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MODELLING ANIMAL SYSTEMS PAPER

Aspects of rumen microbiology central to mechanistic modelling of methane production in cattle

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SUMMARY

Methane, in addition to being a significant source of energy loss to the animal that can range from 0·02 to 0·12 of gross energy intake, is one of the major greenhouse gases being targeted for reduction by the Kyoto protocol. Thus, one of the focuses of recent research in animal science has been to develop or improve existing methane prediction models in order to increase overall understanding of the system and to evaluate mitigation strategies for methane reduction. Several dynamic mechanistic models of rumen function have been developed which contain hydrogen gas balance sub-models from which methane production can be predicted. These models predict methane production with varying levels of success and in many cases could benefit from further development. Central to methane prediction is accurate volatile fatty acid prediction, representation of the competition for substrate usage within the rumen, as well as descriptions of protozoal dynamics and pH. Most methane models could also largely benefit from an expanded description of lipid metabolism and hindgut fermentation. The purpose of the current review is to identify key aspects of rumen microbiology that could be incorporated into, or have improved representation within, a model of ruminant digestion and environmental emissions.

INTRODUCTION

Methane (CH_4), produced as a result of microbial digestion, represents an energy loss to the animal. The energy lost through CH_4 production can range from 0·02 to 0·12 of gross energy intake, varying with the type of diet fed (Johnson & Johnson 1995). While most of the research in the past on CH_4 production has focused on methane emissions from an energetic inefficiency standpoint (e.g. Coppock *et al*. 1964; Moe & Tyrrell 1979; Belyea *et al*. 1985), attention has now shifted towards its contribution to climatic change and global warming (e.g. Johnson & Johnson 1995; Benchaar *et al*. 2001; Boadi *et al*. 2004).

In 1997 the Kyoto protocol, the goal of which is to reduce global greenhouse gas emissions, was opened for signatures worldwide. It is now signed by more than 170 countries globally, and has committed developed countries to reduce their emissions by an average of 5% below 1990 levels by 2012 (UNFCCC 2007). Globally, agriculture produces approximately 0·20 of the projected anthropogenic greenhouse gas effect, mostly due to CH_4 and nitrous oxide production (Kebreab *et al*. 2006*a*). On a worldwide basis, the livestock sector produces 0·37 of anthropogenic CH_4 (Steinfeld *et al*. 2006). As a result of the Kyoto protocol and growing concern for the global

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environment, it has become the focus of recent research in animal science to understand CH_4 production in ruminants and how its production can be manipulated to reduce overall emissions.

Methane is produced predominantly in the rumen (0·87) and to a small extent in the large intestine (0·13) of ruminants (Murray *et al.* 1976; Torrent & Johnson 1994). Conversion of feed material to CH_4 in the rumen involves the integrated activities of several different microbial species, the final step being carried out by methanogenic archaea (Hobson & Stewart 1997; Whitford *et al.* 2001). The formation of acetate and butyrate, largely as the result of fermentation of structural carbohydrate (although reasonable amounts of butyrate are produced from soluble carbohydrates), results in production of hydrogen gas (H_2), a substrate methanogenic archaea use to reduce CO_2 (Hegarty 1999; Moss *et al.* 2000). The end result of this reaction is the production of CH_4. Propionate, on the other hand, largely produced with fermentation of non-structural carbohydrates, serves as a competitive pathway for H_2 use in the rumen and is accompanied by a decrease in overall CH_4 production (Hegarty 1999; Moss *et al.* 2000). While the type of carbohydrate present in the diet appears to determine the microbial population present and thus the volatile fatty acid (VFA) profile, other major mechanisms that appear to influence total CH_4 production, either directly on methanogens or indirectly through changes in digestion rate, are rumen pH (Sutton *et al.* 1986; Shabi *et al.* 1999) and passage rate (Okine *et al.* 1989; Hegarty 2002).

Empirical models based on commonly measured dietary inputs are fairly successful in predicting CH_4 emissions (Ellis *et al.* 2007). However, the impact of mitigation strategies to reduce CH_4 emissions has to be assessed holistically, and empirical models lack the biological basis for such an assessment. When dealing with the complex digestive processes in the rumen, it has become useful to develop mathematical models of digestion to both increase understanding of a complex system and to identify areas where knowledge is lacking and more research is required to improve prediction or understanding. Adding a dynamic CH_4 prediction component to these dynamic models has been accomplished (Argyle & Baldwin 1988; Benchaar *et al.* 1998; Mills *et al.* 2001), although limitations still exist in the accuracy of the CH_4 predictions. It is likely that further modifications of the models, incorporating other aspects of the rumen ecosystem, are required to improve the prediction of CH_4 production. It is worth noting that the purpose of these models is not to model the rumen microbial ecosystem *per se*, but to model rumen function and hindgut fermentation with a view to predicting nutrient supply to the host animal and emissions to the environment. This, however, requires detailed knowledge of the biology of the system. Therefore, it is the purpose of the present paper to review current rumen models that predict CH_4 production and to address aspects of methanogenesis and rumen fermentation that may be relevant to improving modelling of CH_4 production. The biology of the system will be considered and attempts to represent biology mathematically will be discussed. Discussion of areas for future work include competition for substrate within the rumen, interspecies H_2 transfer, the role of protozoa, VFA stoichiometry, pH, the effects of supplemental dietary fat and hindgut fermentation.

METHANE MODELS

Dynamic mechanistic models of CH_4 production already exist in the literature, and they attempt to account for the most important features of ruminal digestion and fermentation that will influence CH_4 produced by the animal. It should be noted that, while the current review will focus on aspects of microbiology that could be relevant to modelling CH_4 production, many have not yet been incorporated into models. The models evaluated herein attempt to describe aspects of digestion relevant to the nutritional status of the animal and to its environmental emissions, and not all aspects of rumen microbiology that may be relevant to CH_4 prediction are covered. Therefore, they may benefit from some modifications and these will be discussed. The models described by Benchaar *et al.* (1998) and Mills *et al.* (2001) will be reviewed.

Benchaar et al. (*1998*)

Benchaar *et al.* (1998) reviewed two dynamic, mechanistic models (Baldwin *et al.* 1987; Dijkstra *et al.* 1992) and incorporated into each a H_2 balance submodel previously developed by Argyle & Baldwin (1988), from which CH_4 production can be estimated.

The H_2 sub-model of Argyle & Baldwin (1988) included as inputs: (1) H_2 produced from fermentation of carbohydrates to VFA and (2) H_2 produced from fermentation of amino acids to VFA (Fig. 1). Each input has associated equations to determine the net balance of H_2 produced from acetate and butyrate production, and H_2 utilized by propionate and valerate production. As outputs the model includes: (1) H_2 used for the biosynthesis of microbial cell components, as a function of microbial growth with or without preformed amino acids (according to Reichl & Baldwin (1975)), as well as (2) H_2 used for biohydrogenation of unsaturated fatty acids, a function of the amount of lipid ingested, the proportion of long chain fatty acids and the moles of H_2 required for saturation. Methane production is estimated from the H_2 balance (model inputs minus outputs) (H_y) (mol) as CH_{4rumen} (Mcal/d) $= (H_y/4) \times 0{\cdot}211$, which assumes 4 moles H_2 are required to produce 1 mol of CH_4, and

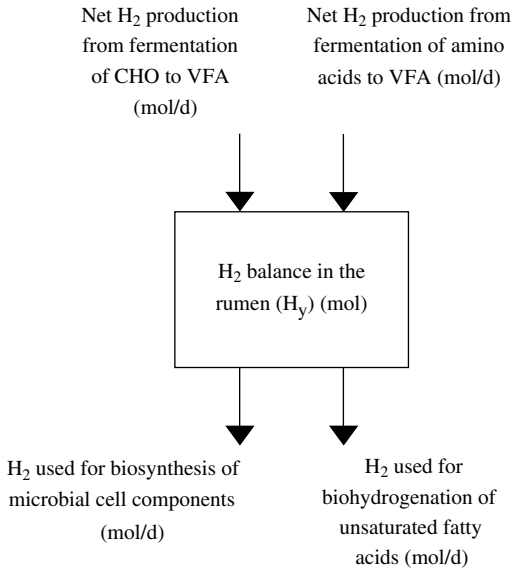

Fig. 1. Hydrogen gas balance model of Argyle & Baldwin (1988) as used in Benchaar *et al.* (1998) and incorporated into Baldwin *et al.* (1987) and Dijkstra *et al.* (1992). Methane production is estimated from H_y as CH_{4rumen} (Mcal/d) = (H_y/4)*0·211, which assumes 4 moles H_2 are required to produce 1 mol of CH_4, and 0·211 is the heat combustion of CH_4 in Mcal/mol (Benchaar *et al.* 1998).

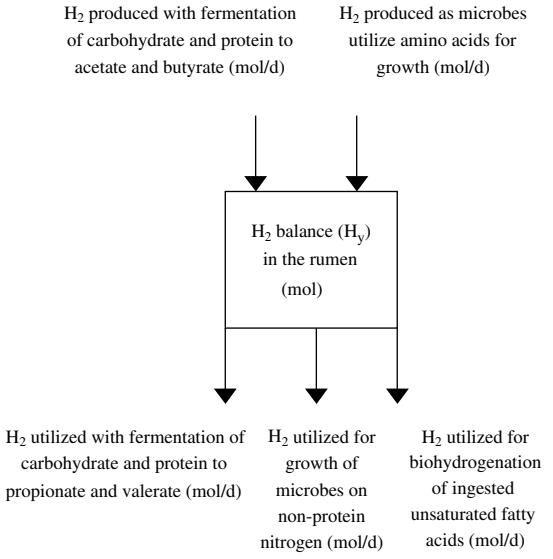

Fig. 2. Hydrogen gas balance model developed by Mills *et al.* (2001) and incorporated into the Dijkstra *et al.* (1992) rumen model. Methane production is estimated from H_y as CH_{4rumen} (Mcal/d) = (H_y/4)*0·211, which assumes 4 moles H_2 are required to produce 1 mol of CH_4, and 0·211 is the heat combustion of CH_4 in Mcal/mol (Benchaar *et al.* 1998).

0·211 is the heat combustion of CH_4 in Mcal/mol (Benchaar *et al.* 1998). In this model H_y is a zero pool, meaning that whatever H_2 is produced but not used for the two outputs is utilized for CH_4 production by methanogens. While the Baldwin *et al.* (1987) model used VFA stoichiometry developed by Demeyer & Van Nevel (1979) and Czerkawski (1986), the Dijkstra *et al.* (1992) model used the VFA stoichiometry of Murphy (1984) and Murphy *et al.* (1982).

Benchaar *et al.* (1998) showed that the modified Baldwin *et al.* (1987) model, with the H_2 sub-model, over-predicted CH_4 production (root mean square prediction error (RMSPE) as a percentage of the observed mean = 36·9 %, bias = 72·4 % and random error = 20·1 % of RMSPE). The authors suggested this could be due to an over-estimation of the amount of structural carbohydrate degraded in the rumen, giving rise to increased acetate production and therefore increased H_2 production, which would increase the resultant CH_4 production. However, Donovan & Baldwin (1998) stated that incorrect input parameters within the study were likely the major source of error for Benchaar *et al.* (1998) and their analysis showed a tendency for a slight under-prediction by the Argyle & Baldwin (1988) model.

Evaluation of the modified Dijkstra *et al.* (1992) model, with the H_2 sub-model, showed a tendency for under-estimating CH_4 production, with a RMSPE value of 19·9 % (due to 25·7 % bias and 66·1 % random error). Benchaar *et al.* (1998) suggested the under-estimation of CH_4 production could be due to under-estimation of fibre degradation in the rumen by the model. In both cases it seems that the under-estimation of CH_4 production is likely related to inadequate prediction of VFA production and VFA profile inputted into the H_2 sub-model, however, other sources of error may also be contributing.

Mills et al. (*2001*)

Further to the work of Benchaar *et al.* (1998), Mills *et al.* (2001) also undertook mechanistic modelling of CH_4 production in the rumen and the hindgut, with the goal of improving the representation of methanogenesis within the Dijkstra *et al.* (1992) model. In addition to adding a different H_2 sub-model and a representation of hindgut fermentation to Dijkstra *et al.* (1992), Mills *et al.* (2001) also updated the VFA stoichiometry of the model to that of Bannink *et al.* (2000, 2006) and modified the parameters used to determine H_2 utilization during microbial growth compared to Benchaar *et al.* (1998) or Baldwin *et al.* (1987). The H_2 model is illustrated in Fig. 2, and inputs to the H_2 sub-model developed included: (1) H_2 produced with acetate and butyrate

(lipogenic VFA) during fermentation of carbohydrate and protein and (2) H_2 produced as microbial populations utilize amino acids for growth. Outputs of the model include: (1) H_2 utilized with production of propionate and valerate (glucogenic VFA), (2) H_2 utilized for growth of microbes on non-protein nitrogen and (3) H_2 utilized for biohydrogenation of ingested unsaturated fatty acids. Methane production is calculated by the same methods used by Benchaar *et al.* (1998), from H_y.

The model of Mills *et al.* (2001) with the new H_2 sub-model over-estimated CH_4 production using a literature database, with a RMSPE % of 15·4, of which 0·73 was random. On an individual cow data database (named CEDAR), however, CH_4 was under-estimated, with a RMSPE % of 12·4, of which a significant portion was due to bias (0·48) and deviation of the regression slope from unity (0·46). Mills *et al.* (2001) suggested the difference in results between the two databases was due to differences in dry matter intake (DMI) and milk production level. The animals in the CEDAR trials were all lactating cows and had a higher DMI compared to the literature database. These results showed an improvement over Benchaar *et al.* (1998) in terms of RMSPE analysis. One major change in the Mills *et al.* (2001) paper compared to Benchaar *et al.* (1998) is the inclusion of hindgut fermentation and its contribution to CH_4 production by the animal. This resulted in an increase in the average model-predicted CH_4 production. New VFA stoichiometry was also utilized by Mills *et al.* (2001), switching from the original Murphy *et al.* (1982) to Bannink *et al.* (2000) and this may also have contributed to better predictions. Details of the current state of VFA prediction will follow in a subsequent section.

The H_2 sub-model of Mills *et al.* (2001) was also used in the model of Kebreab *et al.* (2004) and subsequently evaluated on an independent database of lactating and lactating plus dry cow data by Kebreab *et al.* (2006*b*). Methane production was under-estimated for the lactating cows with a RMSPE value of 23·7% (0·97 due to random error), while it was overestimated for the lactating plus dry cow data with a RMSPE of 29·0% (0·89 due to random error). Better prediction on the lactating cow database is likely because the Mills *et al.* (2001) and Kebreab *et al.* (2004) models were developed on high producing dairy cows and not dry cows.

While the Mills *et al.* (2001) and Benchaar *et al.* (1998) models have achieved some success in predicting CH_4, there appears to be a tendency for under-prediction and in most cases this tendency increases as CH_4 emissions increase. Thus, there is room for further improvement. It is important to note that the model into which the H_2 sub-model is incorporated will significantly alter the predictions from the H_2 sub-model. Therefore, the complete models must be considered and evaluated. Models, like the dynamic processes they intend to represent, are themselves, dynamic and constantly evolving as new information becomes available and is integrated into the model.

There are several areas of focus that repeatedly come up when searching the literature on rumen dynamic models with regards to CH_4 production. The major areas, relevant to the development of an accurate H_2 sub-model and for improving prediction of CH_4 production include: representation of the competition between methanogens and other bacteria in the rumen for substrate, estimation and influence of rumen pH, protozoa and supplemental fat, improvement of the representation of VFA stoichiometry and of postruminal digestion/fermentation. The following sections will discuss current knowledge of the biology of these areas, and how they could be incorporated into a H_2 balance model which aims to improve CH_4 prediction.

SUBSTRATE USAGE

Despite the large number and variety of methanogens within the rumen, as a group methanogens use a small number of simple compounds as substrates, many of which contain single carbons (Zinder 1993). Substrates include H_2 and CO_2, formate, acetate, methanol, methylamines, dimethyl-sulphide and alcohols (Zinder 1993). As a result of specialization for a limited number of substrates, methanogens in the rumen are dependent on the products of other organisms as substrates for their metabolism (Zinder 1993).

Within the rumen, fermentative bacteria hydrolyse and ferment carbohydrates, proteins and lipids to produce acetate, propionate, butyrate and other longer-chain fatty acids (FA) along with H_2 and CO_2. These end-products of microbial fermentation are either absorbed through the rumen wall and used by the animal, or used as substrates for other microbes in the rumen. Propionate, longer-chain FA, some organic acids and alcohols can be further degraded by obligate H_2-producing (proton-reducing) acetogenic bacteria to produce acetate (Czerkawski 1986), although this may be a relatively minor occurrence in the rumen. Zinder (1993) stated that, for slow growing acetogenic FA oxidizers, retention time is not long enough to establish a significant population. When present, these organisms produce H_2 and CO_2 as byproducts of acetate formation and this contributes to the overall H_2 and CO_2 level within the rumen.

Hydrogen gas and CO_2, produced as end products of fermentation, represent the major substrates used by methanogens, and methanogens represent the largest H_2 sink in the rumen. As a result of the preferential use of H_2 by methanogens, the concentration or partial pressure of H_2 in the rumen is generally

kept very low. Rumen H_2 partial pressure may range from 1–10 Pa, and the contribution to the gas phase in the rumen has been estimated at 0·003 by Zinder (1993). Moate *et al.* (1997), however, found less H_2 in the ruminal gas phase of forage fed dairy cows than the 0·001 detection limit of their equipment. Hydrogen gas in the rumen is essentially used up as it is produced.

Although H_2 is the major substrate of methanogens, other substrates can be used and some methanogens grow exclusively on these alternative substrates (Zinder 1993). Most methanogens can use formate as an electron donor in CO_2 reduction as an alternative to H_2, using formate dehydrogenase (Schauer & Ferry 1980), and formate is a common fermentation end product. Acetate, the ultimate end product in many fermentation pathways, can also be used as a substrate for methanogens. Methanol, arising from cleavage of methylated compounds such as pectin (Schink & Zeikus 1980), can be a precursor for methanogens when the animal's diet is high in pectin. Other compounds such as methylamines and methylated sulphides, breakdown products of methylated amino compounds (such as choline and betaine) and methionine, respectively, can also act as methanogen substrates. Short chain alcohols can also serve as electron donors in CO_2 reduction, where secondary alcohols get oxidized to ketones and primary alcohols get reduced to carboxylic acids (Widdel 1986; Zellner & Winter 1987). Most models of CH_4 production do not consider these alternative substrates for methanogenesis and this may contribute to an underestimation of CH_4 production by the models under specific feeding conditions (Donovan & Baldwin 1998; Mills *et al.* 2001).

Knowledge of substrate utilization and preference is far from complete. The application of molecular techniques, in particular 16S rDNA techniques, allowed the further characterization of methanogen diversity within the rumen (McSweeney *et al.* 2007). New yet uncultured archaea have been identified, but substrate use and methanogen activity requires successful attempts to cultivate such archaea (Whitford *et al.* 2001). Methyl coenzyme-M reductase, involved in the reduction of the methyl group bound to coenzyme-M, is crucial in the terminal step of methanogenesis (Denman *et al.* 2005). Measuring these reductase levels and genes controlling them may help to further quantify methanogenic activity in the rumen ecosystem. Even then, it will still remain questionable whether it will reflect *in vivo* scenarios in a quantitative manner.

Substrate preference

Substrate preference can be related to the thermodynamics of the reactions performed. Delta G (ΔG°), or change in free energy (products –reactants), of a reaction indicates how energetically favourable a reaction is. Table 1 summarizes the major reactions methanogens perform using the substrates discussed above, and the corresponding ΔG° value of the reaction. According to Table 1, while use of carbon monoxide is the most energetically favourable reaction for the methanogen to perform ($\Delta G^\circ = -196$ kJ/mol CH_4), only two species of methanogens can perform this reaction (*Methanobacterium* and *Methanosarcina*). In addition, CO levels in the rumen are very low, and CO is toxic to many micro-organisms (Russell & Jeraci 1984). On the other hand, H_2 is highly available in the rumen and releases 145 kJ/mol CH_4 produced, which is more energetically favourable than any alternative reactions, though followed closely by formate. Thus, in an environment with a spectrum of substrates available, methanogens prefer the substrate with which the associated reaction is the most energetically favourable, primarily H_2. In addition, K_m, the affinity constant, is quite low for H_2 use by many methanogens (Table 2), which adds to its preference as a substrate. Due to this strong preference for H_2, much of the CH_4 modelling that has been done to date is based solely on H_2 balance and availability in the rumen, with excess being used completely by methanogens for CH_4 production.

Competition for substrates by methanogens in the rumen

Partial pressure and thermodynamics

The minimum H_2 partial pressure threshold required for a reaction to occur is related to the thermodynamics of the reaction involved. There is an inverse relationship between ΔG° for a reaction and the threshold H_2, or minimum H_2 partial pressure of the system, required for a H_2 utilizing reaction to occur (Zinder 1993) (Fig. 3). Thus, if the H_2 partial pressure is high, it is very energetically favourable for the reactions with H_2 as a substrate to occur (high levels of H_2 substrate, large negative ΔG°); *v.* if the H_2 partial pressure is low, it is less energetically favourable for the reaction to occur (lower level of H_2 substrate, more positive ΔG°). Ungerfeld & Kohn (2006) presented a comprehensive review of the thermodynamic control of rumen processes and Fig. 3 demonstrates this inverse relationship between ΔG° and H_2 partial pressure for sulphate-reducers, methanogens and reductive acetogens, all of which use H_2 as a substrate, along with their threshold H_2 partial pressures. It is apparent that the higher the partial pressure, the more energetically favourable the reactions become.

Acetogens

Two types of acetogens populate the rumen, the reductive acetogenic bacteria which reduce CO_2 to acetate by oxidation of H_2, and obligate proton-reducing acetogens that hydrolyse FA and convert

Table 1. *Methanogenic and competitive organism reactions and reaction thermodynamics*[a]

Organism	Reactants	Products	ΔG°		ΔG
Methanogens			kJ/mol CH_4	kJ	kJ/2H
Methanobacterium and Methanosarcina	Carbon monoxide, water	Methane, bicarbonate, hydrogen	−196	−196	
Hydrogenotrophic methanogens	Formate, hydrogen, water	Methane, bicarbonate	−145	−145	
Most methanogens	Hydrogen, bicarbonate	Methane, water	−135	−135	
Most methanogens	Carbon dioxide, hydrogen	Methane, water	−134	−134	−16·9
Some hydrogenotrophic methanogens	Ethanol, bicarbonate		−116	−116	
Methanosphaera stadtmanii, methylotrophic methanogens	Methanol, hydrogen	Methane, water	−113	−113	
Methanosarcina and other methylotrophic methanogens	Methanol	Methane, bicarbonate, water, hydrogen	−105	−315	
Methanosarcina and other methylotrophic methanogens	Methylamines, water	Methane, bicarbonate, ammonia, hydrogen	−76	−684	
Some methylotrophic methanogens	Methylsulphides, water	Methane, bicarbonate, hydrogen sulphide, hydrogen	−49	−147	
Methanosarcina and Methanothrix	Acetate, water	Methane, bicarbonate	−31	−31	
Competitive Organisms					
Reductive acetogens (e.g. *Acetomaculum ruminis*)	Carbon dioxide, hydrogen	Acetate, hydrogen, water	–	−71·6	−2·2
Sulphate-reducing bacteria (e.g. *Desulfovibrio desulphuricans*)	Sulphur anion, hydrogen	Hydrogen sulphate, water	–	−234	−21·1
Propionate producers (e.g. *Ruminobacter amylophilus*)	Fumarate, hydrogen	Succinate	–	−84	−63·6
Propionate producers (e.g. *Prevotella ruminicola*)	Acrylate, hydrogen	Propionate	–	−94·3	−51·3

[a] Modified from Zinder (1993) and Ungerfeld & Kohn (2006).

them to acetate, CO_2 and H_2 (Mackie & Bryant 1994). Reductive acetogens have the ability to compete with methanogens in the rumen for H_2. However, in the unmodified rumen, very little acetogenesis from H_2 actually occurs because the partial pressure of H_2 is lower than the threshold required for acetogens. A literature search did not reveal any reported K_m values for H_2 use for acetogenesis, so it is unknown to the authors how well acetogens compete with methanogens in terms of affinity for H_2.

The H_2 threshold, or minimum H_2 partial pressure required for uptake of H_2 and thus use of it as a substrate, is much higher for acetogens than for methanogens (Breznak & Kane 1990) (Fig. 3). Methanogens can operate and use H_2 as a substrate at a lower ruminal concentration of H_2 than can acetogens. Thus, acetogens would not be able to compete effectively with methanogens for H_2 unless the 'steady-state' H_2 concentration in the rumen was maintained above their threshold level, e.g. if methanogenesis was inhibited. It is likely that micro-pockets of higher H_2 partial pressure exist within the rumen, but as a whole, it is kept low. Methanogens have the advantage when competing directly with acetogens for H_2, as methanogens can reduce the H_2 partial pressure in the rumen to a level at which they can still use it as a substrate (by using up H_2 as it is produced), while preventing acetogens from being able to use H_2 at these low partial pressures.

Sulphate-reducing bacteria

The sulphate-reducing bacteria in the rumen represent a second population competing for substrate with methanogens, but much less information is available about them. Sulphate-reducing bacteria can use sulphate or other oxidized forms of sulphur (thiosulphate, sulphite and elemental sulphur) as electron acceptors, and use H_2, organic acids, alcohols, amino acids and some aromatic compounds as electron donors (Zinder 1993). Lin *et al.* (1997) showed that sulphate-reducers are a small but significant population of the rumen microflora.

In anaerobic environments where sulphate is not limiting, sulphate-reducing bacteria can out-compete

Table 2. *K_m values for various methanogens, fumarate reducers and sulphate reducing bacteria from Zinder (1993) and Asanuma* et al. *(1999)*[a]

Organism	K_m for H_2 uptake	
Source:	(Zinder 1993)	(Asanuma *et al.* 1999)
Reported units:	μM	mM
Methanogens		
Methanosprillum hungatei	5	–
Methanosarcina barkeri	13	–
Methanobacterium thermoautotrophicum	8	–
Methanobacterium formicicum	6	–
Mean	8	1·6
Fumarate Reducing Bacteria		
Selenomonas ruminantium	–	7·5
Selenomonas lactilytica	–	4·7
Veillonella parvula	–	5·8
Wolinella succinogenes	–	4·0
Fibrobacter succinogenes	–	6·2
Mean		5·6
Sulphate Reducing Bacteria		
Desulfovibrio vulgaris	2	–
Desulfovibrio desulphuricans	2	–
Mean	2	–

[a] This table presents results from two separate studies (Zinder 1993; Asanuma *et al.* 1999). While comparisons can be made within study between K_m values, it appears that either of the studies may have a K_m unit error, as the units provided would result in a 1000-fold difference in values.

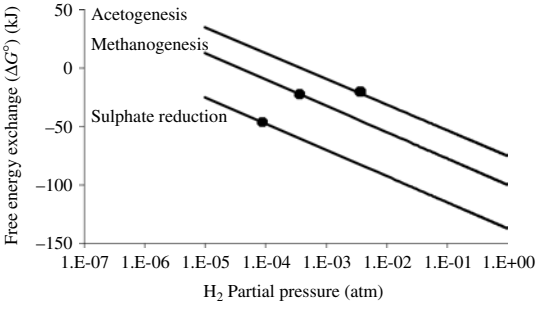

Fig. 3. Relationship between the free energy released from a reaction and the H_2 partial pressure of the system for methanogens, acetogens and sulphate-reducing bacteria, with the solid dots representing the threshold H_2 partial pressure required for the reaction to occur (diagram modified from Zinder (1993)).

methanogens for substrates (H_2, CO_2, formate and acetate) (Zinder 1993). Sulphate-reducers have a lower minimum H_2 threshold than methanogens, meaning they have a competitive advantage for H_2 use in the rumen (Zinder 1993), as well as having a lower K_m value (Table 2, Fig. 3). The amount of sulphate-reduction that actually takes place in the rumen is, however, directly proportional to and limited by the amount of sulphur containing compounds available (Zinder 1993). For a normal/healthy animal diet, the sulphur level is not high enough to allow sulphate-reducers to exceed methanogens as the major H_2 sink in the rumen. It is possible, however, that on a high sulphur diet, sulphate-reducers could increase in numbers and compete with the methanogen population for available H_2. This would not be very desirable though, as increased sulphur intake, and thus high sulphide concentrations in the rumen, can also depress DMI, retard growth rate, decrease copper status and cause the central nervous system disorder polioencephalomalacia (PEM) (Gould 1998). Co-products such as maize gluten feed, modified distillers grains and wet distillers grains are typically higher in sulphur content than unprocessed maize because sulphur is added during the milling process. For example, the sulphur content for dried maize gluten meal, dried maize distillers grains, ground dried maize and maize silage are 8·6, 4·4 and 1 g/kg DM, respectively (National Research Council 2001). With the current interest in using ethanol as a fuel source, the economics of feeding more maize co-products instead of maize has become appealing to many cattle farmers. It is possible that increases in the sulphate content of the diet due to inclusion of more maize co-products may alter H_2 use within the rumen and cause a reduction in CH_4 production. Including sulphate-reducing bacteria in a model of

H_2 balance may thus become more relevant as feeding practices shift.

Propionate production

A more immediately relevant competitive pathway to methanogenesis is the reduction of dicarboxylic acids, including aspartate, malate and fumarate, to propionate in the rumen. These organic acids or their metabolites are reduced by rumen micro-organisms that use either H_2 or formate as electron donors to succinate and then propionate. Ungerfeld & Kohn (2006) showed that reduction of fumarate to succinate, which will result in propionate formation, is actually more thermodynamically favourable than methanogenesis within the range of H_2 partial pressures seen in the rumen. Based on thermodynamics alone (Table 1), it is interesting that propionate formation does not out-compete methanogenesis *in vivo*. While some protection for methanogens may be granted by their frequently observed close associations with protozoa in the rumen and the resulting direct supply of H_2 (Finlay *et al*. 1994; Ushida *et al*. 1997), on a diet rich in ruminally degradable starch or at high intake levels, propionate production does increase, and this is mirrored by a reduction (although not complete elimination) of methanogenesis. On a high forage diet however, CH_4 production is the major H_2 sink in the rumen.

It is possible that the free energy for fumarate reduction (propionate production) cannot be efficiently used for microbial ATP synthesis, and how well the efficiency of ATP utilization for cellular anabolism compares between methanogens and different propionate producers in the rumen is unknown (Ungerfeld & Kohn 2006). In addition, fumarate reduction could also be limited kinetically by the availability of H_2 or fumarate (Ungerfeld & Kohn 2006), and the average K_m value for fumarate reduction using H_2 as a substrate is much higher than that of methanogens using H_2 as a substrate (Table 2). This could provide a competitive advantage to the methanogens. Recent research has also suggested a strong role for pH in determining the balance between methanogenesis and propionate production, and this topic will be discussed in detail in a subsequent section on pH.

Addition of fumarate to the diets of ruminants as a mitigation strategy to reduce overall CH_4 emissions has been explored. Enhanced production of propionate in the rumen (through addition of fumarate or other organic acids) may also be beneficial to the dairy cow in certain situations (e.g. high roughage diet, peak lactation) and increased supply of glucogenic precursors may improve energy balance and reduce severity of ketosis and fatty liver (Van Knegsel *et al*. 2005). In terms of CH_4, if fumarate reduction is thermodynamically more favourable than methanogenesis, but kinetically limited (fumarate reduction has a higher K_m value for H_2 use than does methanogenesis, meaning methanogenesis is more favourable) (Asanuma *et al*. 1999), addition of fumarate to the system could increase the number of fumarate-utilizing bacteria which could, in theory, remove H_2 and reduce CH_4 formation by methanogens. Fumarate reducing bacteria do, however, have a lower K_m value for formate use as an electron donor compared to methanogens (Asanuma *et al*. 1999), and addition of fumarate to the system allows formate-utilizing fumarate-reducers to steal substrate (formate and some H_2) away from methanogens, resulting in a decline in CH_4 production. Studies reviewed by Ungerfeld & Kohn (2006), such as Asanuma *et al*. (1999) and Callaway & Martin (1996), have shown that while CH_4 production is decreased by fumarate addition, the actual depression in CH_4 is less than half what is expected if all fumarate added were metabolized down the succinate pathway. In reality, only a fraction of the added fumarate is metabolized to propionate and some other pathways, such as acetate production, are also stimulated. This is undesirable with respect to mitigating CH_4 emission, as acetate production releases H_2 which counteracts the uptake of H_2 by fumarate and decreases the magnitude of expected CH_4 reduction (Ungerfeld & Kohn 2006). Studies have also investigated other organic precursors of propionate, including malate, which is another intermediate of the succinate pathway.

The addition of malate to batch cultures resulted in a mild inhibition of CH_4 similar to fumarate (Hino & Asanuma 2003) and recoveries of malate as propionate and acetate were approximately the same as for fumarate addition (Martin & Streeter 1995; Callaway & Martin 1996; Carro & Ranilla 2003). Newbold *et al*. (2005) tested fifteen potential precursors of propionate, including fumarate, acrylate, malate and citrate, in short-term batch cultures. Sodium acrylate and sodium fumarate produced the most consistent effect and decreased CH_4 production by between 8 and 17%. In longer term (21 d) *in vitro* incubations, fumarate addition, but not malate, was effective in reducing CH_4 production. However, such *in vitro* results were largely unsuccessful *in vivo*. Wallace *et al*. (2006) reported a large decrease (49–75%) in CH_4 production by lambs upon fumarate supplementation, using a tunnel system to measure gaseous emissions. On the other hand, addition of fumaric acid to diets of steers or heifers by McGinn *et al*. (2004) and Beauchemin & McGinn (2006) did not reduce CH_4 emissions determined using respiration chambers.

While the competition between H_2 utilizing organisms in the rumen appears important in determining CH_4 production, none of the current rumen models account directly for this aspect of rumen microbiology and its influence on the profile of end products. Similarly, none of the current rumen models account for alternative substrate use, such as

formate, and competition for formate as a substrate. It is possible that not accounting for alternative substrate use by methanogens could contribute to under-estimation of CH_4 production seen in some of the models discussed earlier. Similarly, it would be interesting to see whether competition with acetogenic bacteria or sulphate-reducing bacteria for excess H_2 could be included in a model, and whether it would improve the model predictions. Competitive bacteria could share in a variable proportion of the H_2 available when the amount of H_2 going into CH_4 decreases, or during dietary manipulations that alter the amount of methanogenesis taking place. With the search for dietary additives that could serve as mitigation strategies to reduce CH_4 production, inclusion of other variables such as fumarate or malate into a rumen model may also become valuable in explaining observed shifts in ruminal metabolism. All of these areas would represent valuable research areas for mechanistic modelling of ruminal CH_4 production.

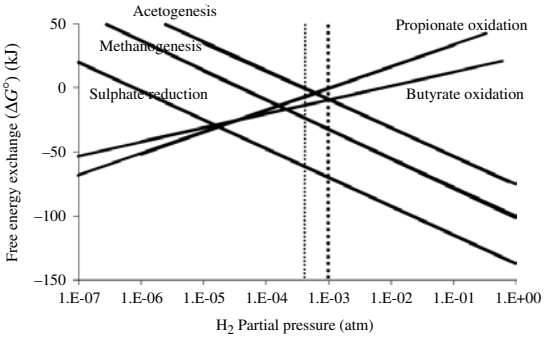

Fig. 4. The relationship between the thermodynamics of a reaction and the H_2 partial pressure, for H_2 producing bacteria (propionate and butyrate oxidation) and H_2 utilizing bacteria (acetogens, methanogens and sulphate reducers), where the vertical lines represent the minimum H_2 partial pressure tolerated for methanogens (left) and for propionate/butyrate oxidation (right).

Interspecies H_2 transfer

Comparing species, sulphate-reducers release approximately 45 kJ/reaction at their minimum H_2 partial pressure threshold, while methanogens and acetogens release approximately 25 kJ/reaction (Fig. 3) at their thresholds. The sulphate-reducers have the lowest threshold H_2 partial pressure among the three, and thus, have an advantage over the other two species provided that sulphate is not limiting. This is rarely the case, however.

Based on Fig. 3, it would seem advantageous to keep the partial pressure in the rumen as high as possible, making H_2 utilization by these bacteria as energetically favourable as possible. This, however, is not the case. Conditions in the rumen must also be made energetically favourable for H_2 producers, for whom accumulation of too much product (H_2) is inhibitory (Immig 1996). For these organisms, low H_2 partial pressure in the rumen makes their reactions more energetically favourable (Fig. 4) and they are limited by a maximum H_2 partial pressure above which their reactions will not occur. For propionate and butyrate oxidation, for example, the maximum H_2 partial pressure is 10^{-4} (Zinder 1993), and is represented by the vertical dotted line to the right in the diagram (Fig. 4). The result of this balance between H_2 production and utilization is a relatively narrow range of H_2 partial pressure maintained in the rumen so that H_2 produced is also used up (between the two dotted lines in Fig. 4) and both reactions are energetically favourable. Through this diagram one can see how, in an unmodified rumen, this balance (keeping the H_2 partial pressure between the dotted lines) keeps the H_2 partial pressure high enough such that sulphate-reducers cannot reduce it further to gain a competitive advantage, and low enough such that acetogens cannot take over either. The H_2 produced by fatty acid oxidizers is utilized by methanogens almost immediately, and this keeps the ruminal H_2 partial pressure low. This is imperative to the proper functioning of the rumen, as an accumulation of H_2 would inhibit fermentation and negatively affect digestion in the animal (Wolin 1975; Hegarty & Gerdes 1998).

Steps involved in this electron transfer between H_2 producing and H_2 utilizing species include: reduction of H^+ to H_2, diffusion into the aqueous phase of the rumen towards a methanogen, and then oxidation of H_2 by the methanogen (Boone *et al.* 1989).

Interspecies H_2 transfer is facilitated for electron transfer from protozoa to methanogens by their frequently observed close associations. This relationship is advantageous to both organisms, as the protozoon can quickly rid itself of H_2 produced and the methanogen is fed H_2 directly without having to search for substrate. It is also advantageous because protozoa selectively retain themselves in the rumen, which is favourable for 'slow' growers such as methanogens (Jouany *et al.* 1988). The relationship between methanogens and protozoa will be discussed next, with a focus on their representation within dynamic mechanistic models and some of the associated limitations.

PROTOZOA

Protozoa release H_2 and CO_2 during the breakdown of starch, sugar and fibre, producing compounds such as acetate and butyrate (see Williams & Coleman (1997) for a review of protozoal metabolism). Methanogens have been observed both attached to

the cell surface, as well as in the cytosol, of these protozoa (Finlay *et al.* 1994; Ushida *et al.* 1997), presumably benefiting from direct interspecies H_2 transfer. The symbiotic relationship between methanogens and protozoa may be responsible for 0·25–0·37 of the rumen CH_4 produced (Finlay *et al.* 1994) and this interaction appears to be a significant consideration when examining factors important to modelling CH_4 production in the rumen. The number of methanogens associated with protozoa, either on the surface or in the cytosol, appears to be regulated at least in part by the H_2 partial pressure in the rumen (Finlay *et al.* 1994). Methanogens tend to live free during a meal when the H_2 partial pressure is higher and substrate is readily being fermented in the rumen. On the other hand, more methanogens tend to associate with protozoa when the H_2 partial pressure is low, i.e. between meals, when substrate is less readily available (Tokura *et al.* 1997). Tokura *et al.* (1997) reported that the number of methanogens associated with protozoa increased 100 to 1000-fold after a meal is finished. This makes sense, since when substrate is readily available such as during a meal, H_2 is also readily available, and protozoa will also fill with substrate. Protozoa continue to digest the food after the meal has finished (Williams & Coleman 1988). Methanogens then benefit from associating with protozoa particularly between meals, because they can directly receive the H_2 produced as a result of protozoal fermentation of stored substrate. It also means that through their associations with protozoa, methanogens are perfectly capable to follow H_2 gradients within the rumen.

In terms of biomass, protozoa represent a fraction comparable to that of bacteria in the rumen (Sylvester *et al.* 2004), suggesting they have an important role in rumen fermentation processes. Numerous defaunation experiments have demonstrated however, that protozoa are nonessential to the ruminant (Williams & Coleman 1997). The modifications caused by elimination of protozoa are generally large, but not systematic (see Eugene *et al.* 2004). Protozoa, either directly or indirectly, influence ruminal retention time and passage rate, rumen volume, numbers and types of rumen bacteria present, overall concentration and proportion of VFA present, nitrogen recycling, pH, concentration of ammonia and DM digestibility (Williams & Coleman 1988; Nagaraja *et al.* 1992; Jouany 1996; Williams & Coleman 1997). The exact impact of the presence or absence of protozoa may depend on the diet being fed and the type and number of protozoa present in the rumen. In animals fed a low-protein diet, the presence of protozoa seems to have a negative effect on growth and performance (Bird & Leng 1978), whereas in animals fed a high grain diet, protozoa may play a beneficial role through their ability to influence starch and lactic acid metabolism (Hungate 1978; Veira 1986; Nagaraja *et al.* 1992). The molar proportion of propionate often increases and butyrate often decreases upon defaunation (Eugene *et al.* 2004), suggesting reduced CH_4 production. Observations confirm that CH_4 production is reduced in defaunated cattle (Demeyer *et al.* 1982), either through methanogens being removed from the rumen with the protozoa or by a decrease in available substrate. However, the whole system, including both direct and indirect effects, must be evaluated when considering defaunation as a mitigation strategy.

In the rumen, protozoa feed on smaller organisms, particularly bacteria, which are an important source of nitrogenous compounds for protozoal growth (Williams & Coleman 1997). A requirement for live bacteria for protozoal growth is manifested in cultures longer than 2 to 4 days (Dehority 2003). Unfortunately, protozoal dependence on live bacteria confounds *in vitro* culture results (Dehority 2003), and thus *in vitro* data on protozoa, and their subsequent influence on the bacterial population and interactions with methanogens have been difficult to collect. *In vivo* work has been challenging due to the absence of a reliable protozoal marker, separating protozoa from the bacterial fraction (Broderick & Merchen 1992; Firkins *et al.* 1998). Thus, most information to date on the role of protozoa in the rumen has been inferred indirectly, through measuring differences between faunated and defaunated animals as discussed above. This severely limits the research that has been done on protozoal metabolism to date. Recently however, Sylvester *et al.* (2004) developed a promising assay for measuring protozoal biomass in rumen digesta by targeting and quantifying the DNA encoding 18SrRNA (rDNA). This methodology may aid in increasing the number and types of experiments investigating the role of protozoa in the rumen that can be undertaken.

More accurate representation of protozoa in digestion models than already attempted (Dijkstra 1994) will require more research to determine their exact role and function in the rumen. An accurate understanding of the role of ruminal protozoa would aid not only in better CH_4 predictions under varying rumen conditions, but also other aspects of rumen digestion and nutrient assimilation. In the Mills *et al.* (2001) and Dijkstra (1994) models, consideration of protozoal dynamics is partly based on unconfirmed assumptions due to lack of experimental results. For example, the Dijkstra (1994) model is sensitive to the maintenance requirement of protozoa (fixed in the model) and some indirect justification for the maintenance requirement was obtained from data on rate of endogenous amylopectin utilization in protozoa, assuming that this endogenous utilization rate represents maintenance requirements. Another sensitive attribute of the protozoa sub-model is bacterial engulfment by protozoa. Information on preference

by protozoa for certain bacteria types due to species or adherence to substrate is also largely lacking (Dijkstra *et al*. 2008), in contrast to other systems (including soil and marine ecosystems) in which much more information is available on the response of individual bacterial species to protozoal predation (Matz & Kjelleberg 2005). It is also not known whether the nutritional state of protozoa affects what they engulf. Another important aspect of the model requiring development is rate of passage of protozoa from the rumen, which will affect the model predictions. The model currently assumes protozoa pass at a rate proportionate to the fractional solid passage rate (0·45). However, this assumption has been questioned in recent *in vivo* experiments with frequently fed cows (Karnati *et al*. 2007) and the knowledge that protozoa can sequester themselves within the rumen (Abe & Iriki 1989). A series of new experiments would provide raw data for the development of an improved protozoa sub-model.

A dynamic mechanistic model of rumen metabolism which includes a more detailed description of protozoa would provide: (i) greatly enhanced understanding of the requirement for nutrients by ruminant livestock and opportunities to optimize dietary nutrient inputs; (ii) better understanding of the interaction between bacteria and protozoa which would lead to better estimation of total microbial flow to the duodenum; (iii) a nutrient-based feed evaluation system that considers protozoal requirements and the impact of dietary manipulation such as defaunation on animal production efficiency and CH_4 production. Increasing our knowledge of methanogen-protozoa interactions would aid in increasing our understanding of the defaunation-CH_4 production level relationship, and is an area worthy of further research. The effect of defaunation on CH_4 production could be attributed to removal of methanogens associated with protozoa during defaunation, resulting in lower methanogen numbers, as well as reduced substrate availability, due to the absence of protozoa which produce substrate for methanogens. Removal of protozoa will also alter the VFA profile produced in the rumen, and may result in a lowering of ruminal pH (Nagaraja *et al*. 1992) due to removal of their buffering capacity. Incorporation of these effects into a rumen model would allow mitigation strategies that result in defaunation to be evaluated more accurately.

VFA PROFILE

Volatile fatty acids, the major end product of fermentation in the rumen, represent the primary energy source available to the animal, where acetate, propionate and butyrate account for more than 0·95 of the VFA found in rumen fluid (Bannink *et al*. 2006). While acetate and butyrate are primarily used as precursors for longer-chain FA synthesis in the ruminant, propionate is predominantly used as a glucose precursor (Bannink *et al*. 2006). Hydrogen gas is produced as a result of acetate and butyrate formation and is utilized in the production of propionate. Since H_2 represents the major substrate for methanogenesis, it is important to be able to accurately predict the VFA profile created in the rumen as a result of the diet fed, which will in turn largely predict the H_2 balance of the system.

On a high forage diet, the acetate : propionate : butyrate ratio in rumen fluid is typically in the proportion 70 : 20 : 10. The ratio of VFA produced is a function of the microbial populations present, which are largely dictated by diet and type of carbohydrate being fermented. On a high grain diet, typically high in non-structural carbohydrate and low in structural carbohydrate, the growth/development of propionate producing bacteria is favoured and the proportion of propionate produced is increased at the expense of acetate (Bannink *et al*. 2006). This results in an alternative sink for H_2, and methanogenesis does indeed decrease with high concentrate diets. Diets that encourage the development of a large protozoal population are accompanied by an increase in butyrate rather than propionate production (Williams & Coleman 1997), resulting in production of H_2, which can be used as substrate for methanogenesis and increased CH_4 production. If intake is increased, increasing the amount of substrate available, a shift in the fermentation pattern away from acetate and towards propionate production occurs, in order to dispose of excess H_2 (Dijkstra 1994). Accurate prediction of the rumen VFA profile and production rates of individual VFA for a given diet likely represent the biggest sources of error in predicting CH_4 produced in the rumen.

Accurate determination of VFA is important for predicting nutrient availability to the animal, as well as being imperative to accurate prediction of CH_4. The balance between H_2-utilizing propionate production and H_2-producing acetate and butyrate production has an important role in determining the H_2 available in the rumen for utilization by methanogens. A number of methods for estimating rates of VFA production have been developed over the years. Predictions, however, of VFA molar proportions by these stoichiometric coefficients are still largely inaccurate. Bannink *et al*. (1997) stated that this inaccuracy was due to either inadequate representation of VFA production or inadequate representation of VFA absorption through the rumen wall. It is very common that concentrations of VFA in the rumen are measured and this gives an indication of the balance between the rate of VFA production and the rate of VFA absorption. While these relative concentrations of VFA appear to be a reliable indicator of the rate of VFA production when forage

diets are fed, it appears they are less reliable with high concentrate diets (France & Dijkstra 2005). Concurring with this, Bannink *et al.* (2000, 2006) found that the standard error of coefficient estimates were higher for concentrate-rich diets compared to those for roughage-rich diets, indicating more variation that could not be explained. This is likely due to differences in the relative fractional absorption rates of individual VFA, as well as to differences in production rates of individual VFA (Lopez *et al.* 2003), as the fermentation rate changes (Bannink *et al.* 2006). Recently, Bannink *et al.* (in press) developed a mechanistic model of absorption and intra-epithelial metabolism of VFA that addresses some of the inadequacies in representation of VFA absorption.

While others have worked on the problem of accurately predicting the VFA profile in the rumen (Argyle & Baldwin 1988; Pitt *et al.* 1996; Friggens *et al.* 1998; Kohn & Boston 2000; Nagorka *et al.* 2000; Sveinbjörnsson *et al.* 2006), they do so with varying levels of improvement over Murphy *et al.* (1982). A full review of these papers can be found in Dijkstra *et al.* (2008) and will not be repeated here. In a recent attempt to improve VFA representation, Bannink *et al.* (2006) developed a model of VFA production in the rumen of lactating dairy cows fed either high forage or high concentrate diets, based on Murphy *et al.* (1982). In this model, the amount of starch, hemicellulose, cellulose, other carbohydrates (soluble and rapidly fermentable carbohydrates such as pectin) and protein convert to acetate, propionate, butyrate and other VFA, by rumen microbes, calculated in all combinations. As has occurred in previous attempts to model VFA production, Bannink *et al.* (2006) observed that variation in predicted values was smaller than in observed values. This, however, was attributed to statistical methods and the necessity for molar proportions of VFA to add up to unity. Generally, predicted VFA followed the pattern of observed VFA. Bannink *et al.* (in press) further developed the model to include pH-dependent stoichiometric parameters for rapidly fermentable carbohydrates. These coefficients likely represent an improvement over Murphy *et al.* (1982) because of the extensive and wide database used to derive the coefficient values, and it would be interesting to see them challenged on an independent dataset and applied in one of the current rumen models.

The Mills *et al.* (2001) model (which uses the VFA stoichiometry of Bannink *et al.* 2000, 2006) could be improved by replacing the current VFA stoichiometry in the model with the updated VFA stoichiometry of Bannink *et al.* (in press). Evaluation of the model should be conducted using an independent database, evaluating results with the current VFA stoichiometry and then again with the new Bannink *et al.* (in press) stoichiometry. The performance of other stoichiometry sets (e.g. Murphy *et al.* 1982; Argyle & Baldwin 1988; Pitt *et al.* 1996; Friggens *et al.* 1998) could also be evaluated for comparison, though some of them were previously assessed by Bannink *et al.* (1997). Getting the VFA stoichiometry correct may be the most influential modification within a model of H_2 balance with the aim of predicting CH_4 production. Inaccurate prediction of VFA levels can lead to an over- or under-prediction of CH_4 production in the rumen contributing to bias of the results, and deviation of the regression slope of predicted *v.* observed VFA from unity may transfer through to the predicted *v.* observed CH_4 relationship as well.

RUMEN pH

When the ratio of fermentation end products, acetate:propionate, declines in the rumen, CH_4 production also declines (Russell 1998). A decline in the acetate:propionate ratio typically occurs when the diet is switched from a high forage to a high grain one, and is the result of increased ruminal propionate production (Russell 1998). Switching the diet from high forage to high grain also causes a drop in rumen pH, an effect that is greater with increasing DMI. Methane and propionate production represent competitive pathways for H_2 use in the rumen and while at high pH and on a forage based diet CH_4 production out-competes propionate production for H_2 usage, when the diet is switched to high concentrate resulting in a higher fractional rate of substrate degradation and a lower pH, CH_4 production declines and propionate production increases. The exact mechanism regulating this shift is unclear. It is likely to be regulated by some combination of substrate availability and pH.

Substrate availability suggests that acetate is high and propionate low in forage-fed animals because acetate-producing bacteria prefer structural carbohydrates as substrates, and thus flourish and outcompete propionate producers (as their preferred substrate is in low quantities). Since acetate results in a net production of H_2, methanogens flourish with a high availability of substrate in the rumen on high forage diets. On the other hand, when the diet is high in non-structural carbohydrates (high concentrate), propionate producing bacteria outcompete acetate forming bacteria because their substrate is now in abundance and they tend to be more acid tolerant (Russell 1991). This then provides competition for H_2 as a substrate with methanogens, and could explain the lower CH_4 production levels in the rumen of animals fed a mainly concentrate-based diet. As stated earlier, Ungerfeld & Kohn (2006) raised the issue that reduction of fumarate to succinate, the first step in propionate production, is more thermodynamically favourable than methanogenesis. It is possible that when the diet is forage based,

methanogens out-compete propionate producers because non-structural carbohydrates are low and pH is high. When non-structural carbohydrates are high and pH low, however, such as with a high concentrate diet, propionate producers out-compete methanogens because (1) their substrate is now available, (2) they have an advantage of being able to survive in lower pH environments and (3) it is more energetically favourable than methanogenesis. However, methanogenesis is not completely eliminated on high concentrate diets. Johnson & Johnson (1995) reported that CH_4 production is roughly 0·02 of gross energy intake on high concentrate diets. This might be explained by their lower K_m values for H_2 use compared to propionate producing bacteria (Table 2).

An alternative argument to explain these shifts in microbial populations is that they are regulated by pH. Van Kessel & Russell (1996) showed that methanogenesis was sensitive to rumen pH, with methanogenesis decreasing dramatically in forage fed cows at $pH < 6{\cdot}5$ and virtually halting at $pH < 6{\cdot}0$. The inhibitory effect of low pH was supported by a variety of observations, which included the fact that addition of a base to the ruminal fluid of a concentrate fed cow resulted in resumption of CH_4 production (Van Kessel & Russell 1996). If methanogenesis were inhibited at low pH, it would allow H_2 to be used by propionate producing bacteria, allowing them a competitive advantage in a lower pH.

Van Kessel & Russell (1996) discussed possible mechanisms for the inhibition of methanogenesis at low ruminal pH, mainly that VFAs were causing a pH-dependent inhibition of methanogenesis. In other species of bacteria (e.g. *Escherichia coli*), which are relatively acid-tolerant and able to grow in low pH, placement in a rumen fluid solution with $pH < 6{\cdot}0$ resulted in their inability to grow (Hollowell & Wolin 1965). Hollowell & Wolin (1965) ascribed the inhibitory effect of rumen fluid to the concentration of VFA. VFAs are commonly used as food preservatives because they are able to inhibit bacterial growth at low pH values. Associated VFA, present at low pH values, easily cross cell membranes (Russell 1992). If the intracellular pH is greater than the extracellular pH, VFA will cross the cell membrane, dissociate and accumulate in the intracellular compartment, eventually causing cell death. Because the concentration of VFA in the rumen is relatively high, particularly at low pH levels associated with high concentrate diets, even a modest increase in the ΔpH (intracellular *v.* extracellular) can lead to a dramatic increase in uptake by bacteria. It is possible, then, that ruminal methanogens are inhibited by the toxicity of VFA anions at low pH, while they remain non-toxic at higher pH values. This relationship between pH and VFA association/dissociation is described by the Henderson–Hasselbalch equation, which states that $pH = pK_a + \log_{10}[A^-]/[HA]$ (where pK_a is the acid dissociation constant, $[A^-]$ is the concentration of conjugate base and [HA] is the concentration of acid) (Po & Senozan 2001), and it is likely that this mechanism has some role in inhibiting methanogenesis at low pH.

The shift in fermentation pathways from acetate and butyrate production at high pH towards propionate production at low pH could at least in part be explained by the sensitivity of methanogens to rumen pH and an inability to survive at low pH values. On the other hand, this cannot fully account for the shift in fermentation end products, as in monocultures, where fermented starches and sugars result in high propionate levels and a reduction in pH will shift VFA production towards propionate, even in the absence of methanogens (Bannink *et al*., in press). This indicates that at least part of the effect is not related to methanogens. More starch, sugar or a lower pH will automatically shift the rumen bacterial population towards species like *Streptococcus bovis*, thus increasing propionate production. While pH has a strong influence, it seems that more H_2 will not go to propionate production unless there is enough substrate available (starch and sugars) to start with, allowing propionate producing bacteria to flourish.

Recently, Bannink *et al*. (in press) derived changes in stoichiometric coefficients of VFA produced from fermentation of starch and of soluble sugars at low pH that confirm the increase in propionate production at the expense of acetate and butyrate. The changes in propionate and butyrate production depended on the type of diet and the type of substrates involved (sugars or starch). In all cases, a strong decline in acetate at low pH explained much of the CH_4 reduction. When these coefficients were included in mechanistic models such as the Mills *et al*. (2001) model, a decrease of rumen pH indeed resulted in simulated reduction of CH_4 emission (Bannink *et al*. 2005). Incorporation of the effect of pH on rumen VFA stoichiometric coefficients would improve VFA prediction, which would in turn improve prediction of CH_4 production in the rumen.

Rumen pH is also a critical indicator of rumen function (Baldwin 1995). On either side of a narrow pH range (6·0–7·2), microbial activity appears to be affected sufficiently to produce adverse effects on nutrient recovery by the host animal, although, even in healthy animals, pH may temporarily drop lower during the day depending on ration and feeding pattern. What seems to be important is the amount of time spent at sub-optimal pH, rather than the lowest value reached (Alzahal *et al*. 2007*b*). A major issue of concern when incorporating rumen pH into a mechanistic model is accurate prediction of pH, without which, incorporation of the effects of pH change on the rumen microbiology cannot be realistically accomplished. The problem of predicting rumen pH can be overcome by input of observed

pH values into a model, although even that contains error due to incomplete mixing of the rumen, and measuring ruminal pH through direct sampling is invasive and not practical at the farm level. Also, the feed intake pattern, mixing of the ration and grazing *v.* stall-feeding would all become influential factors. Predicting rumen pH from VFA concentrations has been shown to be relatively unsatisfactory over a wide range of diets fed in practice (e.g. see Allen 1997) and, especially in rumen models that stimulate diurnal variation, representation of rumen pH requires further detail (Chilibroste *et al.*, in press). Alternatively, Alzahal *et al.* (2008) predicted rumen pH from measured rumen temperatures. Imamidoost & Cant (2005) presented an attempt at formulating equations to predict rumen pH entirely mechanistically according to the rate:state formalism (Thornley & France 2007), and monitoring ruminal pH in animals has been automated with the advent of continuous indwelling pH systems (Dado & Allen 1993). The future of incorporating pH effects into models for on farm application lies in developing a non-invasive, real-time method to monitor rumen pH (Alzahal *et al.* 2007*a*). As an option, predicting rumen pH from measured eructated sulphur gases (e.g. H_2S, dimethly sulphide (DMS)) appears to hold some promise, as rumen pH changes alter the balance between ionic and non-ionic forms of H_2S, and gaseous emissions differ accordingly (Elliott-Martin *et al.* 1997). It has been proposed that real-time monitoring of sulphur gas (e.g. H_2S, DMS) emissions and other eructated gases in ruminant breath will provide a mechanism to monitor rumen pH remotely using Blue Tooth communication technology (Warren *et al.* 2004; Alzahal *et al.* 2007*a*). An established relationship between the proportions of these eructated gases and pH could be used to construct an improved submodel of rumen pH that would be incorporated into the larger rumen model, thus significantly improving predictions.

SUPPLEMENTAL FAT

Fats are typically added to the diet of ruminants to increase energy concentration of the diet and meet the elevated energy requirements of high producing animals. Addition of dietary fat can also enhance milk yield and modify the FA composition of milk fat, partly related to the various FA intermediates formed in the rumen (Jenkins & McGuire 2006). Saturated long chain FA (LCFA) are of particular interest because of human health concerns over their contribution to the development of cardiovascular disease (Grundy 1990) and cancer (Parodi 1997). This area is of interest to ruminant nutritionists and nutritional modellers because fermentation in the rumen has a profound effect on the type of LCFA presented to the small intestine for absorption and subsequently the LCFA composition of milk and meat. The ability to model these processes could result in prediction of feeding strategies to produce healthier end products for human consumption.

The addition of FA to the diet, particularly those of medium (C12–C16) (Dohme *et al.* 2000; Odongo *et al.* 2007) and unsaturated long (>C16) (McGinn *et al.* 2004; Beauchemin & McGinn 2006) carbon chain length have also been shown to depress CH_4 production. These longer chain FA have the capacity to hold more H_2 atoms and thus may be more able to influence the H_2 balance in the rumen when large quantities are included in the diet compared to shorter chain FA. Giger-Reverdin *et al.* (2003) also showed that the degree of saturation of a FA is inversely proportional to its effectiveness in reducing CH_4 production, although there is some uncertainty about this relationship (Johnson & Johnson 1995). Unsaturated FA contain double bonds that can be replaced with single bonds with addition of H_2. As a result, the addition of unsaturated FA to the diet has been suggested as a mitigation strategy for reducing CH_4 emissions from ruminants (Boadi *et al.* 2004). Biohydrogenation of LCFA appears to compete with methanogens for H_2 as a substrate. A direct comparison of how biohydrogenation of FA compete with other H_2 sinks in the rumen in terms of thermodynamics, minimum H_2 partial pressure and K_m (as discussed in earlier sections) is not available. Biohydrogenation of FA, as a fraction of FA liberation after lipolysis, is usually quite high, well over 0·90 for normal diets (linoleic and linolenic acid are hydrogenated 0·75–0·95 and 0·85–1·00, respectively) (Doreau & Chilliard 1997; Harfoot & Hazlewood 1997). When the amount of unsaturated FA in the diet is elevated, however, the efficiency of biohydrogenation (proportion of free FA) starts to decline, and thus the rate of biohydrogenation is influenced by the level of fat in the diet (Beam *et al.* 2000). However, this begs the question that, if methanogenesis was more thermodynamically favourable than biohydrogenation, would these bacteria be able to reach such high biohydrogenation levels? It seems likely that biohydrogenation of FA is very favourable, either enzymatically or thermodynamically, and that addition of unsaturated fat to the diet results in biohydrogenation out-competing methanogenesis for H_2 use. The addition of fat would decrease the activity of fibrolytic bacteria, resulting in a shift in VFA production towards propionate, which would result in a decrease in CH_4 production. Thus, while competition for H_2 has an effect on CH_4 production directly, it is difficult experimentally to separate the effects of biohydrogenation from other factors such as a decrease in fibre degradation caused by fat supplementation. It is interesting that the rate of lipolysis and biohydrogenation also appear to be somewhat pH-dependent, as depression in lipolysis is observed

with high starch diets (Van Nevel & Demeyer 1996). This may make inhibition of fibre digestion the more likely pathway, at least during situations of low ruminal pH. Mechanistic modelling might be able to elucidate this.

It has also been shown that some fats and oils have a direct toxic effect on methanogens in pure and mixed cultures (Prins *et al.* 1972; Dong *et al.* 1997). The toxicity of FA to methanogens is positively related to the degree of unsaturation of the FA (Prins *et al.* 1972; Henderson 1973; Maczulak *et al.* 1981; National Research Council 2001). The addition of large amounts of fat to the diet (>0·05–0·06 of DMI) depresses fibre degradation in the rumen (Dong *et al.* 1997; Mathison *et al.* 1998), shifts the profile of VFA being produced from acetate to propionate (Nagaraja *et al.* 1997; Ungerfeld *et al.* 2005) and depresses overall DMI (Allen 2000). Long chain FA may specifically inhibit ruminal fibrololytic microbes (Maczulak *et al.* 1981) and added fats may also decrease protozoal numbers (Ungerfeld *et al.* 2005). A decline in butyrate production is observed with fat supplementation, which could be the result of decreased protozoal numbers and activity (Nagaraja *et al.* 1997). Decreased protozoal numbers may also have a role in decreasing CH_4 production, since methanogens are often found closely associated with protozoa, benefiting from their H_2 production. Death of protozoa is, however, not likely the principal cause of CH_4 reduction, because the reduction occurs in defaunated as well as faunated animals (Van Nevel & Demeyer 1996; Nagaraja *et al.* 1997).

Since LCFA also represent a non-fermentable substrate, increasing the proportion of LCFA in the diet may contribute to decreasing CH_4 as a proportion of GE intake (Johnson & Johnson 1995) by decreasing the amount of fermentable substrate as a proportion of the entire diet. A coating effect of fat on structural carbohydrates when fed at high levels may also contribute to decreased digestibility in the rumen with fat supplementation.

All of these changes would be responsible for a portion of the decrease in CH_4 production in the fat supplemented animal. The degree to which CH_4 production is decreased is likely to depend, however, on the type of fat fed, the amount, and the composition of the basal diet. Dohme *et al.* (2000) found with *in vitro* work, that CH_4 production was reduced by 34, 21 and 20 % with the addition of 53 g of palm kernel, coconut or canola oil, respectively. Palm kernel and coconut oil are high in medium chain saturated fatty acids, and canola oil is high in unsaturated longer chain fatty acids (see Odongo *et al.* (2007) for recent *in vivo* work). Machmuller & Kreuzer (1999) found that for coconut oil supplemented to sheep at levels of 0·03 and 0·07 of DM, CH_4 production was reduced by 28 and 73 %, respectively. This was attributed to direct inhibition of microbes, lower DMI and lower levels of digestible fibre in the diet. In a study by Dohme *et al.* (2004), lauric, myristic and stearic acid were added to the diet of Brown Swiss dairy cows fed a 3:2 forage to concentrates ratio. Compared to stearic acid, the addition of lauric acid decreased feed intake by 18 %, reduced NDF digestion by 7 % and reduced CH_4 loss (g CH_4 kg/milk) by 16 %. Myristic acid showed little difference to stearic acid, except a reduction in CH_4 of almost 8 %. Odongo *et al.* (2007) showed a 36 % reduction in CH_4 production with the addition of myristic acid to the diet. On the other hand, in a long term study Johnson *et al.* (2002) showed no effect of adding whole cottonseeds or ground canola oilseed at up to 0·056 of DM. It was suggested in the study by Johnson *et al.* (2002) that the level of added fat in the diet may not have been high enough to cause a reduction in CH_4 production, but it may also be due to the type of fat added and its availability in the rumen. It is worth noting that while FA supplementation definitely appears to reduce CH_4 production expressed in, e.g. MJ/d, it may not always substantially reduce CH_4 production expressed per kg of milk, meat or DMI. Lowering the digestibility of the diet would result in a lower milk yield per kg feed consumed, and thus a higher proportion of the ME intake is used for maintenance instead of production.

Despite the impact of dietary fats on rumen metabolism, its representation has received only limited attention in extant models of rumen fermentation. Lipid has not been included in the rumen models of, e.g. Baldwin *et al.* (1970), France *et al.* (1982) and Lescoat & Sauvant (1995). Other models have only a crude representation of ruminal lipid metabolism (Black *et al.* 1981; Baldwin *et al.* 1987; Danfaer 1990; Dijkstra *et al.* 1992; Dijkstra 1994; Dijkstra *et al.* 1996; Mills *et al.* 2001). The Dijkstra (1994) model currently employs a highly simplified representation of lipid metabolism in the rumen, representing the balance between lipid input from diet, outflow to the duodenum and incorporation into microbial lipid. The Mills *et al.* (2001) model has made some advance over this; however, it uses a fixed unsaturated FA part that has a fixed biohydrogenation and no toxic effect of individual FA on various microbial species. Dijkstra *et al.* (2000) made efforts to account for the negative effects of unsaturated FA on fibre degradation and on protozoa, and the depressing effects of high levels of unsaturated long chain FA on CH_4 production. Any of the models attempting to accurately predict H_2 balance and subsequently CH_4 production could benefit from the development or inclusion of an extended lipid sub-model. This would enable prediction of the effect the amount and composition of fat in the diet have on digestion, the nutrient profile available to the animal, as well as evaluate diets for their potential to reduce

absolute CH_4 emissions, emission per kg DMI, or per kg animal product obtained.

POSTRUMINAL DIGESTION/ FERMENTATION

Another area worthy of mentioning is CH_4 production in the hindgut, which is an important factor to consider when developing models of CH_4 production. The rumen does not account for all of the CH_4 produced by the animal and, while its contribution may be relatively small, the hindgut does produce and contribute to overall CH_4 production by the animal. Comparisons of CH_4 production measured using the sulphur hexafluoride tracer gas technique (SF6) (measures only eructated CH_4) to whole animal calorimetry show differences that range from −5–10% (Johnson *et al.* 1994*a*, 1994*b*; McGinn *et al.* 2006; Pinares-Patiño *et al.* 2008), to +1–2% (Boadi *et al.* 2002; Grainger *et al.* 2007). To account for this discrepancy, Basarab *et al.* (2005) adopted an upward adjustment of 3% to all SF6 measurements of CH_4 production to account for hindgut methanogenesis. Other studies report greater discrepancies between SF6 and calorimetry measurements of CH_4 production. Wright *et al.* (2004) found that SF6 CH_4 estimates were consistently more variable, as well as higher by 2–3 times than respiration chamber estimates. In Wright *et al.* (2004), there was no significant correlation between the SF6 methane estimates and the respiration methane estimates ($R^2 = 0{\cdot}11$). It is evident that caution should be used when comparing and combining different types of measurements.

Mills *et al.* (2001) developed a framework for a postruminal digestion/fermentation model. The postruminal model is based on the same structure as the rumen model, with modifications for fractional digestion and passage rates, fluid volume, pH parameters and exclusion of the activity of protozoa, and it aims to account for the extra CH_4 produced by the animal not accounted for in the rumen model. Inputs to the large intestine sub-model are essentially the outflows from the rumen model modified for small intestine digestion. Digesta passage is assumed to be that of a system of complete mixing, however, in reality it is a combination of plug flow and mixing (France *et al.* 1993). While many simplifications are made, it provides the structure upon which modifications can be made to improve representation of hindgut digestion and metabolism further. The simulated contribution of hindgut methanogenesis to total CH_4 production on a range of dairy cattle diets was $0{\cdot}091 \pm 0{\cdot}026$ (Mills *et al.* 2001). Accounting for postruminal digestion may improve estimates of overall CH_4 production by the model when predictions are to be compared against observed data that represent production by the entire animal and not just the rumen. Volatile fatty acid stoichiometry within the hindgut will need to be addressed with the same considerations as those discussed for the rumen. In addition, the balance and competition between H_2 utilizing and H_2 producing micro-organisms may differ in the hindgut compared to the rumen and should be considered. Inclusion of a hind-gut model in the paper of Mills *et al.* (2001) likely contributed to lower RMSPE values and correction of some of the under-estimation of CH_4 production by the model, although it is evident it could still benefit from further expansion.

CONCLUSIONS

The current review has attempted to address aspects of rumen microbiology that are relevant to dynamic models of rumen digestion and gaseous nutrient emissions, particularly those attempting to describe H_2 balance and CH_4 production by the animal. The rumen H_2 sub-models evaluated at the start of this review paper over- or under-predicted CH_4 production to varying degrees depending on the rumen models used, the H_2 sub-model, as well as the database used for evaluation. It is likely that their errors result from combinations of factors discussed in this review that are not fully accounted for in the models, as well as some factors that are not discussed here. Accurate prediction of VFA stoichiometry is likely to have the largest impact on a model of CH_4 prediction, as the VFA profile being produced in the rumen is central to any model of H_2 balance. In addition, many of the topics discussed in this paper will influence the VFA profile produced. However, other factors also appear important. For example, over-estimation of CH_4 production could arise from not accounting for competition for H_2 as a substrate with animals fed specific diets such as those containing maize co-products or high levels of supplemental fat. Under-prediction of CH_4 production could result from not considering alternative electron carriers, such as formate, or not considering hindgut methanogenesis. Consideration of the close interaction of methanogens with protozoa is important, particularly when considering mitigation strategies that involve defaunation. All of these areas represent areas of rumen microbiology relevant to modelling rumen function. Incorporation of these concepts into current models of rumen function should advance knowledge of the complexities of rumen metabolism, including those issues relevant to CH_4 production.

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