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Short communication

Comparisons of manual and automated incubation systems: Effects of venting procedures on *in vitro* ruminal fermentation

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ABSTRACT

Gas produced was measured based on headspace pressures in both manual and automated *in vitro* incubation systems, which were always vented at pre-set fixed times (**FT**) and at a pre-set threshold of fixed pressure (**FP**) respectively. This study was to investigate these two venting procedures (VP) on kinetics of total gas, methane (**CH_4**) and hydrogen gas (**gH_2**), substrate degradation and volatile fatty acid (**VFA**) production. Corn grain, rice straw, rice grain and tall fescue were selected for *in vitro* ruminal batch incubation. Headspace pressure of FT procedure was manually released at incubation times being 2, 4, 8, 12, 20, 28, 36, 48 and 72 h in manual incubation system, while headspace pressure of FP procedure was automatically released at threshold pressure being 9 kPa/bottle in automated incubation system. The FT procedure had particular high venting headspace pressure ($>$ 40 kPa) at some incubation times for corn grain and rice grain. The FT procedure had a lower gas volume ($P<0.001$) and fractional rate of gas production ($P<0.001$) than FP procedure, which was caused by the decreased ($P=0.01$) substrate degradation and increased partial dissolution of CO_2 under higher venting headspace pressure. The FP procedure had higher ($P=0.016$) fractional rate of CH_4 production than FT procedure. The FT procedure had lower final asymptotic gH_2 volume ($P=0.01$) and fractional rate of gH_2 formation ($P=0.002$), and higher propionate production ($P=0.05$), in comparison with FP procedure. The higher propionate production under FT procedure indicated that more H_2 was entering to VFA production, leading to less gH_2 released into the headspace of bottle. In summary, the rate of gas, CH_4 and gH_2 generation could be different between VP and VT procedure, and higher venting headspace pressure caused by FT procedure altered kinetics of gas, CH_4 and gH_2 volume, substrate degradation and VFA production.

1. Introduction

In vitro ruminal fermentation techniques have been widely employed to evaluate the nutritive values of feeds and additives and to examine influences on rumen fermentation and microbial ecology (Rymer et al., 2005; Wang et al., 2011). Recently, *in vitro* ruminal fermentation techniques began to be employed to investigate methane (**CH_4**) and hydrogen (**H_2**) gas (**gH_2**) production, which are important indicators for the ruminal Green House Gas and un-utilized H_2 by methanogens respectively (Wang et al., 2013a; Muetzel et al., 2014).

The first techniques for measurement of gas volume were based on the scale of syringes (Menke and Steingass, 1988). Later, pressure sensor was used to replace the syringe by measuring the cumulative headspace gas pressure (Theodorou et al., 1994; Rymer et al., 2005). Headspace gas pressure should be released to avoid the high pressure conditions ($>$ 45 kPa/bottle), which could lead to a massive partial dissolution of CO_2 in liquid phase and impairment of the activity of ruminal microbes (Theodorou et al., 1994). The headspace pressure could be manual vented at pre-set fixed times (**FT**) (Tagliapietra et al., 2010), or automatically released at a pre-set threshold of fixed pressure (**FP**) (Davies et al., 2000). High headspace pressure is easily avoided for FP procedure, and may happen for the FT procedure, leading to increased partial dissolution of CO_2 (Tagliapietra et al., 2010). The increased partial dissolution of CO_2 caused an elevated available dissolved CO_2 for methanogens, leading to the promoted growth and activity of methanogens (Patra and Yu, 2013), in turn may alter H_2 utilization and volatile fatty acids (VFA) production. However, studies were

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still lacking to study the effect of venting procedures (VP) on total gas, CH_4, gH_2 and VFA production.

In the present study, the FT and FP procedures were performed using manual and automated equipments respectively, and their effects on kinetics of *in vitro* total gas, CH_4, gH_2 production, substrate degradation and VFA profile were investigated.

2. Material and methods

2.1. Two in vitro incubation systems

The manual system: each bottle was connected to the pressure sensors (pressure range from 0 to +100 kPa; accuracy of ±0.1% of measured value) via an extension tube and Luer lok® fitting connected to a 23 g needle inserted through a butyl rubber stopper of the bottle. The headspace pressure of each bottle is read with a frequency of 1 min and recorded in the database during incubation. Each bottle is equipped with T tube that controls the release of the headspace gas pressure. When the T tube was closed, the fermentation gas accumulated in the headspace of bottle. Once reaching the pre-set venting time, the T tube was manually opened and a proportion of vented gas was sampled via a 5 ml syringe to determine CH_4 and gH_2 concentrations in gas chromatography (GC, Agilent 7890 A, Agilent Inc., Palo Alto, CA). As the gas sample injection loop of GC is about 0.5 ml, the volume for sampled gas was set to be more than 2 ml.

The automated system: each bottle was connected to a computer-controlled three-way solenoid valve via an extension tube and Luer lok® fitting connected to a 23 g needle inserted through a butyl rubber stopper of the bottle. Each pressure sensor (pressure range from 0 to +100 kPa; accuracy of ±0.1% of measured value) was equipped in the three-way solenoid valve, so that the pressure sensor could be connected with its corresponding bottle. The other way of three-way solenoid valve was connected to the GC via Luer lok® fitting and extension tube. When the three-way solenoid valve was in the normal closed position, pressure sensor was in line with bottle, so that fermentation gas accumulated in the headspace of bottle. When the three-way solenoid valve was in the open position, the GC was in line with bottle, so that the accumulated gas was vented through 0.5 ml gas sample injection loop attached to the GC column. Once reaching the pre-set threshold pressure, a 12 V signal generated by the computer was employed for gas venting by switching the normal closed position to open position of three-way solenoid valve. Venting event was ended after 15 s of venting, and GC was started to quantify the vented gas composition by switching sample injection loop in line with the column for 50 s. The CH_4 and gH_2 concentration were determined by a thermal conductivity detector and a flame ionisation detector respectively. The headspace of bottle was monitored by a computer in real-time, and recorded every 1 min. If more than one bottle reaches the threshold pressure, the bottle with the first reaching is vented first. As the dead volume between bottle and GC is 1 ml, the threshold pressure at least 6 ml (9 kPa/bottle) was set to flush the line (Muetzel et al., 2014).

2.2. The operation of two in vitro incubation systems

A 60 ml of water was injected into a 150 ml bottle, and 1, 2, 4, 8 and 16 ml of air was injected to get their corresponding headspace pressures. Slopes were obtained by linearly regressing the injected gas volume and their headspace pressures for both systems. The standard CH_4 used in GC contained 0.5%, 1%, 5%, 10% and 20% CH_4 in N_2, while standard gH_2 used in GC contained 0.05, 0.1, 0.5, 1 and 5 ml/l gH_2 in N_2. The CH_4 and gH_2 concentrations were calculated from the linear regression of the peak area over the concentration of the respective standard gases.

Table 1
Chemical composition (expressed in g kg^{-1} of dry matter) of the eight substrates used.

	Corn grain	Rice grain	Rice straw	Tall fescue
OM	976	985	935	900
CP	84.1	111	48.9	126
NDF	93.0	84.5	772	679
ADF	39.0	30.5	404	360
EE	29.3	6.10	33.8	44.3

OM, organic matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fiber; EE, ether extract

A leaking test was performed before the experiment to ensure that leaking would not occur during the process of *in vitro* fermentation (Muetzel et al., 2014). An incubation bottle filled with 60 ml of water and 5 ml of air was attached to every sensor. The system continued to measure the pressure over a period of 2 h to detect a decrease in pressure indicating a leak in the system.

2.3. In vitro incubation procedure

Four 65 °C dried substrates, including corn (*Zea mays*) grain, rice (*Oryza sativa L.*) stover, rice (*Oryza sativa L.*) grain and tall fescue (*Festuca arundinaceae*) hay, was selected to cover a large variability of fibre content (Table 1). Organic matter (OM) was measured by ashing at 550 °C for 12 h. The crude protein and ether extract were determined using methods 954.01 and 920.39 in AOAC (1995), respectively. The neutral detergent fibre (NDF) and acid detergent fiber, inclusive of residual ash, were determined using sodium sulfite and heat stable amylase (Van Soest et al., 1991).

About 600 ± 10 mg of each substrate was accurately weighed into a serum bottle and then pre-warmed at 39.5 °C in the incubator. Rumen fluid was collected via rumen cannulae from fistulated Liuyang black goats before morning feeding, which were fed with a mixed diet of rice straw and concentrate (1:1 DM basis) containing 137 g CP kg^{-1} DM and 380 g NDF kg^{-1} DM. The rumen fluid was filtered through five layers of cheesecloth, and then mixed with a pre-warmed McDougall's buffer, with the volume ratio of 1–4. Under a stream of CO_2, 60 ml of the buffered rumen fluid was manually dispensed into each bottle, which was flushed with CO_2 before dispensation. The bottle was capped with a butyl rubber stopper, incubated at 39.5 °C and connected to the gas system by stabbing the 23 g needle through the butyl stopper. Each bottle was conducted with mixed rumen fluid from two donor goats and repeated three times. Each treatment was conducted in triplicate, each with mixed rumen fluid from different two donor goats. The blank corrections were not performed, as venting events were not observed for the blanks after 72 h *in vitro* incubation.

For the FT procedure of manual system, the pre-set sequence of time for venting was 2, 4, 8, 12, 20, 28, 36, 48 and 72 h to meet the vented gas being more than 3 ml. A 3 ml of vented gas was sampled by 5 ml syringe to determine CH_4 and gH_2 concentrations. For the FP procedure of automatic system, the threshold pressure was pre-set to be 9 kPa/bottle. When the pressure in any bottle exceeded 9 kPa/bottle, three-way solenoid valve opened and gas was flushed through a sampling loop at the GC to measure CH_4 and gH_2 concentration.

The incubation was stopped at 72 h. A 2 ml of sample from the liquid phase was collected from each bottle for the analyses of VFA concentrations according to the method of Wang et al., (2014). The residual solution was filtered into the pre-weighed Gooch filter crucibles, dried at 105 °C for 24 h for *in vitro* dry matter