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Effects of preservation procedures of rumen inoculum on *in vitro* microbial diversity and fermentation

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ABSTRACT

Sheep rumen contents were used as inoculum for an *in vitro* semi-continuous incubation system to study whether preservation method affects microbial fermentation pattern. Rumen fluid was filtered and either used immediately as inoculum (CTL) or dispensed into 110 mm × 16 mm tubes, that were stored refrigerated at 6 °C for 4 h (REF) or frozen at −20 °C (FRZ), frozen in liquid N (FLN) or added with 0.04 glycerol and frozen in liquid N (FGL) for 48 h. Frozen inocula were thawed at 39 °C for 2 min before use (16 ml per bottle). Two 24 h incubations with four bottles per treatment were completed. The microbial utilisation of added glycerol after thawing in FGL increased total gas production ($P<0.05$) and 24 h volatile fatty acid (VFA) production ($P<0.05$), and also increased propionate and butyrate proportions at the expense of acetate. The other freezing inocula (*i.e.*, FLN and FRZ) reduced the rate of gas production (as ml/g dry matter per hour), compared with CTL in the first 2 and 4 h of incubation ($P<0.05$), but this was compensated by increased fermentation at 8 and 12 h, respectively. Differences in gas production did not manifest a different VFA pattern at either 6 or 24 h incubation. Bacterial diversity was slightly affected by the preservation process, and the similarity index between untreated inocula and the 24 h incubated CTL samples was 0.690–0.724. Similarity between bacterial communities in FRZ and FLN with that in CTL after incubation was 0.678. The freezing preservation method of rumen inocula for subsequent *in vitro* gas production studies does not affect microbial fermentation pattern or bacterial biodiversity, provided that processing is rapid enough by using a high surface to volume ratio. Freezing in liquid N is more appropriate than at −20 °C.

1. Introduction

In the last two decades, the *in vitro* gas production technique has achieved relevance for nutritional evaluation of ruminant feeds. An important constraint to its applicability is preservation of microbial inoculum when sampling at long distance to the laboratory is required, or if repeated sampling at various times is needed. Preservation procedures that would ensure the maintenance of microbial and fermentative characteristics of inocula have been tested, but results have not been totally convincing.

Abbreviations: DGGE, denaturing gradient gel electrophoresis; DM, dry matter; DMd, *in vitro* DM disappearance; OM, organic matter; VFA, volatile fatty acids.

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Refrigeration of rumen fluid has been proposed as a viable alternative to fresh rumen inoculum for short times. Exposure to temperatures slightly above 0 °C is associated with bacterial synthesis of specific cold shock proteins, leading to a transient metabolic adaptation (Panoff et al., 1998). Hervás et al. (2005) reported that chilling inoculum for 6 h does not affect 48 h *in vitro* gas production, or gas production rate or lag time, but 24 h storage reduced fermentation rate. Refrigeration of rumen fluid inoculum at 6 °C (Robinson et al., 1999) or 18 °C (Jones et al., 1998) up to 48 h did not markedly affect 48 or 72 h *in vitro* digestibility, but no results of differences in the fermentation pattern were reported. Chilling at 0–4 °C in anaerobic conditions maintains the numbers of total and specific fermentative rumen bacteria up to 6 h without major differences with the fresh inoculum, in contrast to frozen and freeze-dried inoculum (Dehority and Grubb, 1980).

Freezing has also been examined (Robinson et al., 1999; Cone et al., 2000; Hervás et al., 2005) and, although it did not greatly affect maximum fermentation, it delayed initiation and affected rate of fermentation. These experiments dealt with large batches of fluid and aimed to freeze at moderate temperatures (*i.e.*, −5 °C to −24 °C), and, in such conditions, freezing may extend for hours. The lethal effect of freezing and thawing occurs through membrane damage and DNA denaturation, and by an increase in the intracellular solute concentration (Panoff et al., 1998). The magnitude of freezing damage is dependant on cell size and membrane structure (Fonseca et al., 2001), and could alter microbial equilibrium, thus affecting the fermentation pattern. In general, freezing temperatures between 0 °C and −30 °C give poor results because of formation of eutectic mixtures causing high intracellular salt concentrations (Perry, 1998), but cellular viability when freezing in liquid nitrogen (−196 °C) is considered to be almost unaffected (Malik, 1991; Perry, 1998). Counts of some bacterial species remained high after long term storage at −80 °C or −20 °C using glycerol as cryoprotectant (Teather, 1982; Gibson and Khoury, 1986).

This research studied the effects of preservation method of ruminal inoculum on *in vitro* microbial fermentation using a novel gas production system with semi-continuous flow of the liquid phase (Fondevila and Pérez-Espés, 2008), in order to determine optimal times between inoculum extraction and *in vitro* incubation.

2. Materials and methods

2.1. Incubation conditions

Rumen inoculum was obtained from two cannulated adult sheep at the Servicio de Apoyo a la Experimentación Animal of the University of Zaragoza (Spain), that were fed 0.8 kg of a 750:250 mixture of alfalfa hay and ground barley grain daily. Rumen fluid was obtained by suction through the cannula 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 the incubation substrate.

The *in vitro* incubation system of Fondevila and Pérez-Espés (2008) with the following modifications was used. 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-mouth (*i.e.*, 8 mm diameter) polypropylene cap (Kartell, Noviglio, Italy). One mouth was provided with a three-way valve for measuring the internal gas pressure as an indicator of microbial fermentation. The other mouth was fitted with a two-way valve at the outside end and a 150 μm pore nylon cloth fixed in the inside end for liquid exchange (Fig. 1).

An approximate amount of 800 mg substrate was introduced into 6 cm × 3 cm sealed nylon bags (45 μm pore size) and incubated in each bottle. Bottles were filled with 80 ml of incubation solution (Theodorou et al., 1994) made with a buffer solution in which the concentration of bicarbonate ions was adjusted to maintain pH at 6.5 (Fondevila and Pérez-Espés, 2008), a macromineral solution and a reduction solution. Trace mineral solution and resazurin were omitted, as suggested by Mould et al. (2005). The incubation media also included 16 ml (0.20 of total volume) of rumen inoculum. The incubation solution was prepared under CO_2, and bottles were maintained at 39 °C in a water bath throughout the incubation.

The pressure of gas produced in each bottle was recorded with a HD8804 manometer with a TP804 pressure gauge (DELTA OHM, Caselle di Selvazzano, Italy) at different incubation times over a 24 h control period. Pressure readings (*i.e.*, mbar) were converted to volume (ml) using a pre-established linear regression between pressures recorded in this type of bottles and known inoculated air volumes at the same incubation temperature, as:

$$\text{Volume} = \frac{\text{Pressure} - 0.1263}{0.15637};\ R = 0.9985;\ \text{SE} = 0.0513;\ n = 72$$

Gas volume at each incubation time was expressed per unit of incubated dry matter (DM). 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 maintained anaerobically at 38 °C. The rate of the liquid phase turnover was adjusted to approximately 0.06/h, by replacing liquid media with incubation solution, according to this schedule: 10 ml every 2 h from 0 to 12 h of 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.