Effect of storage duration on frozen inoculum to be used for the in vitro gas production technique in rabbit

Giampiero Stanco, Carmelo Di Meo, Giovanni Piccolo, Antonino Nizza

Dipartimento di Scienze Zootecniche e Ispezione degli Alimenti. Università di Napoli "Federico II", Italy

Corresponding author: Prof. Antonino Nizza. Dipartimento di Scienze Zootecniche e Ispezione degli Alimenti. Via F. Delpino 1, 80137 Napoli, Italy - Tel. +39 081 4421933 - Fax: +39 081 292981 - Email: nizza@unina.it

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ABSTRACT

The present study aimed to investigate the effect of storage duration of frozen inoculum on fermentation parameters obtained with the in vitro gas production technique. Two non-predigested diets differing in chemical composition and especially crude fibre content (low fibre diet: 13.8%; high-fibre diet: 22.6%) were ground to pass a 1 mm screen and subjected to fermentation with the same inoculum frozen for different periods: after 1 month (inoculum 1), after 2 months (inoculum 2) and after 3 months (inoculum 3). The inoculum used was obtained from the caecal content of 75-day-old NZW rabbits. After defrosting, the caecal content was diluted with the medium 1:1 (V/V) and squeezed through six layers of gauze to obtain the inoculum. The substrate affected several fermentation parameters. In particular, the high-fibre diet had lower potential and cumulative gas production (A = ml/g 220 vs 256; P < 0.01 and OMCV ml/g 185 vs 221; P < 0.01), lower organic matter degradability (OMd 67.2% vs 58.0%; P < 0.01) and production of volatile fatty acids (mmol/g 56.2 vs 49.8; P < 0.01), and took more time to obtain gas production equal to A/2 (B = h 11.8 vs 10.1; P < 0.01) compared with the low-fibre diet. However, the three inocula had very similar gas production kinetics, overlapping values of degraded organic matter (62.4%, 62.7% and 62.7% respectively for inocula 1, 2 and 3) and similar production of VFA (54.0, 52.2 and 52.8 mmol/g, respectively for inocula 1, 2 and 3). This research showed it is possible to use frozen inoculum for at least 3 months and in this time interval obtain the same parameters of in vitro fermentation.

Key words: Gas production, In vitro fermentation, Rabbits, Caecal content.
vs 256 ml/g, $P < 0.01$; OMCV 185 vs 221 ml/g, $P < 0.01$). Anche la produzione di acidi grassi volatili è risultata più bassa con il mangime più ricco in fibra (49.8 vs 56.2 mmol/g tal quale, $P < 0.01$). Il tempo di congelamento dell’inoculo, invece, non ha fatto emergere differenze significative per nessuno dei parametri considerati. Da questa prova emerge che è possibile utilizzare, con la tecnica della produzione cumulativa di gas in vitro, inoculo congelato per almeno 3 mesi ed ottenere nel suddetto intervallo di tempo parametri di fermentazione sovrapponibili.

**Parole chiave:** Produzione di gas, Fermentazione in vitro, Conigli, Contenuto ciecale

**Introduction**

The *in vitro* gas production technique (GPT) is being increasingly used to evaluate the nutritive value of ruminant feedstuffs according to their fermentation kinetics (Pell and Schofield, 1993; Theodorou et al., 1994). The technique uses a milled substrate, an anaerobic medium and an inoculum of a mixed microbial population from the rumen. Recently, it has been applied successfully using other animal species, such as horses (Macheboeuf et al., 1997) and chicken (Kwakkel et al., 1997) as sources of inoculum.

In light of the above research, Calabrò et al. (1999) proposed the *in vitro* gas production technique to predict the nutritive value and to study the fermentation kinetics of rabbit diets. For this research they used fresh rabbit caecal content as inoculum and ten mixed diets as substrates. The authors considered the results obtained encouraging and highlighted the need for better standardisation of the inoculum. Subsequently, to predict *in vivo* digestibility of mixed rabbit diets, Calabrò et al. (2000) used the frozen caecal content of rabbit as inoculum. Using frozen caecal content, as opposed to fresh, allows the researcher more time to determine useful parameters for standardising inoculum and to work on a larger number of substrates. The latter advantage presupposes that the frozen inoculum may be used more than once, thereby supplying repeatable and reproducible results at least over a period of several months.

The aim of the present research was to study the effect of freezing duration of the caecal content. Therefore, two diets with different chemical characteristics were subjected to fermentation at three times with the same frozen inoculum. The first fermentation occurred with inoculum frozen for 30 days, the second and third with the same inoculum frozen respectively for 60 and 90 days.

**Material and methods**

**Substrates**

Two mixed rabbit diets (commercial diets) with different chemical characteristics (diet 1 high fibre, HF and diet 2 low fibre, LF) were used as test substrates. Diet HF is normally used during the final growth period of future reproductive does and diet LF for growing rabbits. Both diets were supplied with robenidine, but no enzyme was added. These diets were ground to pass a 1 mm screen and their chemical composition (Table 1) was determined (AOAC, 1984).

**Donor animals**

The inoculum was prepared with the caecal content of 24 New Zealand White rabbits (75 days old). The animals were fed a fattening diet with the following composition (on dry matter basis): organic matter (OM) 92.2%, crude protein (CP) 15.5%, ether extract (EE) 3.4% and crude fibre (CF) 15.5%. This diet was always administered *ad libitum* for 25 days prior to slaughter. The feed was removed at 8 p.m. on the day before slaughter. Then the caeca were isolated by tying up the two extremities with a nylon string to prevent losses of digesta. The caecal content of 24 rabbits, after mixing, was subdivided into three parts and frozen at -18°C.

After 1 month (inoculum 1), 2 months (inoculum 2) and 3 months (inoculum 3) the caecal content was defrosted and diluted 1:1 (V/V) with a basal medium (Theodorou, 1993). The caecal content diluted with the basal medium was squeezed through layers of gauze to obtain the inoculum. During these procedures, microbial suspension was kept at 39°C under a stream of CO₂.
Gas production

The fermentation kinetics was studied by the vitro gas production technique described by Theodorou (1993) and Theodorou et al. (1994). For each substrate, about 820 mg was incubated with 74 ml of medium, 3.5 ml of reducing solution and 5 ml of inoculum at 39°C for 96 hours. For each substrate and inoculum, 4 replications were made. At each time, gas production was measured out of a total of 36 bottles. Four bottles per inoculum were incubated without the substrates to represent the control (blanks), to correct organic matter losses (OML) and gas production. Pressure and volume of gas produced were recorded 20 times at 2-24 h intervals. The readings were more frequent during the first 48h of incubation when the pressure inside the bottle increases more rapidly. The measurements were effectuated manually with a pressure modified transducer (Theodorou, 1993).

To evaluate the residual OM, at the end of incubation (96 h) the contents of each bottle were filtered through glass crucibles (Schott Duran, # 2) which, after several hot water washings, were dried at 103°C overnight and burned in a muffle at 550°C for 3 h. From each bottle a liquid sample was collected for pH (pH-meter Orion EA 940) and volatile fatty acids (VFA) (gas chromatography Perkin-Elmer mod. 8410, column packed 80/120 Carbopack B-DA/4% CARBOWAX 20M - 2m x 2 mm id) determinations.

The cumulative gas data, related to incubated OM, were fitted to the monophasic model (Groot et al., 1996): \( G(t) = \frac{A}{1+(B/t)^C} \) where \( G \): gas (ml/g) produced at time \( t \); \( A \): potential gas production (ml/g); \( B \): incubation time (h) at which gas produced is equal to \( A/2 \); \( C \): a constant determining the curve sharp. Gas production profiles were described with the model using a non-linear curve-fitting program (NLREG, Sherrod, 1995). It was also possible to calculate the maximum degradation rate (\( R_{max}, h^{-1} \)) and the time at which RM occurs (\( t_{max} \)) using the formula of Groot et al. (1996).

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 \( \mu \) = overall mean, \( S_i = \) substrates (\( i = 1-2 \)), \( I_j = \) inocula (\( j = 1-3 \)), \( (S*I)_{ij} = \) substrate*inoculum interaction and \( \varepsilon_{ijk} = \) residual error. All statistical procedures were carried out using the GLM procedure of SAS (SAS, 1989).

Results

The two diets used had a fairly different chemical composition (table 1). Moreover, in selecting them, particular attention had been paid to the content in crude fibre (CF), which was 226 and 138 g/kg, respectively for diets 1 and 2 and in structural carbohydrates (ADF, NDF and ADL) insofar as they are fractions that can affect in vitro fermentation more greatly. Table 2 reports, for the two diets and the three inocula, the kinetic parameters of fermentation (A, B, TRM, RM), gas production (ml/g) as a function of incubated (OMCV) or degraded (Y) OM, OML(%), VFA production (mM/g of incubated OM) and pH.

Many of the above parameters were significantly affected by the substrate (diet). In particular, the diet with the highest content of structural carbohydrates showed potential and cumulative gas production which was significantly (\( P < 0.01 \)) lower (\( A = \) ml/g 220 vs 256 and OMCV mg/g 185 vs 221) than that with a lower fibre content. Moreover the HF diet took more time than diet LF to obtain a gas production equal to \( A/2 \) (\( B = h \) 11.8 vs 10.1; \( P < 0.01 \)) and attained a lower maximum gas production rate (6.4%/h vs 7.5%/h). In diet LF both organic matter degradability (OML)

| Table 1. Chemical composition of diets |
|---------------------------------------|
| Diet 1 (HF) | Diet 2 (LF) |
| Dry matter g/kg | 891 | 886 |
| Crude protein " | 207 | 185 |
| Crude fibre " | 226 | 138 |
| Ether extract " | 33 | 36 |
| Starch " | 101 | 203 |
| Ash " | 87 | 88 |
| NDF " | 460 | 351 |
| ADF " | 289 | 202 |
| ADL " | 70 | 38 |
| Digestible energy MJ/kg | 8.18 | 10.24 |
67.2% vs 58.0%) and total VFA production (56.2 vs 49.8 mM/g) were considerably higher. Neither VFA molar proportions nor pH were affected by substrates.

The three inocula tested had very similar gas production kinetics, overlapping values of degraded organic matter (62.4%, 62.7% and 62.7% respectively for inocula 1, 2 and 3) and similar VFA production (54.0, 52.2 and 52.8 mmol/g).

Discussion

The results obtained in this test confirm the validity of the GPT as a means of estimating fermentation characteristics of rabbit diets, using caecal content as inoculum. In accordance with previous research (Calabrò et al., 1999 and 2000) where a good match was found between dietary chemical characteristics and fermentation parameters, also in this test we observed a higher organic matter degradability and a higher gas and VFA production for the diet with a lower structural carbohydrate content.

Generally, the VFA molar proportion is affected by the fibre level. For instance, the proportion of butyrate rose when the fibre/starch ratio decreased. In our case, the low production of butyrate with diet LF could be due to the inoculum freezing. In this respect, Calabrò et al. (2000) also observed low butyrate production with frozen inoculum.

The widespread use of the GPT to study rabbit diets is to correlate in vivo organic matter digestibility and parameters obtained with in vitro degradability. Indeed, unlike ruminants where the use of simple feedstuffs allows the GPT to be an effective tool for estimating ruminal fermentation characteristics of carbohydrates, in rabbits the use of mixed diets often with unknown ingredients reduces the interest in many parameters supplied by the GPT. In this respect, many in vitro techniques (Xiccato, 1989) have aimed to find a correla-

Table 2. \textit{In vitro} fermentation characteristics at 96 hours

| Parameter | Diet HF | Diet LF | Inoculum 1 | Inoculum 2 | Inoculum 3 |
|-----------|--------|--------|------------|------------|------------|
| A ml/g    | 220\textsuperscript{a} | 256\textsuperscript{a} | 234 | 240 | 236 |
| B hours   | 11.8\textsuperscript{a} | 10.1\textsuperscript{a} | 11.0 | 10.9 | 11.0 |
| t\textsubscript{RM} " | 3.75 | 4.00 | 3.77 | 3.89 | 3.93 |
| RM "     | 0.064 | 0.075 | 0.069 | 0.069 | 0.069 |
| OM\textsubscript{I} %     | 58.0\textsuperscript{a} | 67.2\textsuperscript{a} | 62.4 | 62.7 | 62.7 |
| OMCV ml/g | 185\textsuperscript{a} | 221\textsuperscript{a} | 200 | 207 | 202 |
| Y "      | 319 | 328 | 320 | 330 | 322 |
| pH        | 6.40 | 6.30 | 6.33 | 6.37 | 6.34 |
| VFA mmol/g | 49.8\textsuperscript{a} | 56.2\textsuperscript{a} | 54.0 | 52.2 | 52.8 |
| Acetic %  | 72.0 | 71.4 | 71.9 | 71.5 | 71.7 |
| Propionic " | 12.6 | 12.0 | 12.4 | 12.3 | 12.3 |
| Butyric " | 11.6 | 11.8 | 11.7 | 11.8 | 11.5 |

\textsuperscript{A,B}: P < 0.01
A = asymptotic gas production; B = time after incubation at which half of A was formed;
RM = degradation maximum rate; tRM = time at which RM occurs; OM\textsubscript{I} = organic matter loss (% of incubated organic matter); OMCV = gas production related to incubated organic matter;
Y = gas production related to degraded OM; VFA = volatile fatty acids.
tion between digestibility and nutritive value obtained in vivo and parameters supplied with in vitro tests. In the specific case of the GPT, Calabrò et al. (1999), using 10 rabbit diets, obtained regression equations able to predict in vivo OM and energy digestibility from in vitro parameters such as organic matter losses and potential gas production (dOM% = -36.3 + 1.0 OMl + 0.146 A, R² 0.725, RSD 3.77; dGE% = -38.7 + 1.019 OMl + 0.148 A, R² 0.744, RSD 3.65). Also Stanco et al. (2003), using 31 mixed diets for rabbits, found a close correlation (r = 0.775) between digestibility in vivo and degradability in vitro of organic matter obtained with the GPT. In this work, the authors report estimation equations of digestibility of organic matter and energy that have good precision (dOM% = -6.80 + 1.078 DMl + 0.456 B, R² 0.691, RSD 2.368; dGE% = -6.75 + 1.073 DMl + 0.455 B, R² 0.681, RSD 2.417). Estimation precision improved appreciably when the diets were grouped by homogeneity of constituents or by the same food producer. Indeed, the 16 diets supplied by the same food producer allowed a much more precise equation to be obtained (dOM% = -10.10 + 1.197 DMl, R² 0.855, RSD 1.809), although it only took dry matter losses into consideration. In both studies the authors expressed, amongst other things, the need to better standardise the inoculum.

The results obtained in this trial with the three inocula appear of great interest. Indeed, the fact of having achieved similar results means that for almost three months the same inoculum can be used on several diets and statistically similar values can be obtained. The importance of this result lies in the fact that with the GPT, also using the automatic method of Davies et al. (1995-2000), it is difficult to test more than 10 diets at the same time. Moreover, with considerable time available, the researcher may conduct measurements in greater depth to better standardise inoculum. Indeed, many researchers (Pascual et al., 2000; Davies et al., 2000; Stanco et al., 2003) that have used in vitro techniques identify in inoculum the factors of greater variability of the results obtained.

Conclusions

These results indicate the validity of the in vitro cumulative gas production technique to describe the fermentation characteristics of rabbit diets used caecal content as inoculum. Moreover, similar results obtained with inoculum frozen at different times assume great importance since it would mean that frozen inoculum could be used for three months and that in this time interval statistically similar in vitro fermentation parameters may be obtained.

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