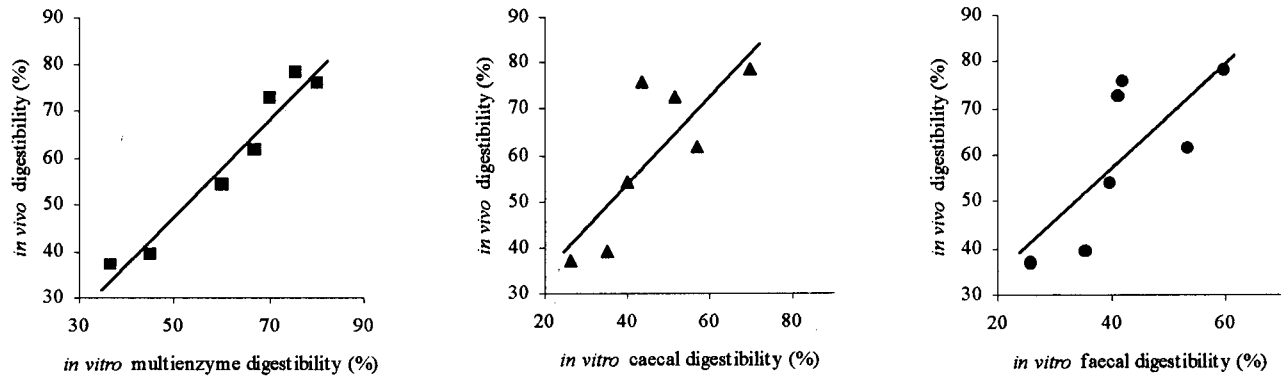

Figure 2 : Relationship between the *in vivo* and *in vitro* DM digestibility values with the different *in vitro* techniques evaluated: ■ multienzyme, ▲ caecal and ● faecal.

Multienzyme and caecal techniques showed adequate precision and repeatability for DM and OM digestibility prediction. However, the more disappointing results over time and the lower precision with the caecal technique indicate that it is necessary to improve its standardisation.

Acknowledgements : The present work was supported by a grant from CICYT (AGF97-1139).

Received : February 11th, 2000
Accepted : June 7th, 2000

REFERENCES

ADERIBIGBE, A.O., CHEEKE, P.R., PATTON, N.M., 1992. *In vitro* rumen and rabbit cecum digestibilities: I. Effects of probiotics and incubation time. *Journal of Applied Rabbit Research,* 15: 948-958.

DE BLAS, C., WISEMAN, J., FRAGA, M.J., VILLAMIDE, M.J., 1992. Prediction of the digestible energy and digestibility of gross energy of feeds for rabbits. 2. Mixed diets. *Animal Feed Science and Technology,* 39: 39-59.

FERNÁNDEZ-CARMONA, J., CERVERA, C., BLAS, E., 1993. Un nuevo método *in vitro* para el estudio de la digestion en el conejo. *XVIII Symposium de Cunicultura, Granoller, Spain,* 43-45.

FERNÁNDEZ-CARMONA, J., CERVERA, C., BLAS, E., 1996. Prediction of the energy value of rabbit feeds varying widely in fibre content. *Animal Feed Science and Technology,* 64:61-75.

LINDGREN, E., 1979. The nutritional value of roughages determined *in vivo* and by laboratory methods. *Report no.45, Upssala, University of Agricultural Science, Dept. Animal Nutrition,* 58: 233-243.

LITTELL, R.C., HENRY, P.R., AMMERMAN, C.B., 1998. Statistical Analysis of Repeated Measures Data Using SAS procedures. *Journal of Animal Science,* 76: 1216-1231.

LOWGREN, W., GRAHAM, H., AMAN, P., 1988. An *in vitro* method for studying digestion in pigs. *British Journal of Nutrition,* 61:689-698.

RAMOS, M., CARABAÑO, R., 1996. Nutritive evaluation of rabbit diets by an *in vitro* method. *Proceedings 6th Congress of World Rabbit Science Association, Toulouse,* 1: 277-282.

STATISTICAL ANALYSIS SYSTEMS INSTITUTE. 1996. User's guide statistics. *Statistical Analysis System Institute Inc., Cary, NC.*

VILLAMIDE, M.J., FRAGA, M.J., 1998. Prediction of the digestible crude protein and protein digestibility of feed ingredients for rabbits from chemical analysis. *Animal Feed Science and Technology,* 70:211-224.

XICCATO, G., TROCINO, A., CARAZZOLO, A., MEURENS, M, MAERTENS, L. CARABANO R., 1999. Nutritive evaluation and ingredient prediction of compound feeds for rabbits by near-infrared reflectance spectroscopy (NIRS). *Animal Feed Science and Technology,* 77: 201-212.