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Comparison of rumen fluid inoculum vs. faecal inoculum on predicted methane production using a fully automated *in vitro* gas production system

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ABSTRACT

The aim of the present study was to compare *in vitro* methane (CH_4) production and other fermentation parameters from different substrates incubated either in rumen or faecal inoculum. Five different substrates were incubated in two different inocula and gas recordings were made using an automated *in vitro* gas production system. The substrates were: particulate matter obtained from reticulo-rumen (RR) digesta and faeces (FC) by wet sieving, timothy hay (H), first cut grass silage (S) and a mixture of grass silage and barley (50:50; SB). One gram of each substrate was incubated either in 60 ml buffered rumen or faecal inoculum taken from Swedish lactating dairy cows for 48 h. The results indicated that *in vitro* total gas production, predicted *in vivo* CH_4 production and the ratio of CH_4 production to total gas production were greater ($P<0.01$) for substrates incubated in rumen inoculum as compared to faecal inoculum. Mean of predicted *in vivo* CH_4 production was greater for substrates incubated in rumen inoculum (23.5 ml/g DM, 29.0 ml/g OM) as compared to faecal inoculum (11.2 ml/g DM, 14.3 ml/g OM). Predicted CH_4 production based on volatile fatty acids (VFA) stoichiometry equations (CH_4VFA) showed no difference in CH_4 per mol VFA ($P=0.44$) between the two sources of inoculum used for all substrates. Molar proportions of propionate were higher and that of butyrate were lower ($P<0.01$) for all substrates incubated in faecal inoculum compared to rumen inoculum. No difference ($P=0.13$) in molar proportions of acetate was observed. Digestibility of neutral detergent fibre (aNDFomD) and true organic matter digestibility (TOMD) were lower ($P<0.01$) for all substrates incubated in faecal inoculum compared to rumen inoculum. It can be concluded that using faecal inoculum tended to give lower values of predicted *in vivo* CH_4 production as compared to rumen inoculum. The discrepancy between observed and stoichiometric CH_4 production suggests an existence of acetogenesis in the hindgut.

1. Introduction

Methane (CH_4) production from ruminants represents approximately 37% of total anthropogenic CH_4 emissions (Meale et al., 2013). Methane is one of the most significant contributors to the greenhouse effect, having effects on climate change and global warming by trapping heat 25 times more effectively than carbon dioxide (Myhre et al., 2013). Total dry matter intake and feed digestibility are among the driving forces affecting CH_4 production in ruminants (Johnson and Johnson, 1995; Yan et al., 2000; Jentsch et al., 2007; Ramin and Huhtanen, 2013). Since *in vivo* studies are very expensive and laborious, *in vitro* techniques have been developed to study ruminant nutrition and fermentation processes (Cone et al., 1996; Rymer et al., 2005; Getachew et al., 2005; Tagliapietra et al., 2010; Pellikaan et al., 2011). Methane production has also been successfully predicted based on the kinetic parameters obtained from the *in vitro* gas production system (Ramin and Huhtanen, 2012). In most *in vitro* studies, rumen fluid is used as an inoculum but fresh faeces from ruminants has also been investigated as an alternative to rumen fluid (Demeyer et al., 1996; El-Meadaway et al., 1998; Mould et al., 2005; Váradyová et al., 2005). Using fresh faeces as an inoculant for the *in vitro* gas production system would have some advantages as rumen cannulated animals are not needed. Methane is mainly produced in the rumen but also in the hindgut of ruminants (Murray et al., 1976). Calculating the hydrogen (H_2) recovery *in vitro* allows studying the existence of other H_2 sinks, especially in the hindgut fermentation of ruminants (Demeyer, 1991). However, measuring actual CH_4 production together with measurements based on volatile fatty acids (VFA) stoichiometry equations (CH_4VFA) allows a better

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understanding of hindgut fermentation in ruminants. The production of VFA and gases are stoichiometrically related (Wolin, 1960). Due to the possible influence of reductive acetogenesis in the hindgut, it can be assumed that stoichiometric calculations will predict a greater amount of CH_4 production from the hindgut (fresh faecal inoculum) than actual produced measurements of CH_4 production. The presence of high mucin and free amino acids concentration in the hindgut as a nutritional substrate, in contrast to the rumen, may lead to an induction of reductive acetogenesis in the hindgut (Demeyer et al., 1996). Fievez et al. (1999) indicated the presence and activity of acetogenic bacteria in the hindgut. The hydrogenotrophic capability and ecological significance of acetogenic bacteria in the rumen has also been investigated (Joblin, 1999). In certain conditions, hydrogen is mainly used by acetogenetic bacteria to produce VFA (mainly acetic acid) and to a smaller extent by methanogens to produce CH_4 (De Graeve and Demeyer, 1990). Data concerning actual measurements of CH_4 production from hindgut fermentation is scarce. We hypothesise that in the hindgut the acetogenesis process is more predominant as compared to the rumen, and that less CH_4 is produced per unit of fermented substrate using faecal rather than rumen inoculum. Therefore, the aim of this study was to compare rumen and faecal inocula in an automated *in vitro* gas production system with different substrates for determination of total gas, predicted *in vivo* CH_4 production from rumen and faecal inoculum, and other fermentation parameters. The second objective was to compare stoichiometric predictions of CH_4 based on VFA production with actual measurements of CH_4 production from the gas *in vitro* system based on two different sources of inocula.

2. Materials and methods

2.1. Sample preparation

Five different substrates varying in digestibility were selected for this study: grass silage (S), grass hay (H), a mixture of grass silage and barley (B) (50:50), pooled samples obtained by wet sieving (0.16–5.0 mm) from digesta particles collected from the rumen and reticulum (RR), and faecal particle matter (FC). The chemical composition of the substrates used in the current study is given in Table 1. The reason for selecting RR was to mimic the substrate entering the hindgut of ruminants and FC was chosen to represent a poorly digestible substrate. Prior to the incubations, samples were dried at 60 °C in a forced air oven for 48 h, and milled through a 1 mm screen using a cutting mill (Retsch, SM2000, Rheinische, Haan, Germany). The substrates were kept in sealed glass jars until required.

2.2. In vitro total gas and methane production measurements

The study was conducted with the permission of the Swedish Ethical Committee on Animal Research. Three cannulated Swedish Red lactating cows, fed a diet consisting of 60% grass silage and 40% concentrate on a dry matter (DM) basis for 3 weeks, were used as rumen fluid donors. Rumen fluid and faecal grab samples were collected at two hours after morning feeding. Rumen fluid and faeces were separately poured in pre-warmed thermos flasks previously flushed with carbon dioxide (CO_2). In this study two approaches were followed to predict CH_4 production: (1) predicted *in vivo* production throughout the fermentation based on the kinetics parameters obtained from the *in vitro* gas production system and a mechanistic rumen model and (2) based on *in vitro* VFA production at 48 h (stoichiometric equations). Rumen fluid from three cows was pooled, filtered through four layers of cheesecloth and flushed with CO_2. Filtered rumen fluid was then mixed with buffered mineral solution (Menke and Steingass, 1988) (20:80 v/v) supplemented with peptone (pancreatic digested casein) (Merck, Darmstadt, Germany) at 39 °C with constant stirring and continuous flushing with CO_2. Peptone was added to prevent nitrogen deficiency (Blümmel and Ørskov, 1992). Prior to the incubation, 1 g of each substrate was weighed into 250 ml serum bottles and placed in shaking water baths at 39 °C. According to Akhter et al. (1999) 500 g of fresh faeces (DM = 152 g/kg) taken from the same cows (pooled) was mixed with a buffer solution with the same ratio as the rumen inoculum (20:80 v/v) and then filtered through a two layer of cheesecloth. The buffer solution for fresh faeces was made by mixing 100 ml of stock solution (Akhter et al., 1999) with 400 ml of deionized water and maintaining at 39 °C until mixed with fresh faeces. One ml of ammonium sulphate (1.0 mol l^{-1}) was added to each bottles filled with buffered faecal inoculum. Finally, 18 bottles were filled with 60 ml of buffered rumen fluid and the other 18 bottles were filled with 60 ml buffered faecal fluid. Each substrate was incubated in three replicates either with rumen or faecal inoculum in two different runs for 48 h with blanks included for each inoculum. The *in vitro* gas production method used in the current study is described by Ramin and Huhtanen (2012) using a fully automated system for gas production measurement. The gases are released from the system by opening of the electric gas valve. Readings were done every 12 min and corrected to the normal air pressure (101.3 kPa) (Cone et al., 1996; Cattani et al., 2014). A logarithmic model of incubation time (h) *versus* CH_4 concentration (%) was developed for each bottle to estimate CH_4 concentration at time intervals of 0.2 h (the gas system recorded total gas production every 0.2 h). Samples of gas (0.2 ml) from the headspace of each bottle were collected with a gas tight syringe (Hamilton, Bonaduz, Switzerland) at 8, 24 and 48 h to be analysed for CH_4 contents by gas chromatography (Varian Chromatography, USA). Methane values estimated for each 0.2 h interval, for each substrate incubated in either rumen or faecal inoculum, were analysed with a two pool Gompertz model (Schofield et al., 1994) by the NLIN procedure of SAS (SAS Inst. Inc., Cary, NC). The resulting estimated kinetic parameters were used as input to run a mechanistic rumen model with a 50 h rumen retention time (20 and 30 h in rumen non-escapable and escapable pools) to predict the *in vivo* CH_4 production at maintenance level of intake separately for each substrate and each source of inoculum. Basically, predictions of CH_4 production from the kinetic parameters are analogous to calculating effective ruminal protein degradability or NDF digestibility for kinetic parameters derived from *in vitro* or *in situ* data. Details of the calculations and the modelling procedure are described by Ramin and Huhtanen (2012). Methane production (ml) based on VFA stoichiometry was predicted according to VFA (mmol) stoichiometry equations (CH_4VFA) as described by Wolin (1960)

$$CH_4VFA\,(ml) = 22.4 \times (0.5 \times acetate - 0.25 \times propionate + 0.5 \times butyrate)$$

Table 1
Chemical composition of the different substrates used in the gas *in vitro* incubations.

Substrate	DM (g/kg)	OM (g/kg DM)	NDF (g/kg DM)
RR[a]	963	962	810
FC[b]	953	954	800
Silage[c]	931	919	552
Hay	956	932	570
Barley	953	971	239

[a] RR: wet sieved digesta from rumen and reticulum (pooled).
[b] FC: wet sieved faecal particle matter.
[c] First cut silage (air-dried DM).