Proceedings of the

4-7 july **2000** – Valencia Spain

These proceedings were printed as a special issue of WORLD RABBIT SCIENCE, the journal of the World Rabbit Science Association, Volume 8, supplement 1

ISSN reference of this on line version is 2308-1910
(ISSN for all the on-line versions of the proceedings of the successive World Rabbit Congresses)

CALABRÒ S., NIZZA A., SANNA C., PICCOLO V.

FERMENTATION KINETICS OF SOME FEEDSTUFFS FOR RABBITS USING THE *IN VITRO* GAS PRODUCTION TECHNIQUE

Volume C, pages 145-152

FERMENTATION KINETICS OF SOME FEEDSTUFFS FOR RABBITS USING THE *IN VITRO* GAS PRODUCTION TECHNIQUE

CALABRÒ S., NIZZA A., SANNA C., PICCOLO V.

Dip. Scienze Zootecniche e Ispezione alimenti - via F. Delpino, 1 - 80137 Napoli, Italy

ABSTRACT

Five feedstuffs for rabbits (alfalfa meal, beet pulp, sunflower meal, high and low fibre content commercial diet) were studied using the *in vitro* gas production technique. The caecal content used as inoculum was obtained from rabbits (75 days old) fed a diet with crude protein 15.5 % and crude fibre 15.5 %. Three inocula were prepared: 1) fresh caecal content diluted 1:1 (v/v) with a medium; 2) frozen caecal content diluted 1:1 (v/v) with a medium; 3) frozen caecal content diluted 1:2 (v/v) with a medium.

Beet pulp showed the highest gas (A: 469 ml/g) and VFA (81.4 mM/g) production and the highest OM degradability (OM loss: 68.2 %). Frozen inoculum degraded less OM (61.6 *vs* 64.9 %) with a higher gas (A: 294 *vs* 210 ml/g) and lower VFA (51.4 *vs* 71.6 mM/g) production and showed a slower fermentation, compared to fresh inoculum (R_M: .051, .060, .072 h^{-1}, for inoculum 3, 2, and 1, respectively). The low butyrate production of frozen inocula was remarkable (2.63 *vs* 3.04 *vs* 13.04 mM/g, for inoculum 3, 2 and 1, respectively). There were few differences in inocula dilution for most parameters. Further studies are required to improve the standardisation of inoculum preparation.

INTRODUCTION

At present, *in vitro* digestibility techniques are not yet able to reproduce reliably the digestibility process in rabbit (Xiccato, 1989), perhaps because the caecal microflora only ferments some of what is undigested, namely from a selected substrate. However, the *in vitro* method does not necessarily have to reproduce the *in vivo* digestibility process, but a good correlation between such parameters is required. In the last few years, the *in vitro* cumulative gas production technique (GPT) used to evaluate the nutritive value of ruminant feedstuffs according to their fermentation kinetics (Pell & Schofield, 1993; Theodorou *et al*., 1994) has been increasingly used. The technique uses a substrate, an anaerobic medium and an inoculum of the rumen microbial population. The measurements of the gas produced at stabilised intervals during incubation, supply very detailed information about the degradation extent and fermentation kinetics of a feedstuff by micro-organisms. In addition, determination of total volatile fatty acids (VFA) and their molar concentration allows us to evaluate the fermentation process more widely, and to make a more accurate comparison of the gas produced from the tested substrates.

The GPT has also been used to predict the nutritive value (Menke & Steingass, 1988) in rumen and to study differences between inocula. Our previous results (Calabrò *et al*., 1999) indicate the possibility of estimating organic matter and energy digestibility using the GPT, with good reliability (R^2: 0.725 and 0.744; RSD: 3.77% and 3.65%) for organic matter and energy, respectively) also in rabbits. Recently, the GPT has been applied successfully using other animal species, like horses (Macheboeuf *et al.*, 1997) and chicken (Kwakkel *et al*., 1997), as a source of inoculum. At present, the main obstacle to GPT diffusion seems to be the variability of the inocula pattern (Williams *et al.*, 1995). Consequently, inoculum standardisation is of great interest in order to obtain comparable and reproducible results. For

this purpose, the possibility of keeping the inoculum with its unchanged fermentation capacity is very important.

In this study, the differences between *in vitro* fermentation characteristics of five rabbit feedstuffs as substrates, and microbial activity induced by the preparation and conservation by freezing of the inoculum, were determined using the gas production technique and caecal content from rabbit as a source of inoculum.

MATERIALS AND METHODS

Substrates. Five substrates were used: three simple feedstuffs (alfalfa meal-AM, beet pulp-BP, sunflower meal-SM) and two commercial diets for rabbits (high fibre content-HF and low fibre content-LF). They were ground to pass a 1 mm-screen and their chemical composition (Table 1) was determined (AOAC, 1984).

Table 1 - *Chemical composition (% DM) of feedstuffs*

	AM	BP	SM	HF	LF
Dry matter	88.8	88.2	88.6	88.8	89.7
Crude protein	19.3	8.28	25.4	18.7	24.6
Crude fibre	28.0	19.6	31.2	14.0	27.0
Ash	10.4	3.54	6.46	8.79	8.83
NDF	44.8	47.2	38.4	35.2	46.7
ADF	34.1	32.2	32.5	22.8	34.6
ADL	9.5	1.9	10.3	3.6	7.5

AM: alfalfa meal; BP: beet pulp; SM: sunflower meal; HF: high fibre content diet; LF: low fibre content diet

In vitro fermentation. The fermentation characteristics of feedstuffs were assessed according the procedure reported by Theodorou *et al.* (1994). About 820 mg of sample was fermented at 39°C in a 120 ml serum bottle, containing 74 ml of a medium (Theodorou, 1993) and 3.5 ml of reducing solution. The bottles were sealed with butyl rubber stoppers and aluminium crimp seals and warmed at 39°C until inoculation. Each substrate was replicated 4 times. The inoculum was prepared with caecal content of 12 New Zealand White rabbits (75 days old). The animals were fed a fattening diet with the following composition: OM 92.2%, CP 15.5%, EE 3.4% and CF 15.5%. This diet was always administered *ad libitum* from 50 days old. The feed was removed at 8.00 a.m., 12 h before slaughtering. Subsequently, the caeca were isolated by tying up the two extremities with a nylon string to prevent losses of digesta. After pH determination, the digesta was sampled for VFA analysis using gas chromatography (Perkin-Elmer mod. 8410, column packard 80/120 Carbopack B-DA/4% CARBOWAX 20M - 2m x 2mm id). Then the caecal content was divided into 2 samples: one, diluted 1:1 (v/v) with the medium, constituted the fresh inoculum, 1. The other was frozen at -18°C for 1 month being subsequently defrosted and diluted with the medium 1:1 and 1:2 (v/v) to obtain the frozen inocula, 2 and 3, respectively. The inocula were squeezed through six layers of gauze. During these procedures, the microbial suspension was kept at 39°C under a stream of CO_2. 5 ml of inoculum were added to each bottle, which was incubated at 39°C. Four bottles for each inoculum were incubated without the substrates to represent the control (blanks).

The measurements of cumulative gas production were carried out by using a manual

system consisting of a pressure transducer connected to a disposable Luer lock three-way syringe valve as described by Theodorou *et al.* (1994). Gas pressure and volume were recorded twenty times at 2-24 h intervals throughout incubation to measure the fermentation kinetics. The readings were more frequent during the first 48 h when the pressure inside the bottle increases more rapidly.

Fermentation was stopped at 96 h. Subsequently, the fermentation liquid was sampled for pH determination and VFA analysis. The bottle contents were filtered through sintered glass crucibles (Scott Duran, # 2) and burned to determine the percentage of disappeared organic matter (OM loss). Cumulative gas and VFA production, and OM residue from the blanks were subtracted from those obtained for the substrates. Gas production was expressed as OMCV (ml/g incubated OM) and Y (ml/g degraded OM); VFA production was expressed as mM/g incubated OM.

The monophasic model of Groot *et al*. (1996) was adopted to describe the kinetics of the gas production profiles:

$$G(t) = \frac{A}{1+\left(\frac{B}{t}\right)^{C}}$$

where G (ml/g OM) = the amount of gas produced per gram of incubated OM, at time t after incubation. A (ml/g OM) = the asymptotic gas production. B (h) = the time after incubation at which A/2 has been formed. C = a constant determining the curve sharp. Gas production profiles were described with the model using a non-linear curve-fitting program (NLREG, Sherrod, 1995). Time at which the maximum degradation rate occurs (t_{RM}, h) and the maximum degradation rate (R_M, h^{-1}) were calculated using the formula (Groot *et al*., 1996):

$$t_{RM} = B(C-1)^{1/C} \qquad RM = \frac{Ct_{RM}^{C-1}}{B^{C}+t_{RM}^{C}}$$

Statistical analysis. Analysis of variance was performed for the data using the model:

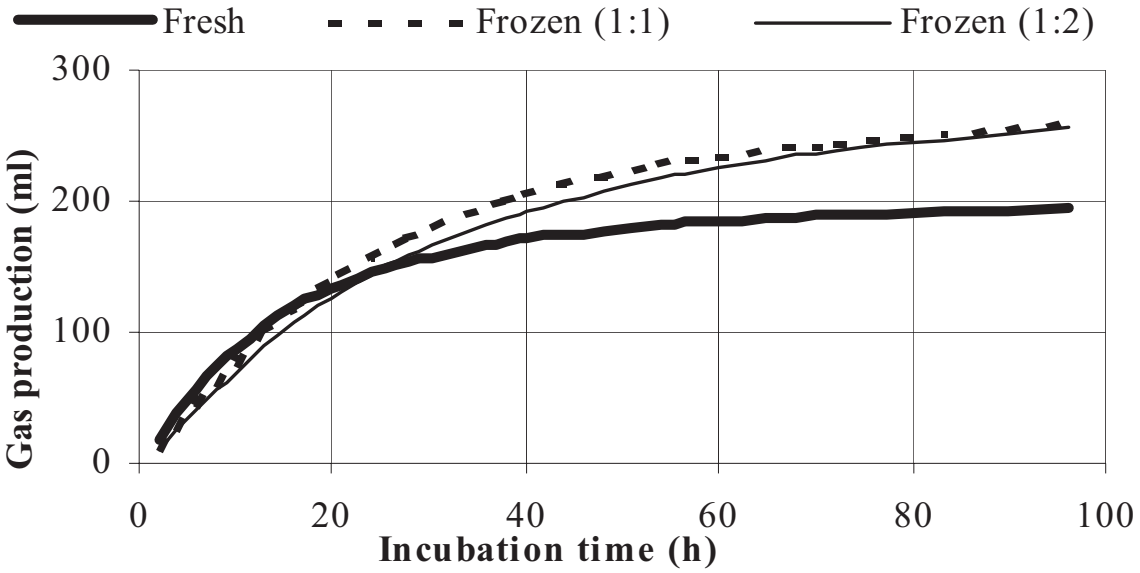
$$y_{ijk} = \mu + S_i + I_j + (S*I)_{ij} + \varepsilon_{ijk}$$

where: μ = overall mean, S_i = substrates (i: 1-5), I_j = inocula (j: 1-3), $(S*I)_{ij}$ = interaction substrate*inoculum and ε_{ijk} = residual error. All statistical procedures were carried out using the GLM procedure of SAS (SAS, 1989).

RESULTS AND DISCUSSION

The pH of the caecal content was 5.41; the total VFA concentration was 64.4. The proportions that each individual fatty acid contributed to the total VFA were: 81.2, 2.5, 15.1%, for acetate, propionate and butyrate, respectively. All the determined parameters presented values normally found in rabbits of the same age fed a similar diet.

Figure 1 - Cumulative gas production profiles for the three inocula



The cumulative gas production profiles (Figure 1) were similar for the two frozen inocula, which supplied a higher gas production compared with the fresh inoculum; however, the fresh inoculum showed a faster fermentation process.

Table 2 shows the gas production kinetics parameters, the end-point measurements and VFA production for all substrates and three inocula preparations. All fermentation parameters were significantly affected by the substrate and the inoculum. As regards the end-point measurements, cumulative gas produced related to incubated (OMCV) and degraded (Y) OM generally followed parameter A. BP showed the highest gas (A: 469 ml/g, OMCV: 256 ml/g, Y: 376 ml/g) and VFA (81.4 mM/g) production with the highest OM degradability (68.2, %). Sunflower meal showed the lowest gas (A: 186 ml/g, OMCV: 115 ml/g, Y: 181 ml/g) and VFA (49.9 mM/g) production with a mid-range OM degradability (64.1 %). Alfalfa meal showed low asymptotic gas production (A: 217 ml/g), the lowest degraded OM (57.0 %) and a low gas yield, associated to good VFA production.

Regarding fermentation kinetics, beet pulp, as reported by Calabrò *et al.* (*in press*) with buffalo inoculum, showed the slowest fermentation process, confirming that it is a highly degradable feedstuff, albeit at slow rates. According to their chemical composition, LF diet showed higher gas production (A: 251 *vs* 224, P>.01), OM degradability (66.5 *vs* 57.8, P>.01) and faster fermentation with higher VFA production, compared to HF diet. It is worth noting that OM degradability is always in agreement with ADL content. By contrast, sunflower meal showed high ADL content and mid-range degradability. This result is due to the fact that ADL is distributed non-uniformly in sunflower OM, but is concentrated in the hull. In confirmation of the above, the kinetics were quite rapid (B, t_{RM}, R_M).

At the end of incubation, all substrates recorded pH values normally found in rabbit caeca. However, BP showed the lowest (6.02) pH, due to higher VFA production. The high VFA and acetate (78.0 % total VFA) production for beet pulp could be connected to the high hemicellulose and pectin content of this feedstuff. These results confirm *in vivo* trials (Fraga *et al*., 1991; Bellier, 1994; Jehl & Gidenne, 1996) where the authors reported an increased

Table 2 - *In vitro fermentation characteristics at 96 h*

	Substrates					Inoculum			I*S		
	AM	**BP**	**SM**	**HF**	**LF**	**1**	**2**	**3**		**MSE**	**D. F.**
A, *ml/g*	217^{C}	469^{A}	186^{D}	224^{C}	251^{B}	210^{B}	294^{A}	303^{A}	***	222	35
B, *h*	19.4^{B}	46.7^{A}	13.1^{C}	11.4^{D}	10.2^{D}	13.0^{C}	21.6^{B}	25.9^{A}	***	5.34	35
t_{RM}, *h*	2.99^{C}	16.9^{A}	2.29C	3.11^{C}	5.98^{B}	5.30^{Bb}	6.49^{Aa}	6.94^{A}	***	1.91	29
R_M, h^{-1}	$.046^{C}$	$.027^{D}$	$.064^{B}$	$.079^{A}$	$.090^{A}$	$.072^{A}$	$.060^{Ba}$	$.051^{Bb}$	***	.0001	29
OM loss, *%*	57.0^{C}	68.2^{A}	64.1^{Bb}	57.8^{C}	66.5^{ABa}	64.9^{B}	61.5^{A}	61.7^{A}	***	6.44	34
OMCV, *ml/g*	125^{Da}	256^{A}	115^{Db}	148^{C}	185^{B}	153^{B}	170^{A}	174^{A}	***	102	35
Y, *ml/g*	223^{D}	376^{A}	181^{E}	257^{C}	278^{B}	239^{B}	274^{A}	276^{A}	***	220	34
pH	6.39^{B}	6.02^{C}	6.45^{A}	6.45^{A}	6.38^{B}	6.40^{B}	6.29^{A}	6.31^{A}	***	.0014	35
VFA, *mM/g*	55.3^{B}	81.4^{A}	49.9^{B}	50.2^{B}	54.8^{B}	71.6^{A}	51.4^{B}	51.9^{B}	***	52.6	30
Acetic, *% tot*	75.6^{ABb}	78.0^{Aa}	72.4^{Cc}	71.5^{C}	70.2^{Cb}	67.7^{B}	75.8^{B}	77.1^{A}	***	102	30
Propionic, "	11.9^{Cc}	9.87^{D}	14.9^{A}	12.8^{BCb}	13.7^{Ba}	10.2^{C}	14.4^{A}	13.2^{B}	***	21.8	30
Butyric, "	10.2^{ABb}	8.75^{BCc}	7.21^{Cd}	11.4^{Aa}	11.0^{A}	18.1^{A}	4.88^{C}	6.18^{B}	***	1.23	30

A: potential gas production; B: time after incubation at which A/2 gas has been formed; t_{RM}: time at maximum rate; R_M: maximum fractional rate; OM loss: organic matter degradability; OMCV: gas production related to incubated OM; Y: gas production related to degraded OM; VFA: volatile fatty acids related to incubated OM. Substrates: AM: alfalfa meal; BP: beet pulp; SM: sunflower meal; HF: high fibre content diet; LF: low fibre content diet. Inoculum: 1) fresh; 2) frozen diluted 1:1 (v/v); frozen diluted 1:2 (v/v). I*S: inoculum substrate interaction. MSE: mean square error; D.F.: degrees of freedom. A,B,C,D,E and a,b: samples with different letters in the same row are significantly different from each other ($P < 0.01$ and $P < 0.05$, respectively). ***: $P < 0.0$

caecal VFA concentration in diets with high beet pulp content. Moreover, also Marounek *et al*. (1995) reported high caecal pectinolytic activities followed first by xylanosic and then by cellulasic activity. Acetic was the most widely produced acid in all substrates followed by propionic. Normally, the caecal content in adult rabbits, fed a commercial diet, shows more butyrate than propionate. An inverse tendency is reported (Piattoni *et al*., 1997; Gidenne & Bellier, 1992) only in fastened animals, due to lower butyrate production. In an *in vitro* trial Piattoni *et al*. (1997) reported similar propionate and butyrate (C_3: 110 and C_4: 120 mM/M VFA) when the substrates consisted of cellulose. In our case, the low butyric production may be due either to the use of caecal content by 12 hours fastened rabbits or to frozen process of inoculum. The clear differences in butyric between fresh (18.1 % total VFA) and frozen inocula (4.88 and 6.18 % total VFA) could be caused by damage to butyric microflora during the freezing process.

Few differences were found in frozen inocula dilution for most parameters. With regard to fermentation kinetics, the 1:2 dilution slowed the fermentation process (B: 25.9 *vs* 21.6 h, P<.01, and R_M: .051 *vs* .060 h^{-1}, P<.05, per inoculum 3 and 2, respectively) but degraded OM with the same efficiency (OM loss: 61.7 *vs* 61.5 %) of the 1:1 dilution. Moreover, we found similar values for A (303 *vs* 294 ml/g), OMCV (174 *vs* 170 ml/g) and Y (276 *vs* 274 ml/g). Otherwise, inoculum 3 significantly reduced (P<.05) propionate production, though VFA concentration was the same. The fresh inoculum showed A (210 ml/g), OMCV (153 ml/g) and Y (239 ml/g) values lower (P<.01) than frozen inocula, regardless of dilution. Moreover, fresh inoculum, compared to frozen inocula, degraded more OM (OM loss: 64.9 *vs* 61.6 %) at a higher rate, especially compared to the 2:1 dilution (R_M:

.072, .060 and .051 h^{-1}; t_{RM}: 5.30, 6.49 and 6.94 h, for inocula 1, 2 and 3, respectively). Total VFA production was significantly higher (P<.01) with the fresh inoculum, which also provided more acetate and butyrate. The non-correspondence, in inocula comparison, between VFA and gas production was both unexpected and hard to explain, especially as propionate production shows no difference between fresh and frozen inocula. It is worth noting the high butyrate production that, with fresh inoculum, was almost double that of propionate (18.1 *vs* 10.2 % total VFA) and similar to those observed in animal caecum (Bellier *et al.,* 1995; Gidenne, 1995; Tortuero *et al.,* 1994), while with frozen inoculum production was very low. All fermentation parameters showed significant inoculum x substrate interaction. However, alfalfa meal and the HF diet supplied the same VFA and acetate with the three inocula.

CONCLUSIONS

In vitro fermentation parameters showed significant differences in feedstuffs and the observed differences are in agreement with their chemical composition. Although further investigations (i.e. inoculum and substrate type) are required, the data obtained open up the possibility of using the GPT to describe the fermentation kinetics of feedstuffs for rabbits with the caecal content as a source of inoculum.

The frozen inoculum supplied results different from those of fresh inoculum. In particular, the frozen inoculum gave VFA values which conflict with mean data in rabbits (propionate/butyrate ratio). However, these results have to be considered very useful because they afford new operating possibilities. The freezing process of the caecal content allows both preparation of appropriate amounts of inoculum and use for many trials in studying the fermentation kinetics of a large number of feedstuffs as well as broader determination to improve the standardisation of the inoculum. However, further studies are required to investigate the effects of other feedstuffs with frozen inoculum and the consequences of the freezing and deferring process on microbial activity.

Acknowledgements: The authors gratefully acknowledge the receipt of funds from the CNR (National Research Council), N. 97.01686.CT06.

REFERENCES

AOAC, 1984. Official Methods of Analysis of the Association of Official Analytical Chemists, 14th ed. AOAC, Washington, DC.

Bellier R. & Gidenne T. 1992. Caecal cannulation in five-week-old rabbits. An *in vivo* study of the circadian variation of the fermentation pattern. In: Proc. 5th WRSA Congr., Corvallis, OR, vol.B 922-930.

Bellier R. 1994. Contrôle nutrionnel de l'activité fermentaire caecale. Thèse de Doctorat. Ecole Nationale Supérieure Agronomique, Institut National polytechnique de Toulouse, 117 p.

Bellier R., Gidenne T., Vernay M., Colin M. 1995. *In vivo* studio of circadian varations of caecal fermentation pattern in postweaned and adult rabbits. J. Anim. Sci. **73**, 128-135.

Calabrò S., Nizza A., Pinna W., Cutrignelli M.I., Piccolo V. 1999. Estimation of digestibility of compound diets for rabbits using the *in vitro* gas production technique. World Rabbit Sci. **74** (4), 197-201.

Calabrò S., Piccolo V., Infascelli F., Bovera F., Cutrignelli M.I. 2000. Fermentation kinetics of some by-products with the *in vitro* gas production technique. Bubalus Bubalis, *in press.*

Fraga M.J., Perez De Ayala P. 1991. Caraba lapin mise au point et validation d'une nouvelle technique de canulation caecale. Reprod. Nutr. Dev. **32**, 365-376.

Gidenne T. 1995. Effect of fibre level reduction and gluco-oligosaccharide addition on the growth performance caecal fermentation in growing rabbit. Anim. Feed Sci. Techn. **56**, 253-263.

Groot J.C.J., Cone J.W., Williams B.A:, Debersaques F.M.A., Lantinga E.A. 1996. Multiphasic analysis of gas production kinetics for *in vitro* fermentation of ruminant feedstuffs. Anim. Feed Sci. Techn. **64**, 77-89.

Jehl N. & Gidenne T. 1996. Replacement of starch by digestible fibre in feed for the growing rabbit. 2. Consequences for microbial activity in the caecum and on incidence of digestive disorders. . Anim. Feed Sci. Techn. **61**, 193-204.

Kwakkel R.P., Williams B.A., Van Der Poel A.F.B. 1997. Effects of fine and coarse particle diets on gizzard growth and fermentation characteristics of the caecal contents in broiler chickens. In: 11th European Symposium on Poultry Nutrition (WPSA). Faaborg, Denmark.

Macheboeuf D., Jestin M., Martin-Rosset W. 1997. Utilization of the gas test method using horse faeces as a source of inoculum. In: Proc. Intern. Symp. "*In vitro* techniques for measuring nutrient supply to ruminants" BSAS Occasional Publication **22**, 187-189.

Marounek M., Vovk S.J., Skrivanova V. 1995. Distribution of activity of hydrolytic enzymes in the digestive tract of rabbits. Br. J. Nutr. **73**, 463-469.

Menke K.H. & Steingass H. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim Res. Devel., **28**, 7-55.

Pell A.N. & Schofield P. 1993. Computerised monitoring of gas production to measure forage digestion *in vitro.* J. Dairy Sci. **76**, 1063-1073.

Piattoni F., Demeyer D., Maertens L. 1997. Fasting effects *in vitro* fermentation pattern of rabbit caecal contents. World Rabbit Sci. **5** (1) 23-26.

SAS. 1989. SAS User's Guide: Statistics (6th edition). SAS Institute Inc Cary, NC.

Sherrod P.H., 1995. Nonlinear Regression Analysis Program, (NLREG). *Nashville, TN.*

Theodorou M.K. 1993. A new laboratory procedure for determining the fermentation kinetics of ruminants feeds. Ciencia e Investigcion Agraria, **20**, 332-344.

Theodorou M.K., Williams B.A., Dhanoa M.S., McAllan A.B., France J. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Techn. **48**, 185-197.

Tortuero F., Rioperez J., Cosin C. Barrera J., Rodriguez M.L. 1994. Effects of dietary fibre sources on volatile fatty acids production, intestinal microflora and mineral balance in rabbits. Anim. Feed Sci. Techn. **48**, 1-14.

Williams B.A., Bhatia S.K., Boer H., Tamminga S. 1995. A preliminary study using the cumulative gas production technique to compare the kinetics of different fermentation by use of standard substrates. Ann. Zoot. **44**, Suppl. 35.

Xiccato G. 1989. Quale sistema energetico per il coniglio. Riv. Professione Allevatore. **2,** 1-8.