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The nutritional evaluation of forage-based mixed rations in New Zealand using an *in vitro* gas production technique. 1: analytical survey

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Abstract

Greenhouse gas (GHG) emissions from livestock are an important consideration in environmental science. Estimating GHG production can be problematic at a farm or animal level, and requires controlled conditions to produce real data. An *in vitro* gas production technique (IVGPT) was developed to evaluate forage-based total mixed rations in digestion kinetics and GHG production. Two hundred and sixty samples of complete mixed rations (MR), which included a pasture component used in commercial lactating dairy herds, were collected around NZ across three calendar years, 2017-2019. Twenty of the 260 samples were 100% total mixed rations (TMR) with no pasture content. The samples were submitted for proximate analysis as well as IVGPT to generate GHG production figures. The results showed an average total gas production (TGP) of 129.82 ml/g dry matter (DM), 78.6% true digestibility (TDMD), 125.06 mg/g DM microbial biomass (MB), 20.16 g CH_4/kg DM, and 12.8 MJME/kg DM. The average nutrient composition was dry matter (DM) 31.55%, crude protein (CP) 21.85%, neutral detergent fibre (NDF) 44.35%, and starch 7.03%. The IVGPT CH_4 production was negatively correlated to NDF (r=-0.312), ADF (r=-0.193), TGP (r=-0.216), and was positively correlated with TDMD (r=0.250), apparent digestibility (ADMD) (r=0.614), starch (r=0.117) and volatile fatty acids (r=0.538). The MR diet showed a strong positive relationship with ADMD digestibility (P=0.01) and a negative relationship with fibre content (NDF, P=0.01 and ADF, P=0.01). However, CH_4 production reduced linearly with increasing TGP (P=0.01). The results indicated that a greater CH_4 production may be related to higher digestibility of mixed ration.

Keywords: *in vitro* rumen fermentation, digestion kinetics, dairy performance, methane production

1. Introduction

Agriculture is said to account for 48% of NZ GHG emissions (Ministry for Environment, 2021). A pasture-based diet is commonly used for dairy cows in New Zealand. The amount of supplementary or complementary feed provided and pasture access varies considerably between regions and individual farms. Proximate analyses of feeds for starch, sugar, crude protein (CP), fat, fibre (NDF and ADF) and ash content are regularly performed. Hence, it would be interesting to construct a stepwise regression or a principle components analysis to gain further insight into how the mix or nutrients in a diet are influencing GHG production.

The *in vitro* gas production technique (IVGPT) is a valuable tool to evaluate rumen digestibility of feed. Moreover, the ability to measure gas evolution in 'real-time' analysis during *in vitro* fermentation provides data that can then be used to test any relationship between the kinetics of digestion and potential environmental effects. The Gompertz model (Armah *et al.*, 2020) for determining dual 'gas pool' production involves both a Fast Pool or Slow Pool, i.e. rate of feed breakdown and gas evolution during IVGPT, provides insight into the relationship of energy reserves available from starch or sugar (in the Fast Pool) or fibre (in the Slow Pool). Measuring total gas production (defined as the total gas pool; TGP) from fermentation allows individual calculations of carbon dioxide (CO_2) and methane (CH_4) production from volatile fatty acid (VFA) concentrations in the liquor, according to the stoichiometry proposed by Wolin (1960).

There is limited data from grass-based feeding systems in dairy cows in NZ to allow comparisons of kinetics of digestion and enteric CH_4 emission from commercially practical rations. Therefore, this study's objective was an *a posteriori* observation of data accumulated from complete diets from pasture-based dairy cow diets in NZ using IVGPT to evaluate the relationship between diet and CH_4 emissions.

2. Materials and methods

Rumen fluid source

Three dairy cows (live weight (LW) approximately 500 kg), were each fitted with a small (300 mm diameter) rumen cannula. Rumen fluid was taken from one of these cows, depending on which ones were in lactation at the time of sampling. The weekly variation in donor fluid was considered, and so a known standard ration with confirmed characteristics was included as a standard as part of each weekly fermentation to provide a correction factor. These animals were sourced from a commercial dairy herd, and the experimental use of animals was approved by the Animal Ethics Committee at AgResearch Ltd., Ruakura, New Zealand, (AE Application 14879).

Feed sample preparation

All feed ingredients, including pasture, from each of the 260 ration samples used on commercial farms were harvested. The IVGPT samples were dried at 55 °C for 48 h and then ground through a 2 mm sieve in a Wiley Mill grinder (Arthur A. Thomas, Philadelphia, PA, USA). Samples were then mixed at the Alltech Oceania IFM Laboratory (Auckland, NZ) according to each farm's ingredient ratio to provide the full mixed rations (MR) as a completed ration. The MR sample was further ground through 1 mm (nutrient analysis), and 0.5 mm (starch and sugar) sieves.

Nutrient analysis

Dry matter (DM) was determined after drying at 135 °C for 4 h (AOAC, 2006) followed by ash analysis, which was measured as weight lost after furnace ignition at 600 °C for 2 h (AOAC, 1995). Acid detergent fibre (ADF) and neutral detergent fibre (NDF) were measured as described by Van Soest *et al.* (1991). Lignin, fat, and CP were performed according to AOAC (2006) methods. Starch and sugar were measured according to the method of Hall (2015). Soluble protein (SPR) was determined using the method devised by Licitra *et al.* (1996). The estimation of non-fibre carbohydrates (NFC) and total digestible nutrients (TDN) were calculated using NRC (2001) equations. The metabolisable energy (ME) content was determined as digestible organic dry matter × 0.16 (Chay-Canul, 2011).

In vitro gas production technique

Gas production was determined using methods described by Tilly and Terry (1963) and Mould *et al.* (2005). Briefly, each of the 2 mm dried samples were weighed and sealed in a synthetic cellulose bag, placed into a 250 ml glass flask and flushed with CO_2 to clear out any air. The gas was then removed, and the flask kept under vacuum and pre-incubated in a water bath set not to exceed 39 °C.

Rumen fluid was collected into thermos flasks from the cannulated cows at 08:30 h. The flask was transported to the IFM Oceania Laboratory (Auckland, NZ), filtered and mixed with rumen buffer in a ratio of 20:80, and continually flushed with CO_2 for 5 min to create a buffered rumen inoculum. A glass flask was filled with 100 ml buffered rumen inoculum using an auto liquid dispenser and incubated at 39 °C for 48 h. Accumulated gas during incubation was measured and recorded continuously at 7 s intervals using a custom-automated pressure transducer system (Alltech, 2008).

At the end of the fermentation period, the liquid sample was collected, and centrifuged (3,000 rpm, 10 min, 4 °C) to remove any sediment. The liquid phase was used for VFA analysis (Erwin *et al.*, 1961), which was measured by gas chromatography (GC), using column: CP7686; length 25 m, diametre 0.15 mm, film 0.25 μm with split inlet and 7693B Auto-injector. The residue of undigested feed in the synthetic bag was dried and weighed to calculate apparent DM digestibility (ADMD). True DM digestibility (TDMD) was estimated after treating the residue with a modified NDF solution. The MB was calculated as the difference between the amount of ADMD and TDMD measured (Goering *et al.*, 1970).

Gas sampling and methane measurement

Methane evolution was estimated by using a stoichiometry calculation. The liquid fermentation sample was centrifuged at 12.5×*g* for 15 min to separate the residue. The samples were analysed in a GC 7890B, equipped with a flame ionising detector, and compared with a mixed VFA standard (containing acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate).

Gas kinetics

Gas production kinetics were analysed using the Gompertz model described by Schofield *et al.* (1994). The gas production curve was fitted to a two-pool logistical model to estimate degradation rate constants for the fast rate and slow rate and gas production volumes of fast pool and slow pool. Gas production was reported as total gas (TGP; ml/g DM) and split into fast pool and slow pool.

Statistical analysis

Gas production and nutrient data were analysed using the Proc Univariate of SAS Pro (Version 9.4; SAS Institute, Cary, NC, USA). The samples were stratified into deciles and correlations.(Proc CORR) were conducted using methane as the dependent variable, while chemical and fermentation parameters were denoted as independent variables. Significance was attained at $P \leq 0.05$.

3. Results and discussion

Nutrient composition of the mixed rations

The 260 mixed ration samples taken from dairy farms comprised unspecified pasture species and up to 10 other feed ingredients. There were few samples with no pasture inclusion. The nutrient composition of all the feed samples is shown in Table 1. The DM averaged 31.5%, and CP and SPR averaged 21.8 and 39.5%, respectively. The fibre levels, measured as NDF and ADF, were 44.3 and 26.6%, respectively. Starch averaged 7.0% and sugar 4.8%. Other components (average for all samples) included fat (5.4%), ash (9.8%), NFC (18.4%) and TDN at 59.4%. There was a high variation (CV, %) for NFC, starch and sugar, which was likely due to the various carbohydrate ingredients used in the mixed rations between farms.

The major component of mixed rations analysed in this study was pasture, with inclusions between 60-100% in 75% of the 260 mixed rations. The pasture types were not identitied in this study, however ryegrass is predominant in New Zealand dairy production systems and was assumed to be present in the majority of mixed rations.

This study did not investigate the effect of inclusion level of pasture on VFA production, but this should be considered for further investigation. In a relatively small *in situ* study, Van Vuuren *et al.* (1992) observed no change in VFA with varying levels of rye grass dominated pasture inclusion.

Fermentation

The *in vitro* fermentation and digestion rates are shown in Table 2. The results showed that digestion kinetics were 38.5 ml/g DM at the rate of 26%/h for Fast Pool and 91.3 ml/g DM at the rate of 5%/h for Slow Pool gas production, with a lag time of 1.2 hours. The average ADMD and TDMD of the MR were 67.0% and 78.6%, respectively. Rumen bacterial growth, expressed as MB, was 125.06 mg/g DM, and total VFA averaged 21.9 mM. The average major VFA composition was 50.0% acetate, 30.1% propionate, and 15.6% butyrate of the total VFA. Methane averaged 20.1 g/kg DM. The ME content averaged 12.8 MJ ME/kg DM. Interestingly, the digestibility of both ADMD and TDMD had low variation (CV%), and this indicated that the MR diet had similar digestibility even when different ingredients were used This result was similar to those reported by Getachew (2005) that showed 83.0% dry matter digestibility of TMR.

The opportunity presented itself to make an *a posteriori* evaluation of an existing fermentation data set. Hence, gas production and kinetics in this study were observations made on mixed rations composed of a variety of of dietary combinations of ingredients that had not themselves been stratified by digestion kinetics. Total gas production was 129.8 ml/gDM lower than seen in the previous study of Getachew *et al.* (2005) which produced 215 ml/gDM at 48 h incubation of a TMR. Interestingly, the kinetics of the current study were 26.5%/h Fast rate and 5.00%/h Slow rate contrasted markedly with a previous study of Mjoun and Gehman (2015) on TMR from South Dakota, USA which showed 19.9%/h fast rate and 4.5%/h slow rate). The slightly higher Fast rate might have been because of pasture inclusion in New Zealand, which contains higher levels of sugar.

Table 1. Nutrient composition of mixed ration samples.

Parameter[1]	Average	Standard deviation	Minimum	Maximum	CV %
Dry matter (DM)	31.5	8.4	11.9	70.1	26.9
Crude protein, %DM	21.8	3.8	11.7	32.0	17.6
Soluble protein, %CP	39.3	8.2	10.2	68.4	21.0
ADF, %DM	26.6	4.2	16.4	40.7	15.9
NDF, %DM	44.3	6.0	27.9	57.7	13.5
Lignin, %DM	4.6	2.2	0.9	15.7	48.1
Starch, %DM	7.0	6.1	0.0	31.6	88.1
Sugar, %DM	4.8	2.4	0.4	14.9	51.9
Fat, %DM	5.4	1.1	2.5	11.7	20.9
Ash, %DM	9.8	1.5	4.7	16.6	15.5
Non-fibre carbohydrate %DM	18.4	8.6	0.0	44.1	46.9
Total digestion %DM	59.4	7.0	17.2	77.9	11.8

[1] ADF = acid detergent fibre; NDF = neutral detergent fibre.

Table 2. Fermentation production of total gas, nutrient digestibility, kinetics, microbial biomass and greenhouse gas evolution.

Parameter	Average	Standard deviation	Minimum	Maximum	CV %
Fast pool, ml/g DM	38.5	11.7	9.8	74.5	30.4
Fast rate, %/h	26.5	79.0	12.1	82.0	29.9
Slow pool, ml /g DM	91.2	14.5	51.5	144.0	15.9
Slow rate, %/h	5.0	1.0	2.9	12.7	19.2
Lag, hr	1.2	0.6	0.0	4.4	54.1
Total, ml/g DM	129.8	17.1	71.0	180.6	13.2
Aparent dry matter digestibility, %	67.0	64.0	45.3	83.1	9.5
True dry matter digestibility, %	78.6	52.0	60.6	89.1	6.6
Microbial biomass, mg/g DM	125.0	25.2	50.3	185.5	20.2
Volatile fatty acids mM	21.9	2.9	12.7	29.6	13.2
Acetate, %	50.0	3.6	40.4	62.0	7.3
Propionate, %	30.1	2.7	23.4	40.0	9.2
Butyrate, %	15.5	2.8	8.7	23.1	18.0
CH_4 g/kg DM	20.1	3.5	9.8	28.0	17.4
Metabolisable energy, MJ/kg DM	12.8	4.1	9.3	35.0	31.8

The VFA production in this study (21.9 mM) was slightly lower than the study of Mjoun and Gehman (2015), that reported 24 mM in TMR. The methane production showed similar results (20.1 g/gDM, equivalent to 28.0 ml/gDM, data not shown) compared to 26.0 ml/g DM reported by Mjoun and Gehman (2015). An even higher result was shown in the *in-vivo* study of Holter and Young (1992) at 29.2 ml CH_4/g DM. Interestingly, the average methane production from this study was at least 16% lower than previous studies that ranged between 31.0-38.9 ml CH_4/g DM) (Eun *et al.*, 2004; Getachew *et al.*, 2005) with TMR. The possible influence of ingredient selction on such results should be considered in future studies on mitigations of total enteric GHG from ruminants.

Pearson linear correlation coefficient (r) between IVGPT CH_4 production with fermentation parameters and nutrient composition

The relationship between methane and nutrient and fermentation as *r* values are shown in Table 3. Negative correlations were found between ADF (r=-0.193), NDF (r=-312), and Fast Rate (-0.052; P<0.01) with CH_4 g/kg DM. Methane production was negatively correlated (P<0.01) to MB (r=-0.250) and total gas (r=-0.216) but positively correlated with apparent dry matter digestibility (ADMD r=0.614, TDMD r=0.25) and VFA (r=0.538). This suggested that, as digestibility increased, the increasing available energy in diets with higher digestibility characteristics may be partitioned more towards gas and VFA as opposed to microbial biomass.

The negative correlation of methane with the Fast rate may have been because the high rumen degradable material, such as grains, increased propionate production in the fermentation, which reduced proportions of other VFAs, such as acetate and butyrate, as seen in previous studies (Bargo *et al.*, 2003; Garcia *et al.,* 2000; Valk *et al.*, 1990).

Table 3. Pearson linear correlation coefficients (r) between methane production, fermentation and nutrient composition.

Parameter	CH_4 g/kg DM	*P*-value
Dry matter (DM)	0.092	0.13
CP, %DM	0.037	0.54
SPR, %CP	0.078	0.20
ADF, %DM	-0.193	0.01
NDF, %DM	-0.312	0.01
Lignin, %DM	0.000	0.99
Starch, %DM	0.117	0.05
Sugar, %DM	0.319	0.01
Fat, %DM	0.069	0.27
Ash, %DM	-0.037	0.55
NFC, %DM	0.197	0.01
TDN, %DM	0.159	0.01
Fast pool, ml/g DM	0.463	0.01
Fast rate, %/h	-0.052	0.40
Slow pool, ml/g DM	0.350	0.01
Slow rate, %/h	0.091	0.14
Total, ml/g DM	-0.216	0.01
ADMD, %	0.614	0.01
TDMD, %	0.250	0.01
MB, mg/g DM	-0.250	0.01
VFA, mM	0.538	0.01

[1] ADF = acid detergent fibre; ADMD = apparent DM digestibility; CP = crude protein; MB = microbial biomass; NDF = neutral detergent fibre; NFC = non-fibre carbohydrates; SPR = soluble protein; TDMD = true DM digestibility; TDN = total digestible nutrients; VFA = volatile fatty acid.

The decrease in methane as microbial growth increased might have been because the latter was subject to competitive behaviour induced by using starch for cell cultivation, rather than producing fibrolytic enzymes as a secondary metabolite. This indicated that the cow had less energy supplied from fibre.

The types of rumen microbes, such as acetogen and methanogen bacteria, were expected to impact CH_4 production by breaking down feed into acetic acid, generating CH_4 as the final product (Holter and Young, 1992; Weimer *et al,* 1998). Lower levels of CH_4 may have been related to the low population of methanogen bacteria.

Therefore, further study of acetogen and methanogen bacteria, including their populations, is required.

4. Conclusions

The digestibility kinetics of a forage-based, mixed ration diet for lactating dairy cows in NZ was studied using IVGPT technique. The potential for methane production was investigated regarding the relationship between diet type and gas production. The results showed that CH_4 was negatively (P<0.01) related to NDF (-0.312), ADF (-0.312) and positively related to sugar (0.319), starch (0.117), NFC (0.197)and TDN (0.159). Fermentation showed a significant (P<0.01) positive relationship between CH_4 and Fast Pool (0.463), Slow Pool (0.350), ADMD (0.614), TDMD (0.250) and VFA (0.538) but a negative relationship with TGP (-0.216) and MB (-0.250). This indicated that CH_4 production was reduced in mixed diets that had higher fibre levels, which may have interrupted fermentation and reduced total gas production. The greater microbial biomass can reduce CH_4 production while converting feedstuffs to energy in the forms of VFA and MB.

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Conflict of interest

N. Meads and N. Jantasila are employees of Alltech NZ Ltd. R. Tahmasbi is an employee of Alltech Lienert Australia.

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