Reducing *in vitro* rumen methanogenesis for two contrasting diets using a series of inclusion rates of different additives

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Abstract. Eleven individual additives were incubated with either perennial ryegrass or with grass silage+barley grain (50:50) and the *in vitro* methane output was assessed using the gas production technique (GPT). Additives were: fatty acids (lauric, oleic, linoleic and linolenic acids), halogenated methane analogues (bromoethanesulfonate and bromochloromethane), pyromellitic diimide, statins (mevastatin and lovastatin), a probiotic (*Saccharomyces cerevisiae*) and an unsaturated dicarboxylic acid (fumaric acid). Each additive was included at a range of concentrations. Effects on methane output per gram of feed dry matter (DM) incubated (CH_4/DMi) and disappeared (CH_4/DMd), as well as other fermentation variables, were evaluated after 24 h of incubation. The addition of increased concentrations of individual fatty acids, bromoethanesulfonate and pyromellitic diimide caused a dose-dependent decline in methane output (CH_4/DMi, CH_4/DMd), when incubated with either perennial ryegrass or grass silage+barley grain. No methane output was detected for either feed with the addition of ≥ 5 μM bromochloromethane. The statins were ineffective inhibitors of methane output regardless of feed type. For perennial ryegrass, *S. cerevisiae* caused a dose-dependent decline in CH_4/DMd and fumaric acid a dose-dependent decline in CH_4/DMi and CH_4/DMd. The effectiveness of lauric, oleic, linoleic and linolenic acids and bromoethanesulfonate to reduce methane output was more pronounced when incubated with grass silage+barley grain than with perennial ryegrass, and therefore the type of feed is an important component for any future *in vitro* and *in vivo* studies to be undertaken with these additives. Thus, incorporating different feed types in the initial *in vitro* screening protocols of all new additives is recommended.

Additional keywords: *in vitro* total gas production technique, rumen fermentation modifiers, ruminant diets.

Introduction

The ruminant diet has a major effect on enteric methane production (Moss *et al*. 2000). The contribution of enteric methane to greenhouse gas emissions and the substantial loss of ruminant feed energy as methane has prompted research into chemical and biological compounds which can be either an integral part of the animal diet (e.g. dietary inclusion of fatty acids using coconut oil as demonstrated by Lovett *et al*. (2003)) or added as an additive (e.g. ionophoric antibiotics (monensin) as shown by Goodrich *et al*. (1984)) to reduce enteric methane losses. The use of ionophores to manipulate rumen fermentation and, thus, reduce methane emissions is being limited because of human health concerns (Nikolich *et al*. 1994; Moss *et al*. 2000). Consequently, there has been an increasing interest in fatty acids, halogenated methane analogues (HMA), probiotics (predominantly *Saccharomyces cerevisiae* and *Aspergillus oryzae*) and dicarboxylic acids as enteric methane inhibitors. The mode of action of these compounds to reduce ruminant methane emissions are quite variable, with some being directly inhibitory to methanogens (e.g. fatty acids (Zhang *et al*. 2008),

HMA (Prins *et al*. 1972)) and/or protozoa (e.g. fatty acids (Matsumoto *et al*. 1991)), while the same or different compounds cause a shift towards the production of propionate in the rumen (e.g. fatty acids (Machmüller *et al*. 2003), dicarboxylic acid (López *et al*. 1999)). The responses of rumen fermentation to yeast cultures are highly variable and yeast cultures do not consistently decrease methane production *in vivo* (Moss *et al*. 2000). The use of statins (e.g. mevastatin, lovastatin) is a relatively new approach to reduce enteric methane emissions and was demonstrated by Miller and Wolin (2001) who inhibited the growth of pure strains of *Methanobrevibacter* isolated from the rumen.

Regardless of the compound chosen to reduce enteric methane emissions, the basal diet on which the animal is fed appears to be important for the effectiveness of many of the aforementioned compounds. For example, Machmüller *et al*. (2003) found that the decrease in methane emissions achieved in response to myristic acid was twice as large in the rumen of sheep consuming a concentrate- rather than a forage-based diet. For other compounds such as HMA, Cole and McCroskey (1975)

suggested that the level of concentrate supplementation could affect their efficacy as methane inhibitors. The implications of these differences for practical farming systems could be important in countries such as Ireland where grassland is the dominant (~0.9) crop on agricultural land and provides the lowest-cost feed available for ruminant production systems (O'Riordan and O'Kiely 1996; Finneran *et al.* 2012). Concentrate feeds (with grass silage) are fed when livestock are accommodated indoors and these feeds can also constitute a major proportion of the lifetime feeding costs of ruminants (O'Riordan and O'Kiely 1996).

Some limitations of previous research in this area include the absence of data across more than one feed type. There is a need to identify the dietary situations in which an additive is more or less effective. Therefore, the objective of the present study was to determine the dose-rate effects of 11 additives, representing currently recognised or potential rumen fermentation modifiers on *in vitro* rumen methane output for two feed substrates (perennial ryegrass and grass silage+barley grain) that represent the major dietary ingredients fed to ruminants in Ireland and in other countries with similar livestock production systems. The minimum effective concentration of each additive to reduce methane output when co-incubated with either perennial ryegrass or grass silage+barley grain will also be reported, and compared with similar *in vitro* batch studies in the literature.

Materials and methods

Experimental design

In vitro rumen methanogenesis was assessed within randomised complete block ($n = 3$) design experiments with treatments in a 2 by 5, 2 by 6 or 2 by 7 factorial arrangement, dependent on the number of concentration levels of each additive. Two feed types (i.e. perennial ryegrass, grass silage+barley grain) were incubated separately with a range of concentrations (see below) of each additive. Except for yeast, replication was in triplicate for all additives. The probiotic yeast additive, *Saccharomyces cerevisiae* (SC), was incubated in triplicate on two different occasions ($n = 6$ replicates).

Feed preparation

Three feeds (perennial ryegrass, grass silage and barley grain) were oven-dried at 40°C for 48 h and milled through a 1-mm screen. The dried milled grass silage and barley grain were combined at a 50 : 50 ratio on a gravimetric DM basis. The two substrates for fermentation therefore were ryegrass and grass silage+barley grain (GS+B) and both were stored under dry, cool, dark conditions in sealed containers before use. Although the oven-drying and milling preparation of the grass silage was to facilitate using a physically homogeneous sample in the *in vitro* gas production technique (GPT), some loss of silage volatiles is likely to have occurred (Porter and Murray 2001). However, as the same silage sample was used for all batches of incubations, the loss of some volatiles would therefore affect all additive concentrations similarly. In the present study, we were interested in the relative effects across concentrations of an additive, rather than quantifying the absolute outputs of methane.

Duplicate samples of each feed mix were assayed for ash, crude protein (CP), acid detergent fibre (ADF), neutral detergent fibre (NDF), *in vitro* DM digestibility (DMD) and, in the case of GS+B, for its starch concentration. Concentration of water-soluble carbohydrate was determined for perennial ryegrass by using the anthrone method (Thomas 1977). The DMD procedure used was that described by Tilley and Terry (1963), with the modification that the final residue was isolated by filtration through a 1.6-μm Whatman GF/A filter (Whatman, Maidstone, England), rather than by centrifugation. Ash concentration was determined following complete combustion of samples in a muffle furnace at 550°C for 5 h. CP concentration ($N \times 6.25$) was determined using a LECO FP-528 N analyser (Leco Corporation, St Joseph, MI, USA), on the basis of the method #990-03 of the AOAC (1990). The ADF (expressed exclusive of residual ash) and NDF (assayed with heat-stable amylase and sodium sulfite, and expressed exclusive of residual ash) concentrations were determined using an ANKOM fibre analyser (ANKOM Technology, Fairport, NY, USA) according to Van Soest *et al.* (1991). Starch concentration was determined using the method of McCleary *et al.* (1997) in a segmented flow analyser (Bran+Luebbe, Norderstedt, Germany). The chemical composition of the feeds is summarised in Table 1. At the time of the *in vitro* GPT incubations, subsamples (2 g) of each feed were oven-dried at 98°C for 48 h for residual moisture determination.

Additives

Additives co-incubated with each feed were selected on the basis of their actual or potential ability to inhibit methanogenesis using different modes of action and their commercial availability. The additives were as follows: the fatty acids oleic, linoleic, and linolenic (purity of 99%) at 0, 1.25, 2.5, 5 and 10 mL/L, lauric acid (purity of 99%) at 0, 1.25, 2.5, 5 and 10 g/L; HMA 2-bromoethanesulfonate (BES, Na salt) and bromochloromethane (BCM, in a α-cyclodextrin matrix) at 0, 1, 5, 10, 20 and 40 μM; pyromellitic diimide (PMDI, purity of 97%) at 0, 1, 5, 10 and 20 mg/L; the statins mevastatin (purity of ≥95%) at 0, 0.25, 0.5, 1, 2, and 4 μM and lovastatin (purity of ≥98%) at 0, 0.25, 0.5, 1, 2, 4 and 8 μM; probiotic yeast (SC, Yea-sacc[1026]) at 0, 0.1, 0.2, 0.5, 1 and 2 g/L; and the unsaturated dicarboxylic acid fumaric (purity of 99%) at 0, 1, 5, 10 and 20 mM. Concentrations of each additive was based on the *in vitro*

Table 1.   *In vitro* DM digestibility (g/kg) and chemical composition (g/kg DM) of the feedstuffs used
Values of grass silage+barley grain are given on a 50 : 50 gravimetric DM basis

Component	Perennial ryegrass	Grass silage+ barley grain
Neutral detergent fibre	426	326
Acid detergent fibre	215	194
Crude protein	161	116
Water soluble carbohydrates	236	–
Starch	–	320
Ash	100	51
In vitro DM digestibility	845	799

studies of Hristov *et al*. (2004) (fatty acids), Choi *et al*. (2004) (BES, PMDI), Miller and Wolin (2001) (mevastatin, lovastatin), Lila *et al*. (2004) (SC) and López *et al*. (1999) (fumaric acid), with some modifications to favour the likelihood that methane output would be suppressed within the range of concentrations. The concentrations of BCM added were based on those for BES. Additives were purchased from Sigma-Aldrich (St Louis, MO, USA), with the exception of Yea-sacc[1026] which was supplied by Alltech (Sarney, Dunboyne, Co. Meath, Ireland) and BCM which was supplied by Nigel Tomkins (CSIRO, Townsville, Australia). Stock solutions of HMA and PMDI were prepared in distilled water. Statins were dissolved in 70% ethanol as per Miller and Wolin (2001), with ethanol making up no more than 0.28% v/v of the fermentation medium. The equivalent amount of either ethanol or water was added to the respective blank and control-treatment fermentation bottles. Stock solutions were stored at 4°C. The concentration given for each additive was the final concentration in the fermentation medium.

In vitro *fermentation*

Effects of each supplement on rumen fermentation were tested *in vitro* by using the GPT of Theodorou *et al*. (1994), with some modifications made according to Mauricio *et al*. (1999). These modifications were made because the total volume of gas produced and its methane concentration were of interest, rather than a profile of the kinetics of their production. Each dried milled feed (0.5 g) was weighed into individual 160-mL fermentation bottles and incubated with the range of concentrations of each additive. Replication was provided by the use of three different rumen-fluid inoculum sources, obtained from three fistulated adult steers fed a grass silage and concentrate diet (60 : 40 on a gravimetric DM basis). Therefore, additives at each concentration were incubated separately with the rumen fluid of each steer. Samples of rumen digesta were obtained from the rumen before morning feeding and strained separately through four layers of cheesecloth. Artificial saliva was prepared according to McDougall (1948). Each rumen-fluid source was mixed with the artificial saliva at a ratio of 1 : 4. A sample (0.8 mL) of each rumen fluid–artificial saliva mixture was stored at −18°C in eppendorf tubes containing 20 μL of 9 M H_2SO_4 for subsequent ruminal volatile fatty acid (VFA) analysis. The pH of each mixture was determined before incubation and subsequently dispensed (50 mL) with a peristaltic pump into fermentation bottles under CO_2 flushing. Bottles were sealed with butyl rubber stoppers and aluminium crimp caps and placed in an incubator at 39°C for 24 h. Fermentation bottles were manually shaken at the start of incubation (0 h) and at 2 and 8 h during incubation. It was necessary to perform six batches of incubations to test all additives (one batch assessed per week), with two additives incubated per batch. The yeast additive was incubated in triplicate in two separate batches.

Gas measurements and chemical analyses

After 24-h incubation, gas accumulation in the headspace of each fermentation bottle was measured using a pressure transducer (Gems Sensors and Controls, Basingstoke, UK) following the procedure described by Mauricio *et al*. (1999). The total amount of gas produced in each bottle was estimated using the following equation of Mauricio *et al*. (1999):

$$\text{Gas production (mL)} = (vh/Pa) \times Pt,$$

where vh equals head space volume (mL), Pa equals atmospheric pressure (N/m^2) and Pt equals the pressure transducer reading (N/m^2). A sample of gas (0.8 mL) collected in a graduated syringe was transferred to a 2-mL evacuated tight vial (National Scientific Co., Rochwood, TN, USA) sealed with a silicon–teflon septa (Sun Sri, Rochwood, TN, USA) before the determination of methane. Measurement of the methane concentration in a gas sample accumulated during 24 h of incubation was the same procedure as reported by Bodas *et al*. (2008) and Navarro-Villa *et al*. (2011). Although Theodorou *et al*. (1994) recommended that pressures of no more than 483 hPa should be allowed to accumulate in fermentation bottle head space, López *et al*. (2007) found an r^2 of 0.993 between gas volume and head-space pressure within the range 0–1082 hPa in fermentation bottles. Since head-space pressure in the present experiment ranged from 10 to 1212 hPa (mean = 638 hPa), the pressure inside the fermentation bottles was unlikely to affect fermentation in a manner that would influence the relative methane output of the various treatments being evaluated. In addition, as a result of the extensive flushing of both the rumen fluid–artificial saliva and the fermentation bottle headspace with CO_2 until the bottles were sealed, and taking account of the low solubility of methane in the incubation medium (Windholz *et al*. 1976), it was considered that the gas sample collected from the headspace at the end of the 24-h incubation period was representative of the methane produced during the fermentation.

The gas inside each of the fermentation bottles was released and the bottles were cooled to 4°C to terminate fermentation. The bottles were then opened to measure pH and to take a sample of fermentation medium (0.8 mL) for VFA analysis. Finally, all contents remaining in the bottles were filtered through sintered Pyrex glass crucibles (pore Number 1, 100–160 μm, Corning Inc., Tewksbury, MA, USA) and the residue recovered was oven-dried (98°C for 48 h). Apparent DM disappearance (aDMd) was estimated as the difference in the weight of DM incubated and in the weight of DM in the residue expressed as a proportion of the weight of DM incubated. For the HMA, PMDI and statins, both gas production and aDMd for each feed were corrected for endogenous substrates by the inclusion of blank fermentation bottles containing rumen fluid and artificial saliva only. Preliminary studies in our laboratory showed some additives to be fermentable by the rumen microbiota and/or cause total gas production (TGP) to increase in bottles containing rumen fluid–artificial saliva and additive only; therefore, alternative blanks containing rumen fluid–artificial saliva mixture, plus the corresponding additive, were included for each concentration of lauric acid, oleic acid, linoleic acid, linolenic acid, SC and fumaric acid, to correct TGP and aDMd values for each feed from the endogenous substrates and additive.

Methane concentration in the fermentation gas was determined by gas chromatography (GC) using a Shimadzu

GC2014 with AOC 20i autosampler (Shimadzu Corporation, Kyoto, Japan) equipped with a 2.1 m by 5 mm (outer diameter) by 3.2 mm (inner diameter) glass column packed with molecular sieve 5A, 60/80 mesh and a flame ionisation detector. Temperatures were 120°C in the column, 170°C in the injector and 150°C in the detector. The carrier gas nitrogen (N_2) flow rate was 40 mL/min. Hydrogen was simultaneously determined using a thermal conductivity detector at 170°C and a current of 75 mA. Each gas sample (8 µL) was injected into the column and the methane and hydrogen concentrations were calculated by external calibration, using a certified gas mixture (Scott-Marrin Inc., Riverside, CA, USA). Chromatograms were integrated using software GC Solutions version 2.30.00 SU6 (Shimadzu, Kyoto, Japan).

A sample of the rumen fluid–artificial saliva mixture (from each animal) and of the post-incubation medium were assayed for VFA (acetic, propionic, iso-butyric + n-butyric (butyric), iso-valeric + n-valeric (valeric)) by GC using a Shimadzu GC17A with AOC 20i autosampler (Shimadzu Corporation, Kyoto, Japan) equipped with a Chrompack column [2.4 m by 5 mm by 3.4 mm glass packed with 9% carbowax 20M + 1% H_3PO_4 on c-WHP 80/100 mesh (Phenomenex, Cheshire, UK)] and a flame ionisation detector. Temperatures were 150°C in the column and injector and 180°C in the detector. The carrier gas N_2 flow rate was 290 mL/min. Iso-caproic acid (0.04 M) was used as an internal standard, as described by Ranfft (1973). Chromatograms were integrated using software EZstart version 7.2.1 (Shimadzu, Kyoto, Japan).

Calculations and statistical analyses

Calculations were performed as per Navarro-Villa *et al*. (2011). Briefly, the change in total VFA concentration in the medium (ΔtVFA) during incubation was calculated by subtracting the total VFA concentration of the initial inoculum (i.e. rumen fluid–artificial saliva mixture) from the total VFA concentration in the medium after 24 h of incubation. Apparent net production of VFA per g DM incubated (tVFA/DMi) was calculated by multiplying ΔtVFA by the volume of medium (50 mL) and dividing by the weight of feed incubated. Individual VFAs were expressed as molar proportions relative to total VFA (mmol/mol).

Statistical analysis was carried out using the SPSS Version 16 for MS-Windows (SPSS Inc., Chicago, IL, USA). The effects included in the general linear model for each variable were block (rumen fluid source), feed type, additive concentration, feed type × additive concentration interaction and error. Dunnett's test was used to compare the methane output between the control and additive treatments. Polynomial contrasts were used to test for linear and quadratic effects of concentration of each additive when co-incubated with either ryegrass or GS+B. Effects were considered significant when $P < 0.05$ and trends at $P < 0.1$

Results

Fatty acids

Total gas production (TGP) decreased linearly for ryegrass and GS+B with an increasing concentration of lauric, oleic, linoleic and linolenic acids when expressed in g DM incubated (TGP/DMi) ($P < 0.001$; Tables 2–5). With an increasing concentration

of lauric, linoleic and linolenic acids, there was a linear decrease in methane output per g DM incubated (CH_4/DMi) and disappeared (CH_4/DMd) when incubated with either feed ($P < 0.001$; Tables 2, 4, 5). With an increasing concentration of oleic acid, CH_4/DMi decreased linearly ($P < 0.001$) for both feeds (Table 3). Hydrogen output per g DM incubated (H_2/DMi) was detected with the addition of ≥ 5 g/L lauric acid with ryegrass and ≥ 1.25 g/L with GS+B (Table 2). No hydrogen output was detected with the other fatty acids examined in the present study. Apparent DM disappearance (aDMd) decreased linearly ($P < 0.01$) with an increasing concentration of lauric acid for ryegrass (Table 2). A quadratic response in aDMd was found for both feeds with an increasing concentration of linoleic acid ($P < 0.05$; Table 4), and aDMd decreased linearly with an increasing concentration of linolenic acid for both feeds ($P < 0.001$; Table 5). For ryegrass, the pH of the fermentation medium decreased linearly with increasing concentrations of lauric and oleic acids ($P < 0.01$; Tables 2, 3) and a quadratic response was evident for linoleic ($P < 0.01$; Table 4) and linolenic ($P < 0.05$; Table 5) acids. In the case of GS+B, there was a quadratic response in pH with increasing concentrations of lauric ($P < 0.01$; Table 2) and linoleic ($P < 0.05$; Table 4) acids and there was a linear increase ($P < 0.01$) in pH with increasing concentration of oleic acid (Table 3). Total VFA output per g DM incubated (tVFA/DMi) decreased linearly or tended to decrease linearly with an increasing concentration of lauric ($P < 0.001$) and oleic ($P = 0.07$) acids, respectively, for ryegrass (Tables 2, 3). Increasing the concentration of lauric acid incubated with ryegrass and GS+B linearly increased the proportion of acetic acid ($P < 0.01$), caused a quadratic response in the proportion of propionic acid ($P < 0.01$), and linearly decreased the proportion of butyric and valeric acids ($P < 0.01$; Table 2). With increasing concentrations of oleic, linoleic and linolenic acids, the proportion of acetic and butyric acids decreased linearly for both feeds, with the exception of oleic and linolenic acid when added to ryegrass ($P < 0.05$; Tables 3–5). The proportion of propionic acid increased linearly with increasing concentrations of oleic, linoleic and linolenic acids incubated with both feeds ($P < 0.05$; Tables 3–5). For ryegrass, valeric acid decreased linearly with increasing concentration of linolenic acid ($P < 0.05$; Table 5).

The minimum measured concentration of the fatty acids lauric, linoleic and linolenic required to reduce ($P < 0.05$) methane output in ryegrass and GS+B compared with the control treatment was 1.25 g or ml/L (Tables 2, 4, 5). A measured oleic acid concentration of 2.5 ml/L was required to reduce ($P < 0.05$) methane output in both feeds (Table 3).

Halogenated methane analogues (HMA) and pyromellitic diimide (PMDI)

Total gas production (TGP/DMi) decreased linearly ($P < 0.01$) with an increasing concentration of BES and PMDI for both feeds (Tables 6, 8), but with an increasing concentration of BCM, the response in TGP/DMi was quadratic ($P < 0.01$) for ryegrass (Table 7). Methane output (CH_4/DMi, CH_4/DMd) decreased linearly with increasing concentration of both BES

Table 2. Effect of lauric acid on *in vitro* rumen total gas production, methane output, hydrogen output, apparent DM disappearance (aDMd), pH and volatile fatty acids (VFA) after 24-h incubation

For methane output, minimum measured concentration of lauric acid required to significantly reduce methane output is indicated in bold. Hydrogen output data were not amenable to statistical analysis. tVFA/DMi, the apparent total VFA output per g DM incubated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant ($P > 0.05$). s.e.m., standard error of the mean (concentration)

Parameter	Lauric acid (g/L of medium)					s.e.m.	Significance	
	0	1.25	2.5	5	10		Linear	Quadratic
Perennial ryegrass								
Total gas production (mL/g DM incubated)	203	107	82	74	65	2.9	***	***
Methane output (mL/g DM incubated)	36.1	**15.2*****	2.8	0.5	0.3	1.36	***	***
Methane output (mL/g DM disappeared)	48.9	38.5	**6.3*****	1.1	0.9	3.82	***	***
Hydrogen output (mL/g DM incubated)	0	0	0	0.7	0.8	–	–	–
aDMd (g/g)	0.74	0.40	0.45	0.44	0.40	0.032	**	**
pH	6.61	6.70	6.66	6.64	6.59	0.016	**	*
tVFA/DMi (mmol/g DM)	5.51	2.92	2.16	2.25	2.10	0.336	***	***
Acetic acid (mmol/mol VFA)	637	574	586	629	668	13.3	**	*
Propionic acid (mmol/mol VFA)	227	304	321	303	266	17.0	n.s	**
Butyric acid (mmol/mol VFA)	110	98	76	62	59	8.9	**	*
Valeric acid (mmol/mol VFA)	26	24	17	6	6	3.7	**	n.s
Grass silage+barley grain (50:50)								
Total gas production (mL/g DM incubated)	203	139	115	105	90	1.8	***	***
Methane output (mL/g DM incubated)	45.4	**18.0*****	4.3	1.8	0.6	0.85	***	***
Methane output (mL/g DM disappeared)	60.6	**38.6*****	7.6	2.9	1.1	1.56	***	***
Hydrogen output (mL/g DM incubated)	0	0.3	0.9	1.7	1.9	–	–	–
aDMd (g/g)	0.75	0.47	0.57	0.64	0.55	0.035	n.s	n.s
pH	6.60	6.63	6.52	6.51	6.56	0.020	n.s	**
tVFA/DMi[D] (mmol/g DM)	4.38	4.12	3.68	3.33	2.84	0.640	n.s	n.s
Acetic acid (mmol/mol VFA)	624	530	600	628	627	11.0	**	n.s
Propionic acid (mmol/mol VFA)	222	362	326	308	315	9.8	*	**
Butyric acid (mmol/mol VFA)	125	85	61	56	51	2.8	***	***
Valeric acid (mmol/mol VFA)	29	23	13	8	7	1.3	***	***

Table 3. Effect of oleic acid on *in vitro* rumen total gas production, methane output, pH, and volatile fatty acids (VFA) after 24-h incubation

With the addition of 5 and 10 ml/L oleic acid it was not possible to measure apparent DM disappearance (aDMd) accurately. Therefore, methane output per g DM disappeared and aDMd values were not amenable to analysis of variance. Hydrogen was not detected. For methane output, minimum measured concentration of oleic acid required to significantly reduce methane output is indicated in bold. tVFA/DMi, the apparent total VFA output per g DM incubated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; †, $P < 0.1$; n.s., not significant ($P > 0.05$). s.e.m., standard error of the mean (concentration)

Parameter	Oleic acid (ml/L of medium)					s.e.m.	Significance	
	0	1.25	2.5	5	10		Linear	Quadratic
Perennial ryegrass								
Total gas production (mL/g DM incubated)	200	189	177	169	154	3.4	***	*
Methane output (mL/g DM incubated)	28.4	24.1	**17.7****	17.2	14.0	1.35	***	**
pH	6.60	6.58	6.59	6.57	6.55	0.008	**	n.s.
tVFA/DMi (mmol/g DM)	5.13	6.60	3.86	3.10	3.73	0.750	†	n.s.
Acetic acid (mmol/mol VFA)	599	605	570	561	548	16.2	*	n.s.
Propionic acid (mmol/mol VFA)	254	260	288	310	327	24.7	*	n.s.
Butyric acid (mmol/mol VFA)	112	105	105	96	93	9.9	n.s.	n.s.
Valeric acid (mmol/mol VFA)	34	29	37	33	32	3.6	n.s.	n.s.
Grass silage+barley grain (50:50)								
Total gas production (mL/g DM incubated)	202	201	194	173	161	6.0	***	n.s.
Methane output (mL/g DM incubated)	36.3	30.6	**29.3***	20.4	17.7	2.01	***	*
pH	6.60	6.59	6.55	6.57	6.53	0.012	**	n.s.
tVFA/DMi (mmol/g DM)	6.38	8.56	5.73	5.54	6.70	0.896	n.s.	n.s.
Acetic acid (mmol/mol VFA)	612	621	553	529	528	18.6	**	n.s.
Propionic acid (mmol/mol VFA)	236	220	302	341	339	29.7	*	n.s.
Butyric acid (mmol/mol VFA)	120	115	111	96	91	6.0	**	n.s.
Valeric acid (mmol/mol VFA)	32	44	34	33	42	7.4	n.s.	n.s.

Table 4.   Effect of linoleic acid on *in vitro* rumen total gas production, methane output, apparent dry matter disappearance (aDMd), pH and volatile fatty acids (VFA) after 24-h incubation

Hydrogen was not detected. For methane output, minimum measured concentration of linoleic acid required to significantly reduce methane output is indicated in bold. tVFA/DMi, the apparent total VFA output per g DM incubated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant ($P > 0.05$). s.e.m., standard error of the mean (concentration)

Parameter	Linoleic acid (ml/L of medium)					s.e.m.	Significance	
	0	1.25	2.5	5	10		Linear	Quadratic
Perennial ryegrass								
Total gas production (mL/g DM incubated)	200	183	167	154	121	5.4	***	n.s.
Methane output (mL/g DM incubated)	28.4	**18.5***	12.9	9.8	5.2	2.06	***	**
Methane output (mL/g DM disappeared)	35.3	**22.9***	15.2	10.3	7.1	2.61	***	**
aDMd (g/g)	0.80	0.81	0.85	0.86	0.75	0.023	n.s.	*
pH	6.60	6.56	6.55	6.56	6.62	0.014	n.s.	**
tVFA/DMi (mmol/g DM)	5.13	3.89	3.62	4.07	3.61	0.640	n.s.	n.s.
Acetic acid (mmol/mol VFA)	599	556	527	524	526	20.1	*	n.s.
Propionic acid (mmol/mol VFA)	254	313	360	371	368	33.0	*	n.s.
Butyric acid (mmol/mol VFA)	112	98	84	78	77	10.5	*	n.s.
Valeric acid (mmol/mol VFA)	34	33	29	27	28	4.4	n.s.	n.s.
Grass silage+barley grain (50 : 50)								
Total gas production (mL/g DM incubated)	202	185	171	149	131	3.1	***	**
Methane output (mL/g DM incubated)	36.3	**25.4****	15.5	9.8	6.3	1.84	***	***
Methane output (mL/g DM disappeared)	48.2	**34.1****	20.3	12.3	10.4	2.29	***	***
aDMd (g/g)	0.75	0.74	0.76	0.82	0.61	0.044	n.s.	*
pH	6.60	6.58	6.55	6.55	6.60	0.016	n.s.	*
tVFA/DMi (mmol/g DM)	6.38	5.79	5.59	7.60	5.49	1.033	n.s.	n.s.
Acetic acid (mmol/mol VFA)	612	550	500	507	491	29.0	*	n.s.
Propionic acid (mmol/mol VFA)	236	314	393	388	430	39.4	**	n.s.
Butyric acid (mmol/mol VFA)	120	105	78	72	55	7.6	***	*
Valeric acid (mmol/mol VFA)	32	32	29	33	24	4.2	n.s.	n.s.

Table 5.   Effect of linolenic acid on *in vitro* rumen total gas production, methane output, apparent dry matter disappearance (aDMd), pH and volatile fatty acids (VFA) after 24-h incubation

Hydrogen was not detected. For methane output, minimum measured concentration of linolenic acid required to significantly reduce methane output is indicated in bold. tVFA/DMi, the apparent total VFA output per g DM incubated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant ($P > 0.05$). s.e.m., standard error of the mean (concentration)

Parameter	Linolenic acid (ml/L of medium)					s.e.m.	Significance	
	0	1.25	2.5	5	10		Linear	Quadratic
Perennial ryegrass								
Total gas production (mL/g DM incubated)	200	150	110	98	89	5.4	***	***
Methane output (mL/g DM incubated)	28.4	**8.4****	1.7	0.5	0.1	1.21	***	***
Methane output (mL/g DM disappeared)	35.3	**11.6****	3.6	1.0	0.5	1.51	***	***
aDMd (g/g)	0.80	0.70	0.47	0.42	0.33	0.027	***	***
pH	6.60	6.60	6.65	6.65	6.64	0.014	n.s.	*
tVFA/DMi (mmol/g DM)	5.13	4.04	3.58	3.59	3.55	0.520	n.s.	n.s.
Acetic acid (mmol/mol VFA)	599	526	527	527	511	24.3	n.s.	n.s.
Propionic acid (mmol/mol VFA)	254	383	375	378	407	30.4	*	n.s.
Butyric acid (mmol/mol VFA)	112	67	73	69	61	6.0	**	n.s.
Valeric acid (mmol/mol VFA)	34	25	24	25	21	2.5	*	n.s.
Grass silage+barley grain (50 : 50)								
Total gas production (mL/g DM incubated)	202	162	134	124	120	6.5	***	***
Methane output (mL/g DM incubated)	36.3	**14.5****	4.4	0.2	0.2	1.83	***	***
Methane output (mL/g DM disappeared)	48.2	**21.9****	8.8	0.4	0.6	2.03	***	***
aDMd (g/g)	0.75	0.64	0.51	0.48	0.40	0.031	***	**
pH	6.60	6.59	6.63	6.60	6.57	0.014	n.s.	n.s.
tVFA/DMi (mmol/g DM)	6.38	5.83	7.23	5.68	5.45	0.973	n.s.	n.s.
Acetic acid (mmol/mol VFA)	612	514	498	469	473	22.9	**	*
Propionic acid (mmol/mol VFA)	236	387	399	456	458	30.6	**	**
Butyric acid (mmol/mol VFA)	120	71	70	51	46	7.2	***	**
Valeric acid (mmol/mol VFA)	32	28	33	24	23	3.5	n.s.	n.s.

Table 6.   Effect of bromoethanesulfonate (BES) on *in vitro* rumen total gas production, methane output, hydrogen output, apparent dry matter disappearance (aDMd), pH and volatile fatty acids (VFA) after 24-h incubation

For methane output, minimum measured concentration of BES required to significantly reduce methane output is indicated in bold. Hydrogen output data were not amenable to statistical analysis. tVFA/DMi, the apparent total VFA output per g DM incubated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant ($P > 0.05$). s.e.m., standard error of the mean (concentration)

Parameter	BES (µM)						s.e.m.	Significance	
	0	1	5	10	20	40		Linear	Quadratic
Perennial ryegrass									
Total gas production (mL/g DM incubated)	204	198	193	187	185	181	3.2	**	*
Methane output (mL/g DM incubated)	27.4	29.4	20.9	**18.5***	16.7	14.1	1.90	***	*
Methane output (mL/g DM disappeared)	37.1	38.7	**27.6***	24.0	21.5	18.3	2.36	***	**
Hydrogen output (mL/g DM incubated)	0	0	1.8	2.2	2.4	2.5	–	–	–
aDMd (g/g)	0.73	0.76	0.76	0.77	0.78	0.79	0.010	**	n.s.
pH	6.43	6.42	6.45	6.45	6.44	6.46	0.010	n.s.	n.s.
tVFA/DMi (mmol/g DM)	6.61	6.62	6.48	6.56	6.49	6.22	0.156	n.s.	n.s.
Acetic acid (mmol/mol VFA)	621	569	565	560	551	550	9.4	**	*
Propionic acid (mmol/mol VFA)	252	279	284	292	297	294	7.7	*	*
Butyric acid (mmol/mol VFA)	97	113	111	108	110	114	5.3	n.s.	n.s.
Valeric acid (mmol/mol VFA)	31	39	40	40	42	42	1.8	**	*
Grass silage+barley grain (50 : 50)									
Total gas production (mL/g DM incubated)	207	197	191	189	187	189	1.8	***	***
Methane output (mL/g DM incubated)	39.3	**29.5***	22.8	21.8	19.0	16.8	1.69	***	***
Methane output (mL/g DM disappeared)	53.5	**39.8***	30.4	28.8	25.0	22.4	2.31	***	***
Hydrogen output (mL/g DM incubated)	0	0	4.1	4.2	4.3	5.3	–	–	–
aDMd (g/g)	0.73	0.74	0.75	0.76	0.76	0.75	0.008	n.s.	*
pH	6.43	6.43	6.46	6.43	6.44	6.46	0.010	n.s.	n.s.
tVFA/DMi (mmol/g DM)	6.49	6.50	6.37	6.11	6.35	6.34	0.146	n.s.	n.s.
Acetic acid (mmol/mol VFA)	603	555	541	515	507	503	11.4	***	**
Propionic acid (mmol/mol VFA)	251	281	300	309	317	318	9.6	**	**
Butyric acid (mmol/mol VFA)	113	125	119	134	135	135	5.2	*	n.s.
Valeric acid (mmol/mol VFA)	33	39	39	42	41	43	1.9	*	n.s.

and PMDI incubated with either feed ($P < 0.001$; Tables 6, 8). Hydrogen was detected with the addition of ≥ 5 µM BES and BCM for both feeds (Tables 6, 7), and ≥ 10 mg/L PMDI for ryegrass and ≥ 5 mg/L PMDI for GS+B (Table 8). The aDMd increased linearly ($P < 0.01$) with an increasing concentration of BES for ryegrass and a quadratic response ($P < 0.01$) was evident for GS+B (Table 6). It increased linearly with BCM for GS+B only ($P = 0.05$; Table 7) and a linear and quadratic response was evident with PMDI for ryegrass only ($P < 0.05$; Table 8). There was a linear decrease in pH with an increasing concentration of BCM for ryegrass ($P < 0.05$) and the response was quadratic ($P < 0.05$) for GS+B (Table 7). The tVFA/DMi decreased linearly with an increasing concentration of PMDI for both feeds ($P < 0.01$; Table 8), but this variable was unaffected ($P > 0.05$) by the addition of either BES or BCM (Tables 6, 7). The proportion of acetic acid decreased linearly ($P < 0.01$) and the proportion of propionic acid increased linearly ($P < 0.01$) with an increasing concentration of both HMA and PMDI incubated with ryegrass or GS+B (Tables 6–8). With the exception of BES incubated with ryegrass, the proportion of butyric acid increased linearly with increasing concentrations of HMA and PMDI for both feeds ($P < 0.05$; Tables 6–8). The proportion of valeric acid increased linearly with an increasing concentration of BES incubated with both feeds ($P < 0.05$; Table 6), and there was a quadratic response with increasing BCM concentration for

ryegrass only ($P < 0.05$; Table 7). In contrast, increasing concentration of PMDI incubated with GS+B linearly decreased the proportion of valeric acid ($P < 0.05$; Table 8).

Compared with the control treatment, a measured BES concentration of 5–10 µM and 1 µM reduced ($P < 0.05$) methane output in ryegrass and GS+B, respectively (Table 6). Methane output could not be detected with a BCM concentration ≥ 5 µM for either feed (Table 7). A similar measured concentration of PMDI (5 mg/L) was required to reduce ($P < 0.01$) methane output in ryegrass and GS+B (Table 8).

Statins

Neither mevastatin (0.25–4 uM) nor lovastatin (0.25–8 uM) reduced methane output from either ryegrass or GS+B. ($P > 0.05$; Tables 9, 10). The proportion of valeric acid increased linearly with increasing concentration of mevastatin when incubated with GS+B ($P < 0.05$; Table 9). No other variables in Tables 9 and 10 were significant ($P > 0.05$) with the addition of mevastatin or lovastatin, respectively, for either feed type.

Saccharomyces cerevisiae (SC)

Total gas production (TGP/DMi) and CH_4/DMd decreased linearly ($P < 0.05$) with an increasing concentration of SC incubated with ryegrass, and CH_4/DMi showed a tendency

Table 7.   Effect of bromochloromethane (BCM) on *in vitro* rumen total gas production, methane output, hydrogen output, apparent dry matter disappearance (aDMd), pH and volatile fatty acids (VFA) after 24 h incubation

Methane and hydrogen output data were not amenable to statistical analysis. tVFA/DMi, the apparent total VFA output per g DM incubated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; †, $P = 0.05$; n.s., not significant ($P > 0.05$). s.e.m., standard error of the mean (concentration)

Parameter	BCM (µM)						s.e.m.	Significance	
	0	1	5	10	20	40		Linear	Quadratic
Perennial ryegrass									
Total gas production (mL/g DM incubated)	200	199	172	173	176	177	4.8	*	**
Methane output (mL/g DM incubated)	29.9	29.6	0	0	0	0	–	–	–
Methane output (mL/g DM disappeared)	40.5	38.8	0	0	0	0	–	–	–
Hydrogen output (mL/g DM incubated)	0	0	21.0	26.2	27.2	28.1	–	–	–
aDMd (g/g)	0.74	0.76	0.79	0.78	0.77	0.78	0.014	n.s.	n.s.
pH	6.53	6.51	6.48	6.48	6.47	6.48	0.013	*	*
tVFA/DMi (mmol/g DM)	6.55	6.50	6.03	6.23	6.02	6.14	0.295	n.s.	n.s.
Acetic acid (mmol/mol VFA)	625	581	543	532	527	524	17.8	**	*
Propionic acid (mmol/mol VFA)	235	256	275	287	294	295	10.4	**	*
Butyric acid (mmol/mol VFA)	105	125	136	137	137	138	7.4	*	n.s.
Valeric acid (mmol/mol VFA)	34	38	45	43	42	43	1.8	*	*
Grass silage+barley grain (50 : 50)									
Total gas production (mL/g DM incubated)	202	209	186	193	192	195	5.2	n.s.	n.s.
Methane output (mL/g DM incubated)	33.9	36.8	0	0	0	0	–	–	–
Methane output (mL/g DM disappeared)	50.5	50.4	0	0	0	0	–	–	–
Hydrogen output (mL/g DM incubated)	0	0	31.1	35.8	36.8	39.0	–	–	–
aDMd (g/g)	0.67	0.73	0.72	0.72	0.75	0.76	0.002	†	n.s.
pH	6.55	6.51	6.47	6.44	6.44	6.45	0.016	*	*
tVFA/DMi (mmol/g DM)	6.55	6.99	6.62	6.27	6.34	6.29	0.301	n.s.	n.s.
Acetic acid (mmol/mol VFA)	603	607	540	490	476	481	18.9	***	**
Propionic acid (mmol/mol VFA)	228	225	279	308	323	316	12.2	***	***
Butyric acid (mmol/mol VFA)	129	130	141	161	159	164	8.9	**	n.s.
Valeric acid (mmol/mol VFA)	40	38	39	41	42	39	2.7	n.s.	n.s.

($P = 0.06$) to decrease (Table 11). Total VFA output (tVFA/DMi) increased linearly with an increasing concentration of SC for GS+B ($P < 0.05$; Table 11). The proportion of valeric acid increased linearly with an increasing concentration of SC for both feeds ($P < 0.05$; Table 11). Compared with the control treatment, the addition of SC reduced methane output only in ryegrass and not in GS+B. The minimum measured concentration of SC required to reduce ($P < 0.01$) methane output in ryegrass was 0.1 g/L (Table 11).

Fumaric acid

When ryegrass was incubated with an increasing concentration of fumaric acid, TGP/DMi and methane output (CH_4/DMi, CH_4/DMd) decreased linearly ($P < 0.05$; Table 12). For both feeds, an increasing fumaric acid concentration caused a linear decrease ($P < 0.05$) in aDMd and pH and a linear increase ($P < 0.05$) in tVFA/DMi (Table 12). With an increasing concentration of fumaric acid, there was a linear decrease in the proportions of acetic, butyric and valeric acids and a linear increase in the proportion of propionic acid for both feed types ($P < 0.001$; Table 12). The minimum measured concentration of fumaric acid required to reduce ($P < 0.05$) methane output in ryegrass ranged from 5 to 10 mM. Fumaric acid did not reduce ($P > 0.05$) methane output in GS+B compared with the control treatment (Table 12).

For the relevant additives, the standard error of the mean and significance of two-way interactions are shown in Table 13 for variables that had significant feed by additive concentration interactions.

Discussion

Perspective

The effects of 11 additives on *in vitro* rumen methane output, VFA profiles and other fermentation characteristics were assessed with perennial ryegrass and GS+B which differed in fibre and CP concentration, but had a high *in vitro* DMD (Table 1). The individual additives representing actual or potential rumen fermentation modifiers were chosen on the basis of their reported ability to suppress rumen methanogenesis, as well as their commercial availability. The efficacy of medium-chain fatty acids to suppress methane output *in vivo* is known to be feed-type dependent (Machmüller *et al.* 2003), and therefore, the present study can assess the sensitivity of the *in vitro* GPT to register differences between the two contrasting feeds supplemented with different additives. The yeast, *S. cerevisiae* (SC), was the organism chosen to represent probiotics because of its use as a feed additive on commercial farms. The use of statins could be an alternative way to reduce methane production, but they have not, to our knowledge, been screened with contrasting feeds. To strengthen the representativeness of the comparison of ryegrass and GS+B with different additives, a range of concentrations of each were used. The minimum measured effective concentration of each additive to reduce methane

Table 8. Effect of pyromellitic diimide (PMDI) on *in vitro* rumen total gas production, methane output, hydrogen output, apparent dry matter disappearance (aDMd), pH and volatile fatty acids (VFA) after 24-h incubation

For methane output, minimum measured concentration of PMDI required to significantly reduce methane output is indicated in bold. Hydrogen output data were not amenable to statistical analysis. tVFA/DMi, the apparent total VFA output per g DM incubated. **, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant ($P > 0.05$). s.e.m., standard error of the mean (concentration)

Parameter	PMDI (mg/L of medium)					s.e.m.	Signficance	
	0	1	5	10	20		Linear	Quadratic
Perennial ryegrass								
Total gas production (mL/g DM incubated)	203	200	181	167	168	6.1	**	*
Methane output (mL/g DM incubated)	32.4	30.0	**18.5***	8.8	0.1	1.63	***	**
Methane output (mL/g DM disappeared)	42.5	39.1	**24.8**	12.4	0.2	2.31	***	**
Hydrogen output (mL/g DM incubated)	0	0	0	12.4	23.5	–	–	–
aDMd (g/g)	0.77	0.76	0.74	0.71	0.73	0.010	*	*
pH	6.42	6.42	6.44	6.46	6.42	0.026	n.s.	n.s.
tVFA/DMi (mmol/g DM)	6.55	6.17	6.16	5.43	5.35	0.232	**	n.s.
Acetic acid (mmol/mol VFA)	614	558	549	519	503	16.6	**	n.s.
Propionic acid (mmol/mol VFA)	260	304	310	329	343	11.3	**	n.s.
Butyric acid (mmol/mol VFA)	95	108	108	121	127	7.5	*	n.s.
Valeric acid (mmol/mol VFA)	31	29	33	32	27	1.7	n.s.	n.s.
Grass silage+barley grain (50 : 50)								
Total gas production (mL/g DM incubated)	206	205	185	185	187	3.7	**	**
Methane output (mL/g DM incubated)	38.6	35.4	**20.2***	8.7	0.1	2.24	***	**
Methane output (mL/g DM disappeared)	52.5	47.6	**27.8***	11.7	0.2	2.81	***	**
Hydrogen output (mL/g DM incubated)	0	0	2.4	19.5	35.4	–	–	–
aDMd (g/g)	0.73	0.74	0.73	0.75	0.72	0.012	n.s.	n.s.
pH	6.42	6.41	6.44	6.45	6.40	0.030	n.s.	n.s.
tVFA/DMi (mmol/g DM)	6.60	6.34	6.28	6.22	5.42	0.242	**	n.s.
Acetic acid (mmol/mol VFA)	599	582	499	517	457	19.3	***	n.s.
Propionic acid (mmol/mol VFA)	249	267	329	318	358	12.5	***	n.s.
Butyric acid (mmol/mol VFA)	117	115	141	135	156	11.1	*	n.s.
Valeric acid (mmol/mol VFA)	34	35	31	30	29	1.8	*	n.s.

output in the present study was compared with that in similar *in vitro* 24-h batch-digestion studies in the literature.

Differences in methane output between feeds

Under the assay conditions of the *in vitro* GPT employed in the present study, a numerically greater methane output was found for GS+B than ryegrass in the control fermentation bottles, which agrees with the results of other similar *in vitro* studies (Klevenhusen *et al*. 2008; Mc Geough *et al*. 2011; Navarro-Villa *et al*. 2011) but not with some *in vivo* studies (Beauchemin and McGinn 2005; Mc Geough *et al*. 2010). These contradictory results have been discussed in greater detail by Mc Geough *et al*. (2011).

Fatty acids

Fatty acids inhibit methane production through the provision of an alternate means of electron disposal, as well as by direct toxic effects on ruminal microoganisms and protozoa (McAllister *et al*. 1996; Zhang *et al*. 2008). With 9–25% of methane production in the rumen attributed to protozoa-associated methanogens (Newbold *et al*. 1995), the elimination of protozoa will simultaneously reduce the associated methanogens (Jouany 1994). Propionate-producing gram-negative bacteria are not significantly inhibited by fatty acids (Van Nevel and Demeyer 1988); thus, the reduction in methane

production shifts fermentation towards the production of propionic acid (McAllister *et al*. 1996).

Agreeing with the findings of an *in vivo* study of Machmüller *et al*. (2003) for myristic acid, in the present study, the highest methane-suppressing effect of lauric acid (on a DM incubated basis) was achieved when it was added to GS+B, a feed with a relatively lower content of structural carbohydrate than in ryegrass. This result confirmed the sensitivity of the *in vitro* GPT to assess an inhibition effect on rumen methane output across two contrasting feeds. However, using the rumen simulation technique (Rusitec), Machmüller *et al*. (2001) showed that methane output with lauric acid supplementation was independent of diet type (high vs low structural-carbohydrate diet), but feed-type dependent when supplemented with coconut oil, which is known for its high content of lauric acid (Beare-Rogers *et al*. 2001).

Methane output declined with the addition of lauric acid, with a corresponding numerical increase in hydrogen output. Depressing the hydrogen-consuming process of methanogenesis by medium chain fatty acids can enhance the *in vitro* release of gaseous hydrogen (Machmüller *et al*. 2001). The reduced TGP and methane output with increasing lauric acid supplementation, as well as alterations to the VFA profile, would suggest that lauric acid restricted the activity of the bacterial and/or the protozoal populations. Therefore, the observed decrease in methane output can be partially explained by the lower extent of *in vitro* fermentation.

Table 9. Effect of mevastatin on *in vitro* rumen total gas production, methane output, apparent dry matter disappearance (aDMd), pH and volatile fatty acids (VFA) after 24-h incubation

Hydrogen was not detected. Methane output was not significantly reduced by mevastatin (0.25–4 µM) vs the control treatment. tVFA/DMi, the apparent total VFA output per g DM incubated. *, $P < 0.05$; n.s., not significant ($P > 0.05$). s.e.m., standard error of the mean (concentration)

Parameter	Mevastatin (µM)						s.e.m.	Significance	
	0	0.25	0.5	1	2	4		Linear	Quadratic
Perennial ryegrass									
Total gas production (mL/g DM incubated)	196	201	203	194	197	201	4.3	n.s.	n.s.
Methane output (mL/g DM incubated)	31.3	28.9	29.9	29.4	27.4	28.6	1.27	n.s.	n.s.
Methane output (mL/g DM disappeared)	41.0	37.6	39.0	38.7	35.7	37.2	1.60	n.s.	n.s.
aDMd (g/g)	0.77	0.77	0.77	0.76	0.77	0.77	0.010	n.s.	n.s.
pH	6.63	6.65	6.62	6.64	6.61	6.63	0.020	n.s.	n.s.
tVFA/DMi (mmol/g DM)	6.52	6.23	6.37	6.41	6.33	6.32	0.280	n.s.	n.s.
Acetic acid (mmol/mol VFA)	628	626	629	628	623	623	7.2	n.s.	n.s.
Propionic acid (mmol/mol VFA)	243	243	243	242	244	244	7.8	n.s.	n.s.
Butyric acid (mmol/mol VFA)	102	97	99	99	99	100	3.1	n.s.	n.s.
Valeric acid (mmol/mol VFA)	27	35	30	31	34	33	2.5	n.s.	n.s.
Grass silage+barley grain (50 : 50)									
Total gas production (mL/g DM incubated)	202	205	205	202	202	204	2.1	n.s.	n.s.
Methane output (mL/g DM incubated)	34.0	35.4	34.9	34.1	33.3	34.8	1.13	n.s.	n.s.
Methane output (mL/g DM disappeared)	47.0	50.7	47.8	48.5	46.1	48.6	1.44	n.s.	n.s.
aDMd (g/g)	0.72	0.70	0.73	0.70	0.72	0.72	0.013	n.s.	n.s.
pH	6.64	6.62	6.63	6.62	6.64	6.68	0.019	n.s.	n.s.
tVFA/DMi (mmol/g DM)	6.26	6.25	6.04	6.40	6.20	6.32	0.230	n.s.	n.s.
Acetic acid (mmol/mol VFA)	625	633	626	624	618	624	6.8	n.s.	n.s.
Propionic acid (mmol/mol VFA)	222	219	223	218	224	221	10.9	n.s.	n.s.
Butyric acid (mmol/mol VFA)	122	119	122	119	120	118	6.2	n.s.	n.s.
Valeric acid (mmol/mol VFA)	31	29	29	38	38	37	2.8	*	n.s.

Table 10. Effect of lovastatin on *in vitro* rumen total gas production, methane output, apparent dry matter disappearance (aDMd), pH and volatile fatty acids (VFA) after 24-h incubation

Hydrogen was not detected. Methane output was not significantly reduced by lovastatin (0.25–8 µM) vs the control treatment. tVFA/DMi, the apparent total VFA output per g DM incubated. n.s., not significant ($P > 0.05$). s.e.m., standard error of the mean (concentration)

Parameter	Lovastatin (µM)							s.e.m.	Signficance	
	0	0.25	0.5	1	2	4	8		Linear	Quadratic
Perennial ryegrass										
Total gas production (mL/g DM incubated)	196	200	198	198	198	198	197	2.8	n.s.	n.s.
Methane output (mL/g DM incubated)	31.3	28.2	28.9	27.8	28.3	30.5	28.0	1.14	n.s.	n.s.
Methane output (mL/g DM disappeared)	41.0	36.5	37.7	36.0	36.6	39.4	35.7	1.54	n.s.	n.s.
aDMd (g/g)	0.77	0.77	0.77	0.77	0.77	0.77	0.78	0.008	n.s.	n.s.
pH	6.63	6.63	6.63	6.63	6.64	6.63	6.62	0.018	n.s.	n.s.
tVFA/DMi (mmol/g DM)	6.52	6.22	6.42	6.38	6.17	6.29	6.24	0.225	n.s.	n.s.
Acetic acid (mmol/mol VFA)	628	621	622	616	622	627	620	4.9	n.s.	n.s.
Propionic acid (mmol/mol VFA)	243	249	247	249	241	246	250	6.0	n.s.	n.s.
Butyric acid (mmol/mol VFA)	102	98	99	103	100	97	100	3.0	n.s.	n.s.
Valeric acid (mmol/mol VFA)	27	32	32	31	36	30	30	1.6	n.s.	n.s.
Grass silage+barley grain (50 : 50)										
Total gas production (mL/g DM incubated)	202	203	201	199	203	201	201	2.7	n.s.	n.s.
Methane output (mL/g DM incubated)	34.0	34.8	31.2	34.3	34.2	32.9	33.7	1.25	n.s.	n.s.
Methane output (mL/g DM disappeared)	47.0	47.8	42.4	47.4	46.6	46.5	47.6	1.78	n.s.	n.s.
aDMd (g/g)	0.72	0.73	0.74	0.72	0.73	0.71	0.71	0.011	n.s.	n.s.
pH	6.64	6.63	6.65	6.64	6.64	6.64	6.64	0.013	n.s.	n.s.
tVFA/DMi (mmol/g DM)	6.26	6.23	6.08	6.19	6.27	5.93	6.11	0.196	n.s.	n.s.
Acetic acid (mmol/mol VFA)	625	630	617	622	621	616	618	6.7	n.s.	n.s.
Propionic acid (mmol/mol VFA)	222	218	230	225	227	229	229	10.0	n.s.	n.s.
Butyric acid (mmol/mol VFA)	122	116	123	124	119	123	124	6.9	n.s.	n.s.
Valeric acid (mmol/mol VFA)	31	36	30	28	32	32	29	2.9	n.s.	n.s.

Table 11. Effect of *Saccharomyces cerevisiae* (SC) on *in vitro* rumen total gas production, methane output, apparent dry matter disappearance (aDMd), pH and volatile fatty acids (VFA) after 24-h incubation

Hydrogen was not detected. For perennial grass methane output, minimum measured concentration of SC required to significantly reduce methane output is indicated in bold. Methane output for grass silage+barley grain was not significantly reduced by SC (0.1–2 g/L) vs the control treatment. tVFA/DMi, the apparent total VFA output per g DM incubated. *, $P < 0.05$; ***, $P < 0.001$; †, $P < 0.1$; n.s., not significant ($P > 0.05$). s.e.m., standard error of the mean (concentration)

Parameter	SC (g/L of medium)						s.e.m.	Significance	
	0	0.1	0.2	0.5	1	2		Linear	Quadratic
Perennial ryegrass									
Total gas production (mL/g DM incubated)	199	194	192	194	193	184	2.3	***	n.s.
Methane output (mL/g DM incubated)	31.6	**25.7****	26.7	26.8	26.9	25.8	1.17	†	n.s.
Methane output (mL/g DM disappeared)	41.1	**32.5*****	35.2	35.4	35.0	33.2	1.32	*	n.s.
aDMd (g/g)	0.77	0.79	0.76	0.76	0.77	0.78	0.020	n.s.	n.s.
pH	6.56	6.56	6.56	6.57	6.56	6.55	0.023	n.s.	n.s.
tVFA/DMi (mmol/g DM)	6.38	6.54	6.50	6.47	6.76	6.82	0.197	n.s.	n.s.
Acetic acid (mmol/mol VFA)	612	615	619	620	620	624	6.7	n.s.	n.s.
Propionic acid (mmol/mol VFA)	248	243	238	236	236	229	7.4	n.s.	n.s.
Butyric acid (mmol/mol VFA)	109	109	108	108	106	105	1.9	n.s.	n.s.
Valeric acid (mmol/mol VFA)	31	33	34	35	38	42	1.9	***	n.s.
Grass silage+barley grain (50 : 50)									
Total gas production (mL/g DM incubated)	200	201	201	202	201	196	2.1	n.s.	n.s.
Methane output (mL/g DM incubated)	36.8	36.2	36.6	35.5	34.9	33.4	1.72	n.s.	n.s.
Methane output (mL/g DM disappeared)	52.5	48.3	48.7	48.8	48.6	46.1	1.90	n.s.	n.s.
aDMd (g/g)	0.70	0.75	0.75	0.73	0.73	0.72	0.018	n.s.	n.s.
pH	6.57	6.56	6.55	6.56	6.56	6.55	0.023	n.s.	n.s.
tVFA/DMi (mmol/g DM)	6.33	6.48	6.59	6.73	6.92	6.94	0.223	*	n.s.
Acetic acid (mmol/mol VFA)	604	609	610	604	604	603	4.2	n.s.	n.s.
Propionic acid (mmol/mol VFA)	231	226	225	229	227	228	5.3	n.s.	n.s.
Butyric acid (mmol/mol VFA)	131	131	131	130	131	130	2.1	n.s.	n.s.
Valeric acid (mmol/mol VFA)	35	34	34	37	38	40	1.9	*	n.s.

The extent of the reduction in methane output (CH4/DMi) and in the proportion of butyric acid with increasing concentration of lauric acid was more pronounced for GS+B than for ryegrass, with the opposite effect observed for TGP/DMi. A lower proportion of butyric acid could indicate a decreased number and activity of protozoa (Van Nevel and Demeyer 1988). The greater decline in TGP/DMi for ryegrass than for GS+B may indicate a greater inhibition of cellulolytic bacteria and/or protozoa, evidenced by the numerically lower tVFA/DMi and the more pronounced decrease in aDMd for ryegrass with increasing lauric acid concentration. Lauric acid is known to have a strong inhibitory effect on protozoa (Matsumoto *et al*. 1991; Hristov *et al*. 2004) and cellulolytic bacteria (Demeyer 1981; Klevenhusen *et al*. 2011a), and therefore would be expected to particularly affect the fermentation of more fibrous feeds, as found in the present study. The difference in methane output between the two feeds may reflect the greater decline in pH that occurred for GS+B than that for ryegrass. Although the difference in pH between feeds was not large (pH 0.01 – 0.14) across lauric acid concentrations, the combination of a lower pH and the effect of lauric acid for GS+B may have had a greater effect on methanogens. A decline in rumen pH is known to reduce fibre digestibility (Hoover 1986), alter VFA fermentation profiles towards a lower acetic to propionic (A : P) ratio (Lana *et al*. 1998) and reduce the activity of rumen methanogens (Van Kessel and Russell 1996; Lana *et al*. 1998). In the present study, the A : P

ratio was numerically lower (data not presented) for GS+B than for ryegrass at each incremental increase in lauric acid. Acetate (and butyrate) promotes methane production while propionate formation can be considered as a competitive pathway for hydrogen use in the rumen (Moss *et al*. 2000). An alternative explanation for the more pronounced reduction in methane output and in the proportion of butyric acid with increasing concentration of lauric acid for GS+B could be due to the reduced action of lauric acid because of competitive adsorption between the more fibrous ryegrass and rumen microbes (Harfoot *et al*. 1974; Machmüller *et al*. 2001, 2003). However, in the present study it is not known whether competition between rumen microbes and feed particles could occur between feeds differing in NDF concentration by ≤100 g/kg DM and what affect milling through a 1-mm screen has on the feed-particle structure of both feeds.

A decline in methane output occurred with the addition of linoleic and linolenic acids for both feeds. CH4/DMi also declined with the addition of oleic acid when incubated with both feeds. These outcomes are most likely to reflect the increased proportions of propionic acid and reduced proportions of acetic acid due to there being less competition for metabolic hydrogen for methanogenesis, which is in agreement with Zhang *et al*. (2008). Since the amount of metabolisable hydrogen used in the biohydrogenation process of endogenous unsaturated fatty acids is small compared with other processes (Czerkawski 1972), the main reason for the decline in methane output for both feeds, as

Table 12.   Effect of fumaric acid on *in vitro* rumen total gas production, methane output, apparent dry matter disappearance (aDMd), pH and volatile fatty acids (VFA) after 24-h incubation

Hydrogen was not detected. For perennial grass methane output, minimum measured concentration of fumaric acid required to significantly reduce methane output is indicated in bold. Methane output for grass silage+barley grain was not significantly reduced by fumaric acid (1–20 mM) vs the control treatment. tVFA/DMi, apparent total VFA output per g DM incubated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant ($P > 0.05$). s.e.m., standard error of the mean (concentration)

Parameter	Fumaric acid (mM)					s.e.m.	Significance	
	0	1	5	10	20		Linear	Quadratic
Perennial ryegrass								
Total gas production (mL/g DM incubated)	198	196	197	196	188	2.1	**	n.s.
Methane output (mL/g DM incubated)	33.2	28.4	26.8	**25.0***	24.4	1.85	*	n.s.
Methane output (mL/g DM disappeared)	41.8	35.6	**33.3****	31.5	31.9	2.43	*	n.s.
aDMd (g/g)	0.79	0.80	0.80	0.80	0.76	0.009	*	n.s.
pH	6.60	6.59	6.57	6.54	6.42	0.012	***	n.s.
tVFA/DMi (mmol/g DM)	6.20	6.25	6.70	7.78	7.46	0.341	**	n.s.
Acetic acid (mmol/mol VFA)	599	601	582	559	570	3.8	***	***
Propionic acid (mmol/mol VFA)	261	262	285	330	323	4.2	***	***
Butyric acid (mmol/mol VFA)	112	110	107	86	85	1.8	***	**
Valeric acid (mmol/mol VFA)	28	27	27	24	23	0.3	***	n.s.
Grass silage+barley grain (50 : 50)								
Total gas production (mL/g DM incubated)	198	200	199	192	192	3.5	n.s.	n.s.
Methane output (mL/g DM incubated)	41.2	37.4	35.2	32.2	32.9	2.76	n.s.	n.s.
Methane output (mL/g DM disappeared)	55.5	49.1	47.3	43.1	47.3	4.10	n.s.	n.s.
aDMd (g/g)	0.72	0.76	0.75	0.75	0.70	0.011	*	n.s.
pH	6.60	6.59	6.57	6.53	6.43	0.011	***	n.s.
tVFA/DMi (mmol/g DM)	6.10	6.15	6.50	6.66	7.22	0.335	*	n.s.
Acetic acid (mmol/mol VFA)	605	600	581	559	559	2.8	***	***
Propionic acid (mmol/mol VFA)	234	240	266	300	313	5.0	***	**
Butyric acid (mmol/mol VFA)	132	130	125	114	103	3.6	***	n.s.
Valeric acid (mmol/mol VFA)	30	30	28	27	25	0.5	***	n.s.

Table 13.   Standard error of the mean (s.e.m.) and significance of two-way interactions between feed type (ryegrass vs grass silage+barley grain) and additive concentration for total gas production, methane output, apparent dry matter disappearance (aDMd), pH, volatile fatty acids (VFA) and acetic to propionic (A : P) ratio

For the mean values of each variable for each additive see Tables 2–8 and Tables 11–12. BES, bromoethanesulfonate. BCM, bromochloromethane. PMDI, pyromellitic diimide. SC, *Saccharomyces cerevisiae*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; †, $P = 0.05$; n.s., not significant ($P > 0.05$). s.e.m., standard error of the mean (concentration)

Parameter	Additive								
	Lauric acid	Oleic acid	Linoleic acid	Linolenic acid	BES	BCM	PMDI	SC	Fumaric acid
Total gas production (mL/g DM incubated)	2.098***					2.773*			
Methane output (mL/g DM incubated)	1.097**	0.946**	0.877**	1.279*	1.723*				
Methane output (mL/g DM disappeared)			1.164**	1.347***	2.139*				
aDMd (g/g)	0.029*						0.009*		
pH	0.008***								
Butyric acid (mmol/mol VFA)	4.945*		4.960†	4.526*					
Valeric acid (mmol/mol VFA)								0.863*	
A : P ratio									0.042†

suggested by Zhang *et al.* (2008), is likely to have been due to the direct action of unsaturated fatty acids against methanogens and protozoa associated with methanogens. According to earlier research in this area, the toxicity of unsaturated fatty acids towards microorganisms can be explained on a physiochemical basis, namely that fatty acids form adsorption layers around the bacterial cell, which results in altered cell permeability (Kodicek and Worden 1945) and therefore interferes with nutrient uptake (Galbraith and Miller 1973).

The inhibitory effect of long-chain fatty acids are not just confined to methanogens, but also to gram negative cellulolytic bacteria (Zhang *et al.* 2008). Toxicity of fatty acids against cellulolytic bacteria (Zhang *et al.* 2008) appears to have been greater for linolenic than linoleic acid because TGP/DMi and aDMd were more depressed with the former. With the absence of aDMd data for oleic acid, it is probable that aDMds of both feeds were also adversely affected, as evidenced by the lower proportion of acetic acid and the reduction in TGP

in the present study and the known inhibitory effect on cellulolytic bacteria (Nagaraja *et al*. 1997). In addition, Hristov *et al*. (2004) reported a severe decrease in protozoal numbers with the addition of oleic, linoleic and linolenic acids, particularly by the two latter fatty acids, which would have contributed to some of the declines in methane output observed.

The extent of reduction in methane output with increasing concentration of oleic, linoleic and linolenic acids was more pronounced for GS+B than for ryegrass, reflecting the greater numerical reduction in A:P ratio (data not presented) with GS+B than with ryegrass when fatty acid concentrations were ≥2.5 ml/L. The proportion of butyric acid was reduced to a greater extent for GS+B than for ryegrass with increasing concentration of linoleic and linolenic acids, which may indicate a decreased number and activity of protozoa (Van Nevel and Demeyer 1988).

Comparative *in vitro* batch-digestion studies that co-incubated the fatty acids used in the present study with ryegrass or GS+B could not be found in the literature. However, Zhang *et al*. (2008) reported that the minimum concentration of oleic, linoleic and linolenic acids needed to reduce *in vitro* methane output when co-incubated with Chinese wild rye meal and cornmeal (50:50) was 35 g/kg, which was ~4–8-fold lower than the concentration required to significantly reduce methane output in the present study with either ryegrass or GS+B. Zhang *et al*. (2008) did not report the final volume of the fermentation medium, the ratio of rumen fluid to buffer or the quantity of feed incubated, making it difficult to conclude whether the discrepancy between the studies was due to the different methodologies used or to inherent differences among feeds.

Halogenated methane analogues (HMA) and pyromellitic diimide (PMDI)

The addition of HMA and PMDI to both feeds caused a reduction in methane output and in the proportion of acetic acid, and an increase in the proportions of propionic and butyric acids and hydrogen output, agreeing with observations reported for BES and BCM by Van Nevel and Demeyer (1995) and Goel *et al*. (2009), respectively. HMAs caused a decrease in methane output through changes in the direction of fermentation rather than in the extent of fermentation, whereas PMDI appeared to decrease methane output through a combined effect on the extent and direction of fermentation.

BES is a structural bromine analogue of coenzyme M and interferes with methanogenesis by inhibiting the methyl coenzyme-M reductase in methanogens (Balch and Wolfe 1979) and BCM inhibits methane production by reacting with chemically reduced Vitamin B_{12}, which then inhibits the cobamide-dependent methyl transferase step of methanogenesis (Chalupa 1977; Wood *et al*. 1968). A gradual decrease in methane output was observed with an increasing concentration of BES (1–40 µM), whereas methane output was not detectable at a BCM concentration of ≥5 µM. If there is a connection between this observation and the known persistency of BCM as an inhibitor of methane emissions, as shown *in vivo* (McCrabb *et al*. 1997), as compared with a lack of persistency observed for BES (Van Nevel and Demeyer 1995), the development of future inhibitors of enteric methanogenesis would warrant

closer attention to the mode of action of BCM. The extent of reduction in methane output with increasing concentration of BES was more pronounced for GS+B than for ryegrass. This is in agreement with Lee *et al*. (2009), who found a similar interaction for *in vitro* rumen methane output between feed type (timothy hay vs timothy hay+concentrate, 40:60) and the level of BES supplementation (0, 1 and 5 mM). In the present study, the greater reduction in methane output for GS+B with the addition of BES was not due to differences in the extent of fermentation as measured by aDMd or tVFA/DMi, but to the direction of fermentation with a numerically lower A:P ratio (data not presented) for GS+B than for ryegrass.

PMDI is a potent inhibitor of rumen methanogenesis (Martin and Macy 1985). Although not a HMA, in other reports (Van Nevel and Demeyer 1995; Choi *et al*. 2004) PMDI has been grouped with HMA, primarily because it is thought to share a similar action as BES to inhibit methanogenesis (Martin and Macy 1985). PMDI adversely affected the *in vitro* fermentation of both feeds to a greater extent than did BES or BCM, as evidenced by the lower aDMd for ryegrass and a lower tVFA/DMi for both feeds with PMDI. It is not known why PMDI affected the aDMd of ryegrass and not GS+B. The reduction in aDMd of ryegrass with the addition of PMDI was somewhat surprising and not observed in previous *in vivo* studies (Martin and Macy 1985; Eijssen *et al*. 1990).

Goel *et al*. (2009) reported the reduction of *in vitro* methane output using 5 µM BCM with hay as substrate, compared with between 1 and 5 µM BCM required for ryegrass and GS+B in the present study. In the case of BES, 1 µM significantly reduced methane output in timothy hay and timothy hay+concentrate (40:60) diets (Lee *et al*. 2009), which was similar for GS+B in the present study, but not for ryegrass, which required 5–10 µM BES to significantly reduce methane output. There were no comparative studies in the literature for PMDI.

There has been some concern that HMAs are toxic to livestock. For example, chloral hydrate led to liver damage and death in sheep after prolonged feeding (Nagaraja *et al*. 1997) but there has been no adverse effects on rumen fermentation when BCM was fed to either steers (McCrabb *et al*. 1997) or goats (Abecia *et al.* 2012). Therefore, the toxic effects of all HMA need to be assessed *in vivo* on a compound by compound basis.

Statins

Statins are known to inhibit hydroxmethylglutaryl~SCoA (HMG-CoA) reductase, which prevents the creation of an important precursor, mevalonate, used in the formation of membrane lipids in the methanogenic *Archaea* cell wall (Miller and Wolin 2001). An *in vitro* study by Miller and Wolin (2001) reported that 4 nmol of lovastatin per mL of culture medium (= 4 µM) resulted in 50% growth inhibition of a *Methanobrevibacter* strain, and concentrations ≥10 nmol per mL (= 10 µM) completely inhibited methane formation. Under the conditions of the present study, neither mevastatin (0.25–4 µM) nor lovastatin (0.25–8 µM) elicited a response in methane output. The culture medium used by Miller and Wolin (2001) was synthetic and contained rumen fluid, whereas the medium in the present study contained artificial saliva, rumen fluid and

either ryegrass or GS+B as substrate. The lack of response in methane output in the present study could have been due to degradation or inactivation of the statins by a biological or chemical component in the medium, which was not present in the medium prepared by Miller and Wolin (2001). In a recent *in vivo* trial where sheep were fed 80 mg lovastatin per kg of total dietary DM for 23 days, there was no effect on total daily methane production (Klevenhusen *et al.* 2011*b*); however, with an increased concentration of lovastatin (up to 10 g/kg total DM) in a Rusitec experiment, methane formation was decreased by 42% (Soliva *et al.* 2011). These authors concluded that a dose-dependent effect of lovastatin was possible.

Saccharomyces cerevisiae (SC)

In the present study, SC suppressed CH_4/DMd when incubated with ryegrass and a decreasing trend was evident for CH_4/DMi. One of the most consistently reported responses to SC supplementation is an increase in the number of total culturable and cellulolytic bacteria recovered from the rumen (Nagaraja *et al.* 1997). The increase in the proportion of valeric acid with increasing SC concentration was more pronounced for ryegrass than for GS+B. Changes in the molar proportion of valeric acid accompanying the decrease in methane output for ryegrass indicated a small but specific shift in the fermentation pattern. An increase in valeric acid has been observed in other studies with the addition of SC (Carro *et al.* 1992; Zeleňák *et al.* 1994). The linear increase in iso-valeric acid with increasing concentrations of SC when incubated with both feeds (data not presented) may indicate that iso-valeric acid is a fermentation product of SC. Since iso-valeric acid is known to be a requirement for fibrolytic bacteria (Allison and Bryant 1963; Bryant 1973), its occurrence will be more beneficial to fibrolytic bacteria in breaking down high-fibre feeds.

Martin *et al.* (1989) co-incubated bermudagrass with either 0.4 or 1.0 g/L SC with no effect on *in vitro* methane output. Lila *et al.* (2004) reported no effect of twin-strains of SC (0.33–1.32 g/L) on *in vitro* methane output when co-incubated with sudangrass hay and concentrate (1.5 : 1). Lynch and Martin (2002) co-incubated alfalfa hay and coastal bermudagrass hay separately with either a SC culture (0.35 and 0.73 g/L; Diamond VXP) or SC live cells (0.35 and 0.73 g/L; Saf Agri) for 24–48 h and observed a reduction in *in vitro* methane output only with 0.35 g \L SC live cells with alfalfa hay. In the present study, methane output was reduced with 0.1 g/L SC with ryegrass but not with GS+B. Future studies should examine the potential shifts in the main groups of ruminal microorganisms with the addition of SC when co-incubated separately with ryegrass, GS+B and other feeds under standardised conditions, to explain the discrepancy in methane reduction between different feed types.

Fumaric acid

Fumaric acid is an intermediate in the propionate pathway, in which it is reduced to succinate by fumarate reductase (López *et al.* 1999). In an *in vitro* experiment using the Rusitec system, López *et al.* 1999) demonstrated that the conversion of fumarate via succinate, and thereafter to propionate, was the way by which most (89%) of the added fumarate was fermentated (López *et al.*

1999). Reducing equivalents are needed in this reaction and therefore fumarate provides an alternative electron sink for hydrogen, resulting in a decline in the availability of hydrogen for methanogenesis in the rumen (López *et al.* 1999). The findings of López *et al.* (1999) help explain the reduction in methane output and the increased proportion of propionic acid observed in the present study with increasing concentrations of fumaric acid incubated with ryegrass. Propionic acid also increased with increasing concentrations of fumaric acid with GS+B, but without a corresponding decrease in methane output. Although, the increasing fumaric acid concentration caused a more pronounced decrease in the A : P ratio for GS+B than for ryegrass, this ratio was numerically lower (data not presented) for ryegrass than for GS+B at each incremental increase in fumaric acid, and therefore the increased production of propionate with ryegrass may have reduced the availability of hydrogen for methanogenesis in the present study. García-Martínez *et al.* (2005) found no significant interactions between fumarate (4 and 8 mM) and a high-, medium- and low-forage diets, but the decrease in methane was greater for the high-forage diet (5.1–5.7%) than for either the medium- or low-forage diets (2.9% and 3.8%, respectively). Newbold *et al.* (2005) reported that 8 mM fumarate could significantly reduce *in vitro* methane output in both a high-concentrate and high-forage diet and López *et al.* (1999) showed *in vitro* methane output to be significantly reduced with 5 mM fumarate in a grass hay and concentrate diet (50 : 50). Nothwithstanding the different techniques, substrates and form (salt or acid) of the additive, evidence from the literature would support the reduction of methane output with increasing concentrations (0–20 mM) of fumaric acid co-incubated with GS+B.

López *et al.* (1999) found an increase in DM digestibility and a corresponding increase in cellulolytic bacteria and postulated that one of the beneficial effects of fumarate is an increased fibre digestion. In the current study, the aDMd of both feeds decreased with increasing concentration of fumaric acid, although most of the decline occurred at the highest concentration (20 mM) of fumaric acid added. Newbold *et al.* (2005) also observed an increase in fibre digestion in batch cultures when the sodium salt (fumarate) was added, but no change was evident with the addition of the free acid (fumaric acid).

Conclusions

With increasing concentrations of fatty acids, BES and PMDI, there was a dose-dependent decline in methane output (CH_4/DMi, CH_4/DMd) with ryegrass and GS+B. Methane output could not be detected with a BCM concentration of ≥ 5 µM for both feeds. The statins mevastatin and lovastatin did not reduce methane output with either feed. *S. cerevisiae* reduced methane output (CH_4/DMd) when incubated with ryegrass but not with GS+B. Fumaric acid reduced methane output (CH_4/DMi, CH_4/DMd) only when incubated with ryegrass. The extent of reduction in methane output with increasing concentration of lauric, oleic, linoleic and linolenic acids and BES was more pronounced for GS+B than for ryegrass.

The results presented in the current study are from experiments of a relatively short exposure to the inhibitory

additives, and studies of a longer duration are required to find out whether the effects can be sustained, particularly *in vivo*.

The effectiveness of some additives to inhibit *in vitro* rumen fermentation was both diet and dose dependent. Future *in vitro* and *in vivo* studies using these additives should include diet type as an important component in the experimental design. To identify an effective dose level of each additive, a review of previous dose levels administered *in vivo* should be first consulted, as *in vivo* trials can provide additional information on the effect of an additive dose(s) on feed intake, animal performance, animal health and adaptation effects, which is not available from this or other *in vitro* experiments. Failing to find relevant *in vivo* studies, the minimum concentrations of each additive identified in the present study to reduce methane may prove useful.

Owing to the differing modes of action for many additives to inhibit rumen methanogenesis as shown in this and other studies, incorporating different feed types for the initial *in vitro* screening of all new additives is strongly recommended.

Acknowledgements

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