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Validation of sheep rumen fluid frozen in liquid N as inoculum for *in vitro* gas production trials

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Abstract. The effect of freezing rumen fluid in liquid nitrogen for its use as inoculum for *in vitro* incubation was evaluated in a semi-continuous system. Inoculum was used immediately (RF) or frozen and then thawed and used (TW) or preincubated for 2 (TW2) or 4 h (TW4) before substrate addition. For TW4, a sugars mixture was added. Bacterial diversity was not greatly affected by the inoculum processes. There were no differences on cumulative gas production (ml/g DM) or rate of gas production (ml/g DM per h) between RF and TW along the 36 h incubation (P>0.05). Delayed inocula (TW2 and TW4) produced less gas than RF up to 12h (P<0.05), but differences in fermentation rate were only appreciable at 2, 20 and 24 h (P<0.05). Volatile fatty acids (VFA) concentration at 6 h was higher with RF than TW and TW4, being lowest with TW2 (P=0.007) whereas at 24h it tended (P=0.07) to be higher with TW than with TW2 and TW4. Butyrate and minor VFA proportions differed for RF and frozen treatments at 6 and 24h (P<0.05). Despite of this, rumen fluid frozen in liquid N behaved similarly than fresh inoculum in terms of *in vitro* substrate fermentation. The lower gas volume in TW2 and TW4 might be justified by soluble nutrients utilisation during the first 2 and 4h incubation.

Keywords. Inoculum preservation – Freezing – Gas production – *In vitro.*

***Evaluation du liquide du rumen surgelé dans N liquide comme inoculum pour essais du production du gas* in vitro**

Résumé. *L'effet du liquide du rumen congelé dans l'azote liquide pour son utilisation comme inoculum pour l'incubation* in vitro *a été évalué dans un système semi-continu. L'inoculum a été employé immédiatement (RF) ou gelé et puis dégelé (TW) ou pré-incubé durant 2 h (TW2) ou 4 h (TW4) avant l'addition du substrat. Pour le TW4, un mélange de sucres a été ajouté. La diversité bactérienne n'a pas été considérablement affectée par les manipulations de l'inoculum. Il n'y avait aucune différence sur la production cumulative de gaz (ml/g DM) ou le taux de la production de gaz (ml/g DM par h) entre le RF et le TW durant les 36 h d'incubation (P>0,05). Les inocula retardés (TW2 et TW4) ont produit moins de gaz que le RF jusqu'à 12h (P<0,05), mais les différences du taux de fermentation étaient seulement appréciables à 2, 20 et 24 h (P<0,05). La concentration en acides gras volatiles (VFA) à 6 h était plus haute avec le RF que le TW et le TW4, étant la plus basse avec TW2 (P=0,007) tandis qu'à 24 h elle a tendu (P=0,07) à être plus haute avec le TW qu'avec le TW2 et le TW4. Le butyrate et les proportions mineures de VFA ont différé pour le RF et les traitements gelés à 6 et à 24h (P<0,05). En dépit de, le liquide du rumen congelé dans N liquide s'est comporté de la même manière que l'inoculum frais en termes de fermentation* in vitro *du substrat. Le volume inférieur de gaz dans le TW2 et le TW4 pourrait être justifié par l'utilisation de nutriments solubles pendant les premières 2 et 4 h d'incubation.*

Mots-clés. *Conservation du inoculum – Congélation – Production de gaz* – In vitro.

I – Introduction

As for other *in vitro* techniques, an important constraint for the applicability of the gas production technique is the preservation of inoculum. When experimental procedures require sampling at long distance to the laboratory, or when repeated sampling at different times is needed, an optimum preservation of the inoculum until its utilisation is crucial. Preservation procedures that

would ensure the maintenance of microbial and fermentative characteristics of inocula have been tested, but results have not been totally convincing.

When short intervals of time are considered, refrigeration of rumen fluid has been proposed as a viable alternative to fresh rumen inoculum. Robinson *et al.* (1999) and Hervás *et al.* (2005) reported that chilling inoculum at 0-6ºC in anaerobic conditions for 6 to 6.5 hours does not affect *in vitro* digestibility or gas production parameters, and maintains the number of total and specific fermentative rumen bacteria up to 6 h without major differences with the fresh inoculum,. However, 24 h storage reduced average fermentation rate (Hervás *et al.,* 2005). Freezing has also been tested (Robinson *et al.,* 1999; Cone *et al.,* 2000; Hervás *et al.,* 2005) but, although it did not generally affect in a great extent the maximum fermentation, it delayed the beginning and affected the rate of fermentation. Further, this process reduced cellulolytic and total bacterial counts of the inoculum (Dehority and Grubb, 1980). The lethal effect of the cold shock occurs through cell dehydration to maintain intracellular electrolyte concentration in freezing at low temperatures, or by the formation of intracellular ice in rapid freezing (Mazur 1970). The magnitude of this damage is dependant on cell size and membrane structure (Fonseca *et al.* 2001), and it thus could alter the microbial equilibrium and affect the fermentation pattern, even though Gibson and Khoury (1986) did not observe major differences in the resistance of different bacterial species. However, the recovery of complete metabolic activity of bacterial cells may last for some time. Then, in terms of quantitative comparison of the fermentative capability of the inoculum, this would increase the lag time and the fractional fermentation rate, and probably the total extent of gas production.

We hypothesise that an appropriate procedure for freezing and thawing rumen fluid could minimise the negative effects of low temperatures on inoculum bacteria. Previous experiments have dealt with large batches of fluid that were aimed to freeze at moderately low temperatures (-5 to -24ºC), and in such conditions freezing, as well as the consequent thawing process, may extend even for hours.

In this work, the effect of the preservation method of the inoculum on *in vitro* microbial fermentation was studied using a novel gas production system with semi-continuous flow of the liquid phase (Fondevila and Pérez-Espés, 2008), in order to evaluate the best alternative for long periods of time between inoculum extraction and *in vitro* incubation.

II – Materials and methods

Rumen inoculum was obtained from two cannulated adult sheep given 0.8 kg daily of a 75:25 mixture of chopped alfalfa hay and ground barley grain. Rumen fluid was obtained before the morning feeding, filtered through a double layer of gauze, mixed and immediately transferred to the laboratory. Alfalfa hay ground to 1 mm was used as substrate for the *in vitro* trials.

The *in vitro* incubation system of Fondevila and Pérez-Espés (2008) was used, with the following modifications: the incubation vessels were commercial 100 ml Pyrex glass Erlenmeyer flasks (123 ml total volume) with a 29/32 frosted mouth, fitted with a two 8 mm outlet polypropylene connector stopper (Kartell, Italy). One outlet was fitted with a three-way valve for measuring the gas internal pressure as an indicator of microbial fermentation. The other outlet was fitted with a two-way valve at the outside end and a 150 mm pore nylon cloth fixed in the inside end for liquid exchange.

An approximate amount of 800 mg substrate was included into 150 mm pore size bags and incubated on each bottle, into 6 x 3 cm sealed nylon bags (45 mm pore size). Bottles were filled with 80 ml of incubation solution (Theodorou *et al.,* 1994), including a buffer solution (to maintain pH at 6.5; Fondevila and Pérez-Espés 2008), macromineral solution and a reduction solution, but without trace mineral solution and resazurin as suggested by Mould *et al.* (2005). Incubation

media also included rumen inoculum to a 0.20 proportion of total volume. Incubation solution was prepared under a CO_2 atmosphere, and bottles were maintained at 39ºC in a water bath throughout the incubation.

Pressure of gas produced in each bottle was recorded with a HD8804 manometer with a TP804 pressure gauge (DELTA OHM, Italy) at different incubation times over a 24 h control period. Pressure readings were converted to volume by using a pre-established linear regression. Immediately after gas measurement, fixed volumes of incubation media were extracted by suction through the filter port, and the exact volume was replaced with incubation solution (without rumen inoculum), that was maintained anaerobically at 38ºC. The rate of the liquid phase turnover was adjusted to 0.06/h by replacing liquid media with incubation solution, according to the following schedule: 10 ml every two hours from 0 to 12 h incubation, and 20 ml every 4 h from 12 to 24 h. When necessary, extracted media was sampled for subsequent analysis. Both pressure measurements and fluid replacement were carried out as quickly as possible, without removing the bottles from the water bath.

Filtered rumen fluid was immediately used as inoculum (RF) or dispensed under a CO_2 atmosphere in 16 ml aliquots into polypropylene screw cap tubes that were frozen in liquid N and stored at -80ºC for 48 h. Frozen inoculum was used directly after thawing in 38 ºC water bath (in about 1 min; TW) or inoculated in the incubation bottles for 2 (TW2) or 4 (TW4) h before adding the substrate bags. For TW4, 24 mg of a 1:1:1 mixture of glucose, xylose and cellobiose was added to avoid that a potential nutrient depletion would reduce bacterial growth and viability. Before incubation, frozen inocula were thawed in a 39ºC water bath for 2 min. This experimental design was repeated in two different runs of two bottles per treatment. After 6 and 24 h incubation, 2.5 ml of the removed incubation medium were sampled in 0.5 ml of 0.5 M phosphoric acid with 4-methylvaleric acid (2 mg/ml) as internal standard, and stored frozen for volatile fatty acids (VFA) determination. Another 2 ml sample was also taken at 24 h and immediately frozen until their study of bacterial biodiversity. After the 24 h incubation period, bags with the substrate were washed and dried at 60ºC for 48 h for estimation of the dry matter disappearance (DMd).

Analysis of VFA was performed by gas-liquid chromatography in a HP 5890A apparatus equipped with a capillary column (Tecknokroma WAW80/100, 10% SP-1200 + 1% H_3PO_4). DNA was isolated from frozen and thawed samples using a QIAamp® DNA Stool Mini Kit (QIAGEN Ltd., England) following the manufacturer's instructions. Total genomic DNA was used as a target for amplification of approximately 200 bp of the variable V3 region of the 16S rRNA (Muyzer *et al*., 1993). PCR amplification conditions used were as follows: 1 cycle (94ºC for 4 min), 30 cycles (94ºC for 4 min, 60ºC for 1 min, 72ºC for 1 min), 1 cycle (72ºC for 10 min). DGGE was performed on a C.B.S. Scientific® system following the manufacturer's guidelines. Electrophoresis was performed at a constant voltage and temperature of 80V and 60ºC for 16h. DNA was visualised by silver staining with a commercial DNA Silver Stanning Kit (Amersham Biosciences Sweden). The gel was scanned using a GS-800 Calibrated Imaging Densitometer (Bio-Rad, UK). Scanned DGGE images were analysed with Quantity One® Software (Bio-Rad) and DGGE profiles of samples of the gel were compared using a similarity matrix. Each band position present in the gel was binary coded for its presence or absence within a lane and each lane was compared by using a similarity matrix (Regensbogenova *et al.,* 2004). Trees were constructed by using the NEIGHBOUR program (PHYLIP version 3.6a; F. Felsentein, Seattle, USA). Finally, Shannon index, a measure of diversity, was calculated following the methodology proposed by Magurran (1988): $H = -\sum (p_i \cdot \ln p_i)$, where p_i is the abundance of every species.

The results for each incubation time were compared by ANOVA using the STATISTIX 8 package (Analytical Software, 2003). Each bottle was considered as the experimental unit, and the incubation run was considered as a block. Differences among treatment means were contrasted by the LSD test ($P<0.05$).

III – Results and discussion

Bacterial biodiversity in the fresh and frozen rumen inocula (used for RF and for TW, TW2 and TW$ treatments, respectively) and in the bottles after 24 h incubation, as shown by results of the DGGE analysis, are shown in Fig. 1. Samples clustered in clearly differenced groups. Both inocula presented a 0.96 similarity, but the 24 h incubation process reduces the similarity to the inoculum to 0.62. Differences between RF and the other treatments were small, as it is shown by the 0.71 similarity index. Among frozen and thawed treatments, TW4 was slightly different, probably because the addition of sugars in its 4 h preincubation modified bacterial community. Numbers of bacterial species were not affected by the incubation, as shown by the Shannon diversity index, being only slightly lower in TW samples (average values of 3.584 in the inocula, and 3.277, 3.376 and 3.384 for TW, TW2 and TW4, respectively). These results demonstrate that the freezing/thawing process has a minimal effect on the diversity of the microbial community in the incubation media.

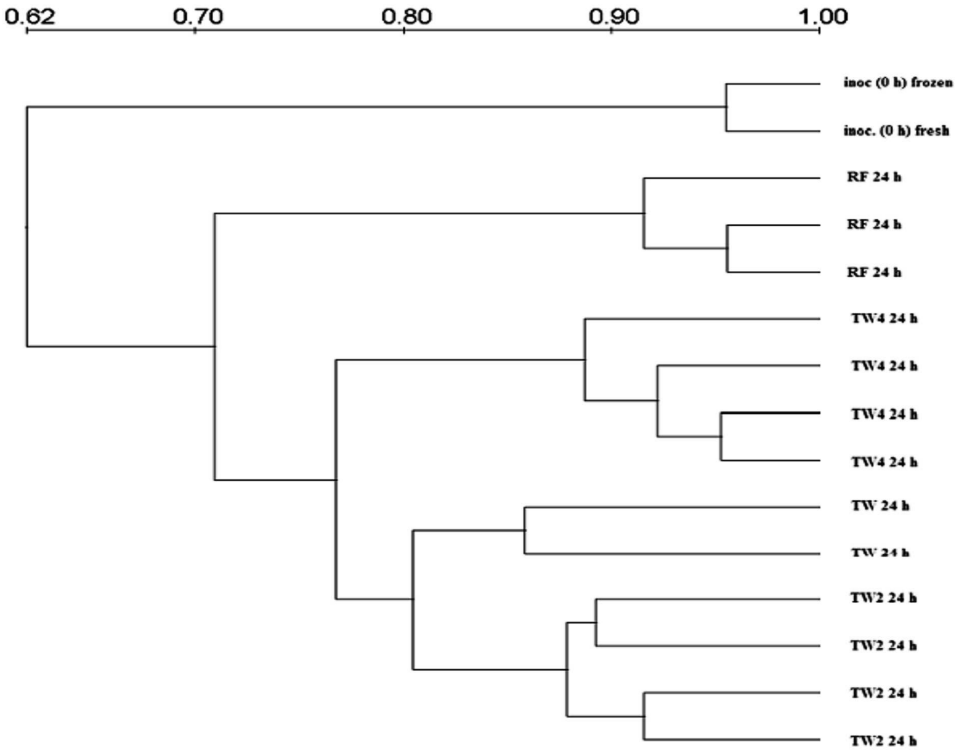

Fig. 1. Distance analysis of the DGGE banding pattern from sheep inocula and the incubation media after 24 h incubation when the inoculum was used without delay (RF) or frozen in liquid N and used immediately after thawing (TW), 2 h later (TW2) or 4 h later with a sugar mixture (TW4).

There were no differences on cumulative gas production (ml/g dry matter (DM); Fig. 2) or rate of gas production (ml/g DM per h; Fig. 3) between RF and TW along the 36 h incubation period ($P>0.05$), except for a higher rate of gas production with TW at 28 h ($P<0.05$) which is difficult to explain. This shows that, despite previous information (Robinson *et al.,* 1999; Cone *et al.,* 2000; Hervás *et al.,* 2005), freezing rumen inocula could be a valuable option for preserve it if the freezing and thawing are carried out procuring for a fast process.

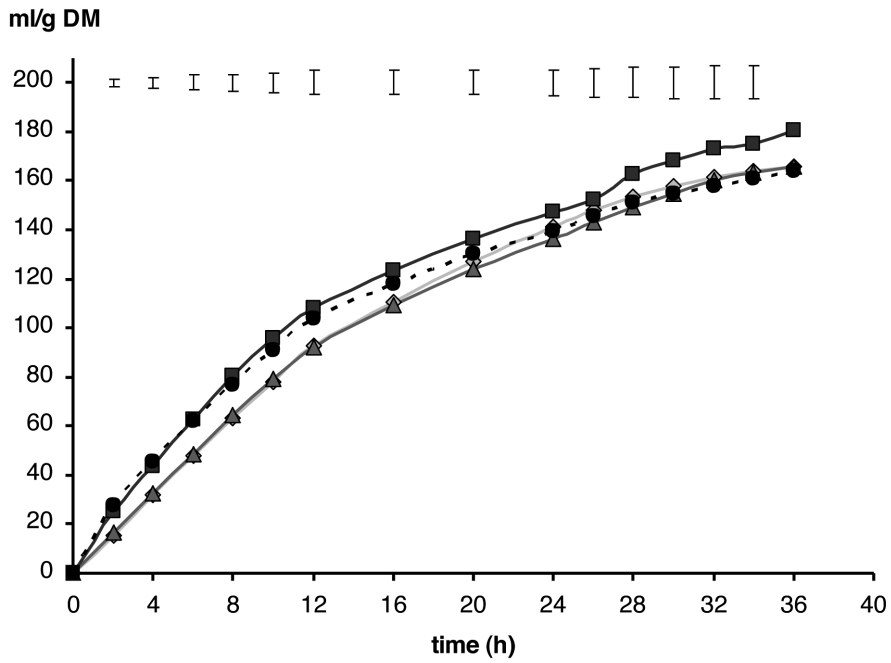

Fig. 2. Gas production pattern with sheep inoculum used without delay (RF, circles) or frozen in liquid N and used immediately after thawing (TW, squares), 2 h later (TW2, triangles) or 4 h later, with a sugar mixture (TW4, diamonds). Upper bars show standard errors of means.

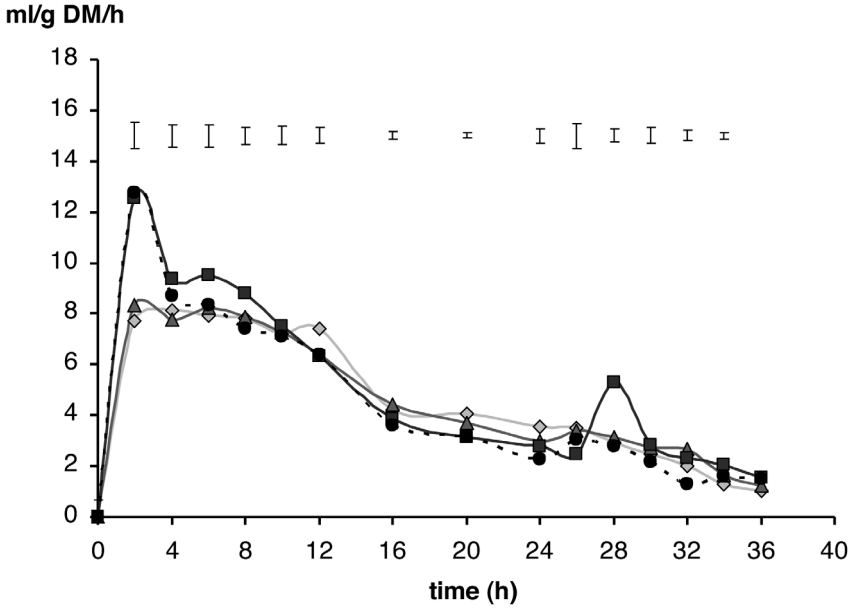

Fig. 3. Rate of gas production with sheep inoculum used without delay (RF, circles) or frozen in liquid N and used immediately after thawing (TW, squares), 2 h later (TW2, triangles) or 4 h later with a sugar mixture (TW4, circles). Upper bars show standard errors of means.

Fermentation of the delayed inocula (TW2 and TW4) rendered less total gas than RF up to 12 h (P<0.05), but differences in the rate of fermentation were only significant at 2 h incubation, indicating a lower gas production with retarded inocula (TW2 and TW4; P<0.001) and at late stages of fermentation, when gas production rate with RF was lower than TW 2 and TW4 at 20 and 24 h of incubation (P<0.001), may be as a consequence of the slightly lower fermentation at earliest stages of incubation. This response may be affected by the fact that soluble nutrients in the rumen inocula could be used by microorganisms during the incubation of inocula without adding substrate for 2 (TW2) or 4 (TW4) h. In this experiment, it is not possible to correct the effect of inoculum self fermentation, since no blank bottles were included in the incubation; however, readings of blank of inoculum for retarded treatments in the first 2 or 4 h incubation were 17.1 ± 2.08 ml and 41.3 ± 2.10 ml for TW2 and TW4, although with some contribution of sugars in the latter.

The DMd after 24 h incubation was higher with RF and TW than with TW2 and TW4 (0.611, 0.555, 0.491 and 0.495, respectively; s.e.m. = 0.0170). Volatile fatty acids (VFA) concentration (Table 1) at 6 h was higher with RF than with TW and TW4, being lowest with TW2 (P=0.007) whereas at 24 h it tended (P=0.07) to be higher with TW than with TW2 and TW4. The VFA profiles at 6 h differed for RF and frozen treatments in terms of butyrate, valerate and branched chain VFA (P < 0.05), but at 24 h TW produced more propionate and less butyrate, valerate and branched VFA than RF, whereas TW2 was different to RF only in propionate proportion (P < 0.05). Although VFA profile showed treatment differences in butyrate and minor VFA, these differences are difficult to interpret because of their lower magnitude, and rumen fluid frozen in liquid N behaved similarly than fresh inoculum in terms of *in vitro* substrate fermentation.

Table 1. Total concentration of volatile fatty acids (VFA, mmol/l) and molar VFA proportions after 6 and 24 h incubation with sheep inoculum used without delay (RF) or frozen in liquid N and used immediately after thawing (TW), 2 h later (TW2) or 4 h later with a sugar mixture (TW4)

	RF	TW	TW2	TW4	s.e.m.
6 h					
Total VFA	36.3 a	32.0 b	27.7 c	31.9 b	1.39
Acetate	0.707 a	0.681 ab	0.674 b	0.670 b	0.0087
Propionate	0.181	0.185	0.182	0.193	0.0044
Butyrate	0.075 b	0.084 a	0.089 a	0.086 a	0.0028
Isobutyrate	0.009 b	0.011 a	0.012 a	0.011 a	0.0004
Isovalerate	0.013 c	0.021 b	0.025 a	0.023 ab	0.0009
Valerate	0.016 b	0.018 a	0.019 a	0.017 ab	0.0007
24 h					
Total VFA	37.5 ab	44.3 a	34.2 b	34.4 b	0.0275
Acetate	0.659 ab	0.677 a	0.638 c	0.654 bc	0.0059
Propionate	0.178 c	0.190 b	0.206 a	0.201 a	0.0031
Butyrate	0.114 a	0.095 b	0.111 a	0.010 ab	0.0044
Isobutyrate	0.011 a	0.008 b	0.010 ab	0.009 b	0.0005
Isovalerate	0.020 a	0.015 c	0.012 ab	0.016 bc	0.0009
Valerate	0.018 a	0.015 b	0.018 a	0.016 b	0.0005

Different letters in the same row indicate differences among treatment means (P<0.05).

IV – Conclusions

Freezing rumen inoculum in liquid nitrogen and in small volumes, followed by fast thawing does not greatly affect microbial biodiversity. To some extent, the volatile fatty acid pattern showed some differences at 6 h incubation, mainly towards a higher butyrate and minor isobutyrate and valerate proportions, but these differences change their trend after 24 h. In any case, these minimum changes in the bacterial communities and VFA pattern do not quantitatively affect the substrate fermentation pattern, indicating that, in any case, the niche of affected species can be occupied by others.

The lower gas volume in delayed treatments, as well as the lower VFA concentration observed, could be caused by the delayed incubation, and might be justified by soluble nutrients utilisation during the first 2 (TW2) and 4 h (TW4) of incubation.

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