

# Relationships among Gas Production, End Products of Rumen Fermentation and Microbial N Produced *in vitro* at Two Incubation Times

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## Summary

This experiment compared linear relationships among end-products of rumen fermentation measured at the time ( $t_{1/2}$ ) at which a feed produces half of its asymptotic gas production) or at 48 h. Meadow hay and corn grain were incubated for  $t_{1/2}$  (16 and 9 h, respectively) or for 48 h into glass bottles. Each bottle (310 ml) was filled with feed sample (0.5 g) and 75 ml of buffered rumen fluid, and incubated at 39.0°C. Gas production (GP) was measured using the ANKOM<sup>RF</sup> System, and gas accumulated in headspace of bottles was released at 3.4 kPa. At  $t_{1/2}$  or 48 h, fermentation fluids were analysed for ammonia N (N-NH<sub>3</sub>), volatile fatty acids (VFA), residual NDF and N bound to residual NDF (N-NDF). Values of GP were also predicted from VFA. Microbial N (MN) was computed as the difference between N present at the beginning and at the end of incubation. At 48 h, the relationship between GP measured and predicted from VFA was weak ( $R^2 = 0.67$ ; equation not shown), whereas the linear relationship was better at  $t_{1/2}$  ( $R^2 = 0.94$ ). At  $t_{1/2}$ , the relationship between N-NH<sub>3</sub> and measured GP was strong ( $R^2 = 0.84$ ), as well as that between MN and measured GP ( $R^2 = 0.92$ ). Conversely, these variables were not well related at 48 h. At  $t_{1/2}$ , the valerate content in rumen fluid was negligible. However, relatively large amounts of valerate were measured after 48 h, probably the result of microbial lysis. Results suggest that relationships among end-products of rumen fermentation can be more accurately evaluated at a substrate-specific incubation time ( $t_{1/2}$ ) rather than at 48 h.

## Key words

rumen, *in vitro* fermentation, end-products of fermentation, incubation time

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## Aim

This experiment examined the validity of evaluating linear relationships among end-products of *in vitro* rumen fermentation (gas, volatile fatty acids, ammonia N and microbial N) at a substrate-specific incubation time rather than uniformly at 48 h. This specific time, defined as  $t_{1/2}$ , is the time at which a feed produces half of its asymptotic gas production, as described by Blümmel et al. (1997).

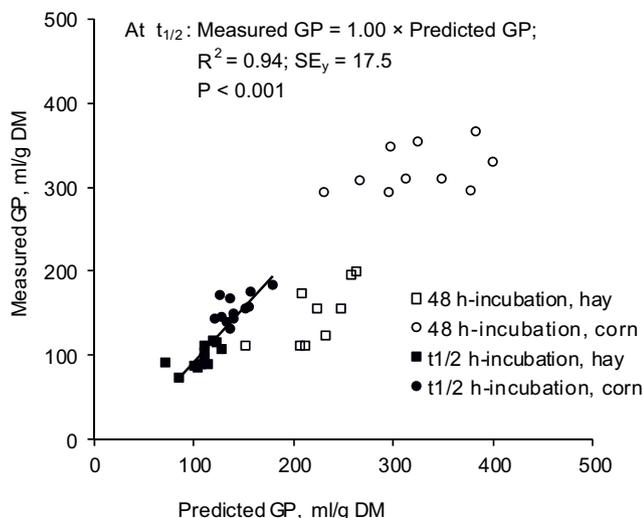
## Material and methods

A preliminary incubation (144 h) was conducted, where samples of meadow hay and corn grain were incubated, in four independent replicates, in individual bottles, to determine the time at which two feeds reached half of their asymptotic gas production ( $t_{1/2}$ ). The  $t_{1/2}$  incubation values were determined as 16 h for meadow hay and 9 h for corn grain. In a second incubation, the two feeds were incubated for the corresponding  $t_{1/2}$  (16 or 9 h) or for 48 h, without (control) or with vitamin E or vitamin C. Results regarding the effects of vitamins are described by Tagliapietra et al. (2013). The experimental design was: 2 incubation times  $\times$  2 feeds  $\times$  4 replications  $\times$  3 treatments (control plus 2 vitamins), for a total of 48 bottles, plus 4 blanks (bottles without feed sample). A buffer solution was prepared according to Menke and Steingass (1988). Rumen fluid was collected by an oral-esophageal probe 2 h before morning feeding from 3 dry Holstein-Friesian cows fed hay *ad libitum* and 2 kg/d of concentrates (Tagliapietra et al., 2012). Once at the laboratory, rumen fluid was filtered through 3 layers of cheesecloth, to eliminate feed particles, and mixed with the buffer solution in a 1 to 2 ratio, under anaerobic conditions (Menke and Steingass, 1988). Each fermentation bottle (310 ml) contained a feed sample (0.5 g) and 75 ml of buffered rumen fluid (headspace = 235 ml). After filling, all bottles were placed into an incubator at  $39.0 \pm 0.5$  °C. Fermentation bottles were not shaken during the incubation. *In vitro* gas production (GP) was measured using ANKOM<sup>RF</sup> System (Ankom Technology<sup>®</sup>, Macedon, NY, USA). This system consists of bottles equipped with a pressure detector (pressure range: -69 to +3447 kPa; resolution: 0.27 kPa; accuracy  $\pm 0.1\%$  of measured values) and an open-closed valve that releases gas accumulated in the headspace of bottles at a threshold pressure of 3.4 kPa (Tagliapietra et al., 2010). Pressure values were converted into gas volumes (ml) using the ideal gas law and corrected for baseline fermentation in the buffered rumen fluid (blanks). The cumulative volumes of GP were fitted using the model of Groot et al. (1996). At the end of incubations ( $t_{1/2}$  and 48 h), two aliquots (5 ml) of fermentation fluid were collected from each bottle, two ml of metaphosphoric acid (25% w/v) were added, and stored at -20°C until analysis of ammonia N (N-NH<sub>3</sub>). The N-NH<sub>3</sub> content was measured according to Bailey (1980) with a potentiometer (Bench pH/ion meter, Oakton Instruments, Vernon Hills, USA) equipped with a specific electrode (pH meter BASIC 20, Crison Instruments, Alella, Spain). VFA profiles were determined using HPLC (Thermo-Finnigan, CA, USA). Values of GP were also predicted from VFA profile, (Blümmel et al., 1997). All fermentation fluids were filtered into F57 filter bags (Ankom technology<sup>®</sup>, Macedon, NY, USA), and analysed for residual NDF without  $\alpha$ -amylase and sodium sulphite (Grings et al.,

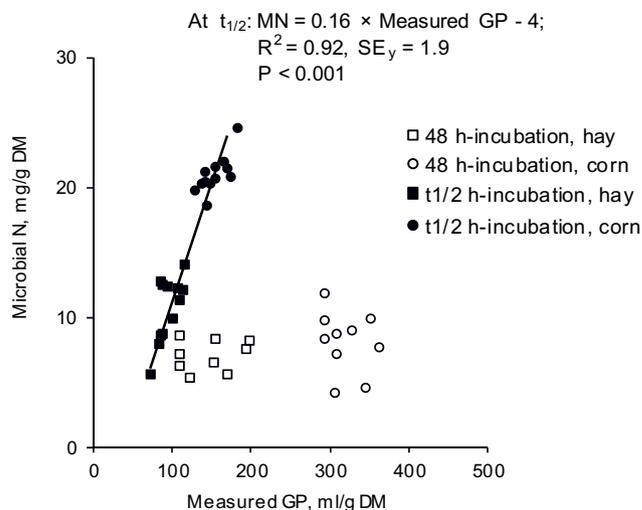
2005) using the Ankom<sup>220</sup> Fibre Analyser (Ankom Technology<sup>®</sup>, Macedon, NY, USA). After that, the N content of residual NDF (N-NDF) was determined using the Kjeldahl method (AOAC, 2003). This N source is not considered to be available for microbial growth (Grings et al., 2005). Microbial N (MN; mg/g DM incubated) was indirectly computed as the difference between N content at the beginning (sum of N supplied by feed and of initial N-NH<sub>3</sub>) and at the end of incubation (sum of N-NDF and of final N-NH<sub>3</sub>), as suggested by Grings et al. (2005). Values of GP, end-products of rumen fermentation and microbial N achieved at  $t_{1/2}$  or 48 h of incubation were compared by linear regression. A linear regression within feed type was also computed. At 48 h of incubation two samples for both the feeds were discarded due to anomalous fermentation process and thus not analyzed.

## Results and discussion

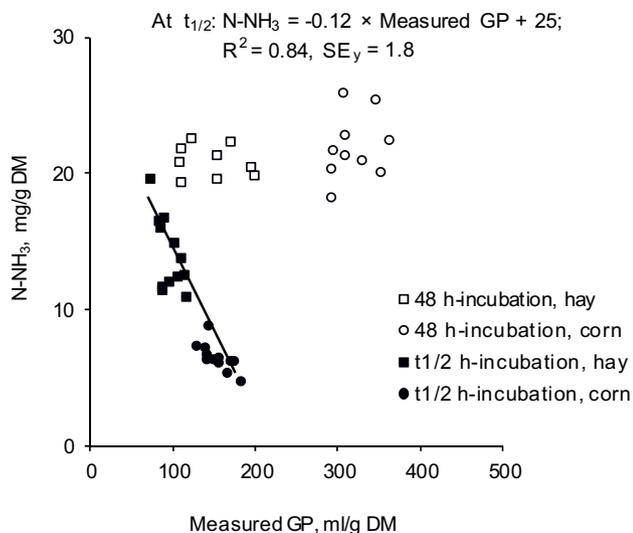
Blümmel et al. (1997) suggested that microbial growth should be evaluated at substrate-specific incubation times, shorter than fixed times (24 and 48 h) used to predict energy value of feeds from feed digestibility or from GP and some feed chemical constituents (Goering and Van Soest, 1970; Menke and Steingass, 1988; Robinson et al., 2004; Tagliapietra et al., 2011), as the kinetics of microbial growth notably differ among substrates. Some studies conducted *in vitro* (Blümmel et al., 2003; Grings et al., 2005; Cattani et al., 2012) evidenced that  $t_{1/2}$ , the time at which a feed produces half of its asymptotic GP, could be a proper time to measure microbial growth and end-products of rumen fermentation (ammonia, VFA). In the current experiment values of measured GP showed some variability (Figure 1) partially due to the effects of the two vitamins on rumen fermentation, as detailed by Tagliapietra et al. (2013). A weak linear relationship ( $R^2 = 0.67$ ; equation not shown) was found between measured GP and predicted from VFA at 48 h, as fermentation of lysed cells probably occurred and altered GP and VFA values (Figure 1). Cone et al. (1997), from *in vitro* incubation of a rapidly fermentable substrate (pure glucose), noted that GP continued at prolonged incubation times (>15 h), even if the substrate was completely fermented, and attributed this pattern to a secondary fermentation of microbial population. In contrast, the relationship between measured and predicted GP was definitely better at  $t_{1/2}$  ( $R^2 = 0.94$ ;  $P < 0.001$ ), irrespective of feed, with a slope equal to 1.00 (Figure 1). This suggests that microbial lysis was low at  $t_{1/2}$ . When incubation lasted 48 h, both the N-NH<sub>3</sub> content of rumen fluid and the estimated MN were not correlated to GP values (Figure 2 and 3). In contrast, when incubation was stopped at  $t_{1/2}$ , the relationship between N-NH<sub>3</sub> and measured GP was close ( $R^2 = 0.84$ ;  $P < 0.001$ ; Figure 2), as well as that between the estimated MN and measured GP ( $R^2 = 0.92$ ;  $P < 0.001$ ; Figure 3). At  $t_{1/2}$  the N-NH<sub>3</sub> content decreased by about 0.12 mg/ml of increased GP, whereas the estimated MN increased by 0.16 mg/ml of increased GP. The linear relationship between MN and measured GP showed a negative intercept, thus the increase of MN was not directly proportional to the increase of GP. At  $t_{1/2}$ , concentration of valerate in rumen fluid was negligible ( $< 0.03$  mmol/g DM; Figure 4). Conversely, amounts of valerate were great and extremely variable at 48 h of incubation (Figure 4). High concentrations of valerate in rumen fluid could be related to lysis of microbial population, as valerate



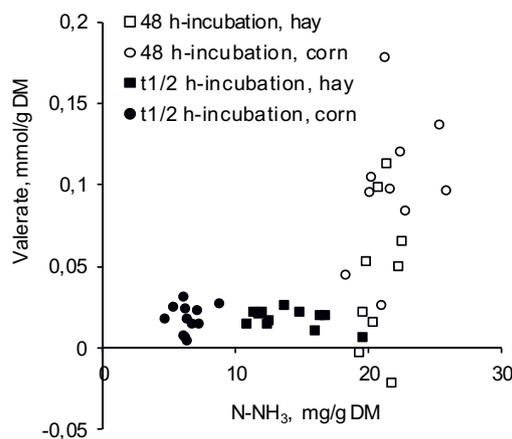
**Figure 1.** Relationships between values of gas production (GP) measured (y) or predicted (x) from VFA after 48 h (□, ○) or t<sub>1/2</sub> (■, ●) of incubation of meadow hay (■, □) and corn grain (●, ○)



**Figure 3.** Relationship between estimated microbial N production (Microbial N; y) and measured gas production (measured GP; x) after 48 h (□, ○) or t<sub>1/2</sub> (■, ●) of incubation of meadow hay (■, □) and corn grain (●, ○)



**Figure 2.** Relationships between ammonia N content in the buffered rumen fluid (N-NH<sub>3</sub>; y) and measured gas production (measured GP; x) after 48 h (□, ○) or t<sub>1/2</sub> (■, ●) of incubation of meadow hay (■, □) and corn grain (●, ○)



**Figure 4.** Relationships between valerate yield (Valerate; y) and ammonia N content in the rumen fluid (N-NH<sub>3</sub>; x) after 48 h (□, ○) or t<sub>1/2</sub> (■, ●) of incubation of meadow hay (■, □) and corn grain (●, ○)

is primarily derived from protein degradation (Hungate, 1966). Linear regression within feed type confirmed that the various end-products of fermentation were not related at 48 h of incubation (data not shown). Conversely, at t<sub>1/2</sub> significant relationships ( $P < 0.001$ ) were found, even if the degree of correlation was weaker than that found when the data of the two feeds were considered in the same linear regression.

## Conclusions

Results of the current experiment suggest that relationships among end-products of rumen fermentation should be evaluated at substrate-specific incubation time (t<sub>1/2</sub>). At prolonged incubation times (48 h) microbial lysis occurs, altering values of GP, VFA, ammonia and microbial N, as well as their relationships. When fermentation process is stopped at t<sub>1/2</sub>, strong relation-

ships among measured GP, ammonia N concentration in the rumen fluid and estimated microbial N growth can be found and microbial lysis is prevented.

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