



## **Review: Reducing enteric methane emissions improves energy metabolism in livestock: is the tenet right?**

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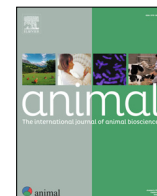
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### Review: Reducing enteric methane emissions improves energy metabolism in livestock: is the tenet right?

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#### ABSTRACT

The production of enteric methane in the gastrointestinal tract of livestock is considered as an energy loss in the equations for estimating energy metabolism in feeding systems. Therefore, the spared energy resulting from specific inhibition of methane emissions should be re-equilibrated with other factors of the equation. And, it is commonly assumed that net energy from feeds increases, thus benefitting production functions, particularly in ruminants due to the important production of methane in the rumen. Notwithstanding, we confirm in this work that inhibition of emissions in ruminants does not transpose into consistent improvements in production. Theoretical calculations of energy flows using experimental data show that the expected improvement in net energy for production is small and difficult to detect under the prevailing, moderate inhibition of methane production ( $\approx 25\%$ ) obtained using feed additives inhibiting methanogenesis. Importantly, the calculation of energy partitioning using canonical models might not be adequate when methanogenesis is inhibited. There is a lack of information on various parameters that play a role in energy partitioning and that may be affected under provoked abatement of methane. The formula used to calculate heat production based on respiratory exchanges should be validated when methanogenesis is inhibited. Also, a better understanding is needed of the effects of inhibition on fermentation products, fermentation heat, and microbial biomass. Inhibition induces the accumulation of  $H_2$ , the main substrate used to produce methane, that has no energetic value for the host, and it is not extensively used by the majority of rumen microbes. Currently, the fate of this excess of  $H_2$  and its consequences on the microbiota and the host are not well known. All this additional information will provide a better account of energy transactions in ruminants when enteric methanogenesis is inhibited. Based on the available information, it is concluded that the claim that enteric methane inhibition will translate into more feed-efficient animals is not warranted.

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#### Implications

The greenhouse gas methane originating from livestock production accounts for a third of anthropogenic methane emissions. Enteric methane from ruminants accounts for the majority of these emissions associated with livestock. Methane is produced during the fermentation of feeds and represents a loss of energy. To accelerate the adoption of mitigation strategies by farmers, it is often highlighted that decreasing methane should improve production. In light of the present knowledge, this argument is not valid and should not be used to reinforce the narrative of sustainably farmed

ruminants. This fact should be considered by decision makers in policy programmes.

#### Introduction

Livestock has a pivotal importance in human nutrition and in sustaining the economy of rural communities, particularly in low- and middle-income countries where it also contributes to food and nutrition security. Direct and indirect jobs from the sector provide livelihood for more than 1.3 billion people (Herrero et al., 2013). Although on a global scale livestock accounts for less than 2% of the gross world product, it contributes a much larger share of the gross domestic product in low-income countries (FAO, 2020). Food products from terrestrial animals provide 29% of the

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daily intake of protein to the world population, but there are large disparities between countries ranging from 49 to 13% on average for high- and low-income countries, respectively (OCDE, 2022). It is noted that animal proteins are rich in essential amino acids and micronutrients like cobalamin (vitamin B12), riboflavin (vitamin B2), retinol (vitamin A), calcium, zinc, and iron that are often deficient in malnourished people (Randolph et al., 2007). The nutritional and economic role of livestock in human societies is associated; however, with a large negative impact on the planet as livestock production uses ~30% of the terrestrial surface for grazing and feed production, consumes a sizable amount of available fresh water, and contributes to global warming through the emissions of greenhouse gases (Steinfeld et al., 2006).

Reduction of emissions of greenhouse gases from agriculture is necessary to achieve climate change mitigation targets agreed by the United Nations Framework Convention on Climate Change. Livestock contributes 14.5% of the global anthropogenic greenhouse gas emissions with enteric methane being the largest single source representing 40% of emissions from livestock (Gerber et al., 2013). This enteric methane comes mostly from ruminants, particularly from cattle due to the number of heads and body size compared to other ruminant species. Methane efficiently traps radiation with a global warming potential of 28-fold greater than CO<sub>2</sub> in a 100-year horizon (GWP<sub>100</sub>) (IPCC AR6, 2022) or 32-fold, according to a revised calculation of radiative forcing (Etmann et al., 2016). As methane is a short-lived atmospheric pollutant with a perturbation lifetime of ~12 years, reducing emissions into the atmosphere can have a positive impact on climate in the medium term, whether using GWP<sub>100</sub> or any other greenhouse gas metrics that can represent better the impact of short-lived climate pollutants (GWP<sub>20</sub> or GWP\* (Lynch et al., 2020)).

Since the 2000s, research on the production of methane in the gastrointestinal tract (GIT) of ruminants, along with strategies to decrease emissions, has steadily increased (Beauchemin et al., 2020), and today, there exist mitigation options that are effective and applicable to different production systems (Arndt et al., 2022). However, the global rate of adoption of those options by producers is lower than 10%, as estimated by Herrero et al. (2016). One reason for why mitigation strategies for reducing enteric methane production are not more adopted is the absence of co-benefits that can compensate the extra cost and management constraints associated with methane mitigation options. An expected co-benefit of reducing methane is to 'recover the energy lost as methane' for productive functions of the host animal. This claim is repeatedly mentioned in scientific papers and the popular press even though it has been shown that it is not a consistent result (Ungerfeld, 2018). Here, we focus on the energetics of methane production, and if and how methane mitigation can be coupled to energetic flows in the ruminant holobiont (the host and its associated microbiota, particularly in the gastrointestinal tract). We identify knowledge gaps and discuss prospective areas of research.

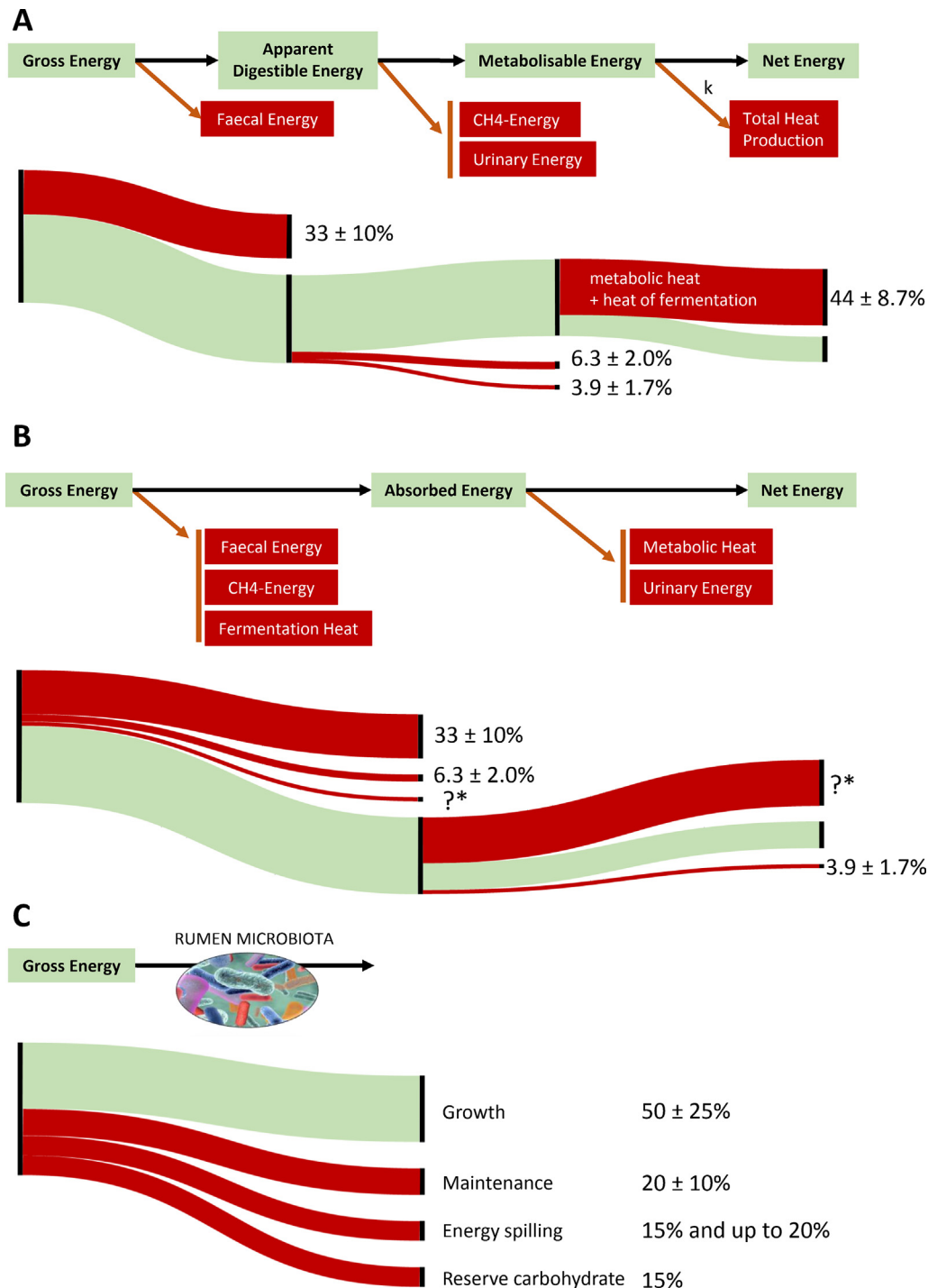
### Enteric methane and energy metabolism in the Holobiont

The production of enteric methane and its relation to the energy utilisation in ruminants has long been known (e.g., Krogh & Schmit-Jensen, 1920; Kriss, 1930). The connection between feed intake and methane emissions was already shown by Kriss (1930); interestingly, the objective of the work reported by Kriss was to solve ventilation problems in cattle barns. The energy eliminated as gas, particularly methane, from the apparently digestible portion of feeds is an important factor (the other one being energy losses in urine) for the estimation of metabolisable energy. Blaxter and Clapperton (1965) in their influential paper collated the first

database of measurements of methane released by animals using close-circuit respiratory chambers, and established relationships between the apparent digestibility of feeds, amount of intake and methane production in cattle and sheep.

The gross energy content of feed, determined by adiabatic calorimetry, is to some extent progressively lost as feeds are fermented and digested, and nutrients metabolised before being assimilated into animal products. Energy losses from feed to animal products are (by order of importance for an average diet) total heat production, faeces, methane, and urine. Traditionally, different steps of energy transactions have been defined (Fig. 1A). Apparent digestible energy is the difference between gross energy inputs ( $18.4 \pm 0.75$  MJ/kg DM, mean  $\pm$  SD from the database used in Inra (2018) that gathers measurements from 1 316 treatments of ruminants in respiratory chambers) and faecal energy output ( $33.3 \pm 10\%$  of gross energy inputs). Subsequent losses in methane ( $6.3 \pm 2.0\%$  of gross energy inputs) and urinary energy ( $3.9 \pm 1.7\%$  of gross energy inputs) result in metabolisable energy ( $56.4 \pm 10\%$  of gross energy inputs). It is noted that all these are average values but lower or higher losses are reported in the literature, i.e. the often-cited Johnson and Johnson (1995) for methane. Metabolisable energy is then transformed into net energy according to a coefficient 'k' of efficiency of energy use, accounting for adenosine triphosphate (ATP) production and use. This coefficient varies with both the nature of diets (i.e. metabolisability or 'q' = metabolisable energy/gross energy) and animal function (i.e. maintenance, milk production, protein and fat deposition). The conversion of metabolisable into net energy is accompanied by energy losses, called energy expenditure or total heat production ( $78.9 \pm 19.5\%$  of metabolisable energy). The latter includes the fasting heat production (basal metabolic rate), the heat increment for maintenance [i.e. energy lost from "food given to a fasting animal in amounts that do not result in a deposition of fat in the body, sparing the oxidation of the tissues of the animal" (Blaxter, 1962a) and heat increment for production. From a nutritional standpoint, the animal energy requirements for maintenance and production are defined based on digestible, metabolisable or net energy depending on the feeding systems, but most of the current feeding systems for ruminants express requirements on a net energy basis (INRA (Inra, 2018), NorFor (Volden, 2011), NASEM (2021)). In the context of this review, it should be stressed that the total heat production that is determined at the whole animal level in respiratory chambers and calculated according to Brouwer (1965) includes the heat of fermentation that arises from microbial fermentation of feeds in the GIT (Blaxter, 1989) besides metabolic heat produced by animal tissues. Total heat production thus originates from the whole holobiont: the microbiome and the animal tissues.

A more physiological approach to energy transactions defines the concept of absorbed energy (Fig. 1B) which is equal to the energy of the summed metabolisable nutrients (Loncke et al., 2011; Ortigues-Marty et al., 2019). Absorbed energy derives from gross energy after accounting for energy losses from methane, fermentation and faeces. There is scarce data on fermentation heat in animals as it is difficult to measure and in the physiological approach, it is considered to be quantitatively similar to urinary energy (Blaxter, 1962b; Loncke et al., 2011). Absorbed energy is close but not similar to metabolisable energy (Fig. 1B). Absorbed energy is transformed into net energy following the metabolism of nutrients in the animal tissues and losses in urine and as metabolic heat. Quantifying fermentation heat, independently of metabolic heat, would be a step forward for a more physiological definition of energy transactions in the holobiont that may help detecting changes in rumen fermentations that can be of nutritional relevance.



**Fig. 1.** Energy flows in ruminants and in the rumen. (A) Traditional approach (source: INRAE, 2018), (B) Physiological approach (source: Ortigues-Marty et al., 2019) and (C) microbial partitioning in the rumen (source: Hackmann & Firkins, 2015). Energy losses are indicated in red in the diagrams. The Sankey flow diagrams depict medium values (average and reported variations when known are reported as percentage of gross energy intake). k = coefficient of energy; \*there are no separate measurement values for metabolic heat and heat of fermentation. The model assumes an energy loss through heat of fermentation similar to losses in urine.

### Inhibition of enteric methanogenesis and animal productivity

Dietary management and supplements are used for decreasing methane emissions. Some of the most effective dietary strategies to decrease rumen methane production can improve animal productivity, but this effect is mostly due to improved nutrition and cannot be directly ascribed to the inhibition of methanogenesis. In the recent meta-analysis by Arndt et al. (2022), dietary strategies that improve animal production decrease methane intensity

(methane per unit of animal product) but not absolute emissions. The decrease in methane intensity when production increases due to a greater availability of metabolisable nutrients is commonly explained by the dilution in the requirements for animal maintenance (Capper et al., 2009).

In studies in which enteric methane production is decreased through the use of different strategies, often various other changes are observed in digestion, metabolism animal production. When deciding which type of studies are adequate for answering the

question of whether or not decreasing methane emissions improves the energy efficiency of the animal, one should consider whether the observed alterations in physiology and metabolism result themselves from the inhibition of methanogenesis or, on the other hand, are co-occurring changes. Changes in diet can affect methane production but at the same time, many other aspects of digestion and metabolism unrelated to methane. Similarly, selecting for low-methane-producing sheep results in animals with smaller rumens and higher passage rates; however, these digestion changes are not a consequence of the animal producing less methane (arguably, lower methane production is in this case the result of such changes in digestive physiology). On the other hand, changes in rumen fermentation and postabsorptive metabolism resulting from specific chemical inhibitors of methanogenesis that are included in small amounts in the diets, so that dietary composition is largely unchanged, are more adequate to answer the question posed. Nutritional strategies to decrease methane production are not further discussed herein because the relationship between decreases in methane production and productivity is confounded by other factors such as intake, digestibility and rumen pH. In contrast, strategies that decrease absolute emissions did not have any positive effect on production (Arndt et al., 2022).

A previous meta-analysis found that strategies aiming at specifically inhibiting rumen methanogenesis did not result in consistent benefits in animal productivity in terms of milk production or growth (Ungerfeld, 2018). We updated that work by adding recent studies that used 3-nitrooxypropanol or the red algae *Asparagopsis* spp. as feed additives. Bromoform contained in *Asparagopsis* and 3-nitrooxypropanol are specific inhibitors of methanogenesis that have shown consistent methane mitigation effects in animal studies (Almeida et al., 2021; Hegarty et al., 2021). A previous study used a 50% methane decrease threshold in at least one treatment to select experiments for meta-regressions analysing biological responses to the inhibition of rumen methanogenesis (Ungerfeld et al., 2022). Here, we opted for a somewhat less stringent threshold of 30% of methane production decrease in at least one experimental treatment, because fewer dairy production studies were available at greater levels of inhibition.

Nine studies and 11 experiments including treatments resulting in 30% or more methanogenesis inhibition, with a total of 34 treatment means, reported responses in body mass gain (BMG), whereas six studies comprising each one experiment with 30% or more methanogenesis inhibition in at least one treatment, with a total of 16 treatment means, reported responses in energy-corrected milk (ECM). Dependent variables BMG and ECM were regressed against the random effect of the experiment, the linear and quadratic effects of methane production, the random interaction between the experiment and methane production, and DM intake as an adjusting co-variable (Kronmal, 1993). Experimental treatment means were weighted by the reciprocal of their SE scaled to unity (St-Pierre, 2001; Sauvant et al., 2008). Non-significant quadratic terms and interactions were removed, and the reduced models were re-fitted.

Inhibiting rumen methanogenesis increased growth only numerically ( $-9.39$  g BMG/mol  $\text{CH}_4$ ;  $P = 0.11$ ; Fig. 2). If eight experiments for which SEs of their treatment means were not available were added to the data set and the regression was rerun without weighting treatment means, a negative relationship between  $\text{CH}_4$  production adjusted by DMI and BMG was obtained ( $-19.8$  g BMG/mol  $\text{CH}_4$ ;  $P < 0.001$ ; not shown); thus, even though a consistent relationship between the inhibition of  $\text{CH}_4$  production and growth efficiency cannot be declared at the conventionally accepted Type I error rate of 0.05, the possibility exists that experiments using more animals and greater power might be able to detect such an association (see next section). On the other hand,

there was no relationship between inhibiting rumen methanogenesis by at least 30% and ECM ( $-6.18$  g/mol  $\text{CH}_4$ ;  $P = 0.91$ ; Fig. 3). These results corroborate the absence of a consistent correlation between productivity and inhibition of methanogenesis.

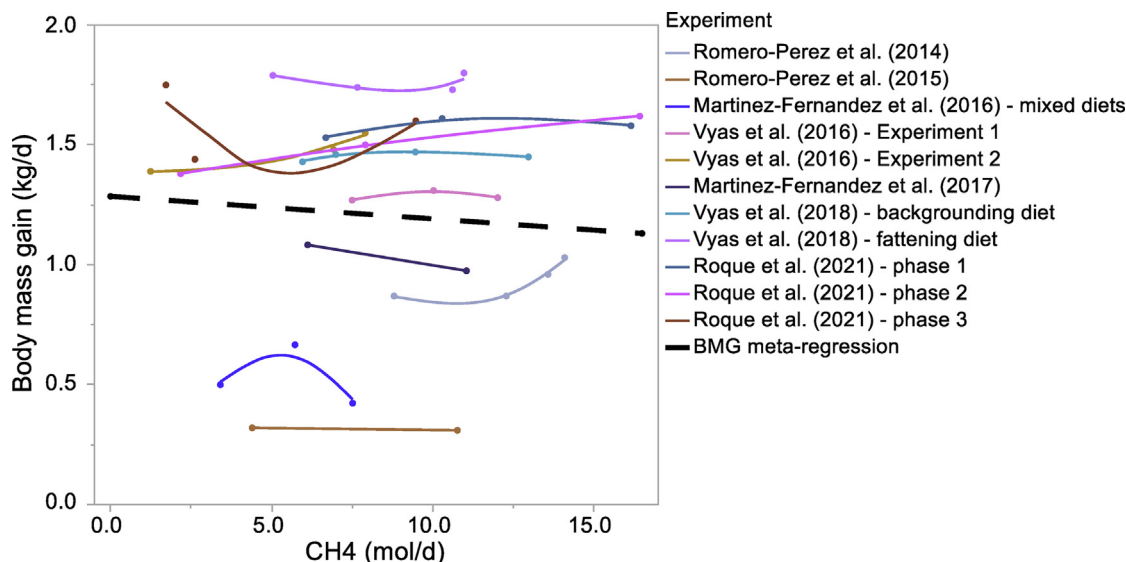
### Energy spared from less methane production: where does it go, and can it be redirected?

From a nutrition perspective, disappointingly, there is no indication that when methanogenesis is inhibited, feed energy is readily re-distributed and used for growth or production (see above; Ungerfeld, 2018). In contrast, from a microbial ecosystem perspective, it is often assumed that inhibiting methanogenesis and the resulting accumulation of  $\text{H}_2$  generally observed would inhibit re-oxidation of microbial NADH and negatively affect key metabolic processes in the animal, reducing the efficiency of feed utilisation (Wolin, 1981). However, similarly to the nutrition paradigm, this negative effect is not observed in vivo; most animal studies do not show detrimental effects on key parameters related to rumen function such as fermentation or digestibility (e.g. Knight et al., 2011; Mitsumori et al., 2012; Kinley et al., 2020; Yu et al., 2021). Meta-analyses do not show relationships between the inhibition of rumen methanogenesis and VFA concentration adjusted by the commonly observed decrease in DMI; effects of inhibiting methanogenesis in vivo on actual flows of VFA production, rather than concentration, have not been reported. Thermodynamic estimations do not suggest that maximal dissolved  $\text{H}_2$  accumulation observed in individual experiments could inhibit re-oxidation of NADH (Ungerfeld et al., 2022). Likewise, the addition of external  $\text{H}_2$  to in vitro systems has not consistently resulted in decreased fermentation (Ungerfeld, 2020). This is positive news, even though it is contrary to the general principle of the inhibitory effect of high  $\text{H}_2$  pressure on individual microbial cells (Wolin, 1981; Hegarty & Gerdes, 1999). Because of this lack of concordance between expected and observed results, it should be acknowledged that the microbial processes involving energy flows are not completely understood in the ruminant holobiont. A better understanding of the fate of feed energy when methanogenesis is inhibited is important for identifying potential levers for improving efficiency.

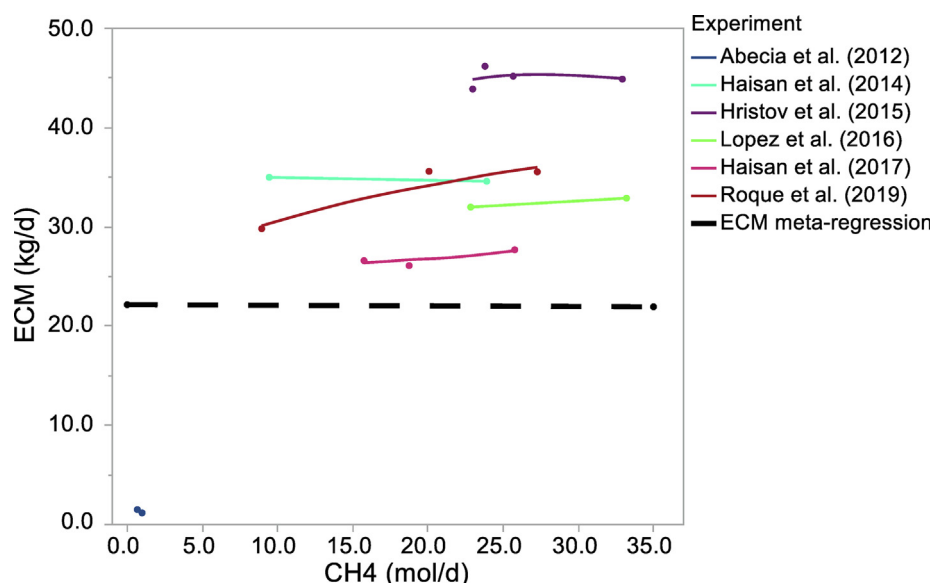
From a theoretical point of view, a reduction in enteric methane emissions, everything else being equal, should impact the energy balance of animals. However, two main reasons may explain why this theoretical gain is not consistently observed. First, the reduction in methane emissions might be quantitatively too small to elicit measurable differences in animal production traits. Indeed, based on simulations using the INRA feeding system (Table 1), a theoretical reduction of 25% of methane emissions in two contrasting production conditions in beef cattle (diets and animals described in Bes et al. (2022), Guarnido-Lopez et al. (2022)) would promote an increase of around 10% in the average daily gain of fattening young bulls, everything else remaining similar. Given the inter-individual variability in average daily gain, this effect size of 10% would only be significantly detectable with a relatively large number of animals (a minimum of 16 individuals per treatment considering that the between-animal variability in average daily gain is close to 10% CV). The other possible reason is that changes in methane emissions are often associated with changes in organic matter digestibility and thus in apparent digestible energy supply.

The theoretical gain in milk production allowed by methane mitigation may also be estimated. We used as an example a study where enteric methane emissions in lactating dairy cows decreased by 132 g/d ( $-31\%$ ) when treated with 3-nitrooxypropanol (Saro et al., 2019). In this study, there was a concomitant decrease in DMI ( $-6.5\%$ ), an effect also observed in other studies (Yu et al., 2021), and no effect on milk production





**Fig. 2.** Relationship between methane ( $\text{CH}_4$ ) production and body mass gain (BMG) in ruminants adjusted by DM intake and the random effect of the experiment in 11 experiments (N treatments = 34) in which rumen methanogenesis was inhibited with chemical compounds by 30% or more in at least one treatment. Fitted meta-regression of BMG against  $\text{CH}_4$  production is shown as a dotted line, with the experiment effect not being considered. For Martinez-Fernandez and Denman et al. (2016), performance results were available for their mixed diet only (Gonzalo Martinez-Fernandez, pers. com.). We meta-regressed BMG against  $\text{CH}_4$  production adjusted by DMI as a co-variable (rather than regressing the ratio of BMG and DMI against unadjusted  $\text{CH}_4$ ; see Kronmal (1993)):  $\text{BMG} = \text{intercept} + \text{experiment}(\text{random}) + \text{DMI intake} + \text{CH}_4 + \text{CH}_4^2 + \text{experiment} \times \text{CH}_4(\text{random}) + \text{error}$ .  $\text{BMG} = 0.20 (\pm 0.33; P = 0.55) + \text{experiment}(\text{random}; P = 0.032) + 0.12 (\pm 0.038; P = 0.004) \text{ DMI} - 0.0094 (\pm 0.0058; P = 0.11) \text{ CH}_4$ . Treatment means were weighted by the reciprocal of their SE adjusted to unity.



**Fig. 3.** Relationship between methane ( $\text{CH}_4$ ) production and energy-corrected milk (ECM) production in dairy cows adjusted by DM intake and the random effect of the experiment in six experiments (N treatments = 16) in which rumen methanogenesis was inhibited with chemical compounds by 30% or more in at least one treatment. Fitted meta-regression of ECM against  $\text{CH}_4$  production is shown as a dotted line. Similar to the body mass weight model presented in Fig. 2, the initial model for ECM was:  $\text{ECM} = \text{intercept} + \text{experiment}(\text{random}) + \text{DMI intake} + \text{CH}_4 + \text{CH}_4^2 + \text{experiment} \times \text{CH}_4(\text{random}) + \text{error}$ .  $\text{ECM} = 15.9 (\pm 4.48; P = 0.007) + \text{experiment}(\text{random}; P = 0.15) + 0.69 (\pm 0.17; P = 0.002) \text{ DMI intake} - 0.0062 (\pm 0.053; P = 0.91) \text{ CH}_4$ . Treatment means were weighted by the reciprocal of their SE adjusted to unity.

(Supplementary Table S1). To keep the comparison between groups similar as for the example on beef production above, we assume in this example that 3-NOP does not modify intake and digestibility, metabolisable energy intake would have increased by 5.86 MJ/d as a result of the reduced methane production. This would have induced an increase in energy produced as milk (3.26 MJ/d, i.e. 1 kg energy-corrected milk), the remaining energy being stored in body reserves (0.63 MJ/d) and lost as heat (1.93 MJ/d) (Inra, 2018). This theoretical increase in milk production would not have been detected in this study that had 14 cows

per treatment as it is similar to the residual error on milk yield observed (SEM = 1.1 kg/d). In addition, the theoretical increase due to decreased methane emissions might be overestimated in the calculation above; according to the meta-analysis of Daniel et al. (2016) an increase in metabolisable energy intake of 5.86 MJ/d, similar to our example, results in an average increase in milk yield of 0.6 kg/d.

These simulations show that moderate (25–30%) inhibition of methane production can, at best, induce modest changes in production that cannot be detected unless a large number of animals

**Table 1**

Simulation of changes in growth performance when methane emissions are reduced by 25% in fattening bulls fed either a grass- or a corn silage-based diet at early or late fattening stage, respectively. All energy conversion coefficients are kept identical within diet for comparison purposes.

Items	Grass silage diet 350 kg BW fattening bulls <sup>1</sup>		Corn silage diet 650 kg BW fattening bulls <sup>1</sup>	
	Reference scenario without inhibition of methanogenesis	-%25 CH <sub>4</sub>	Reference scenario without inhibition of methanogenesis	-%25 CH <sub>4</sub>
DM intake <sup>2</sup> , kg/d	7.04	7.04	10.7	10.7
Gross energy intake, Mcal/d	126.39	126.39	197.53	197.53
Faecal energy <sup>3</sup> , Mcal/d	43.11	43.11	54.82	54.82
Digestible energy intake <sup>4</sup> , Mcal/d	83.28	83.28	142.71	142.71
CH <sub>4</sub> emission <sup>3</sup> , Mcal/d	8.12	6.11	14.73	11.05
Urinary energy <sup>3</sup> , Mcal/d	4.52	4.52	7.62	7.62
Metabolisable energy intake <sup>5</sup> , Mcal/d	70.73	72.82	120.53	123.88
Heat production <sup>3</sup> , Mcal/d				
Total	62.78	63.61	100.44	101.70
Fasting	34.53	34.53	55.24	55.24
Increment for maintenance	16.74	16.74	25.86	25.86
Increment for growth	11.51	12.51	19.33	20.80
Net energy in growth <sup>6</sup> , Mcal/d	8.04	8.87	20.26	22.14
Protein gain <sup>7</sup> , g/d	170	192	174	190
Lipid gain <sup>7</sup> , g/d	102	115	414	452
Average daily gain <sup>8</sup> , g/d	975	1 075 (+10%)	1 386	1 514 (+9%)

<sup>1</sup> Simulated diets corresponded to those used in Guarnido-Lopez et al. (2022) and Bes et al. (2022).

<sup>2</sup> DM and gross energy intakes were estimated by Inration V5 software (<https://app.inration-ruminal.fr/>) from dietary characteristics reported in Guarnido-Lopez et al. (2022) and Bes et al. (2022) and animal characteristic (breed, sex and age).

<sup>3</sup> Energy losses from feed to gain estimated by Inration V5 software (<https://app.inration-ruminal.fr/>) from dietary characteristics reported in Guarnido-Lopez et al. (2022) and Bes et al. (2022) and animal characteristic (breed, sex and age).

<sup>4</sup> Digestible energy intake calculated as gross energy intake minus faecal energy output.

<sup>5</sup> Metabolisable energy intake calculated as digestible energy intake minus energy in CH<sub>4</sub> emissions and energy in urinary excretion.

<sup>6</sup> Net energy in growth calculated as metabolisable energy intake minus total heat production, the latter including fasting heat production and heat increments for maintenance and growth (all coefficients of metabolisable energy use are identical in both the reference scenario and when emissions are reduced by 25%).

<sup>7</sup> Protein and lipid gain estimated by Inration V5 software (<https://app.inration-ruminal.fr/>) from metabolisable energy intake and animal characteristics (breed, sex and age).

<sup>8</sup> Assuming that muscle contains around 20% of proteins and adipose tissue has 80% of lipids.

are used. This is assuming that the energy not accounted as methane is conserved and can be used by the animal. An assumption that has yet to be proven.

## How is methane produced

Structural carbohydrates, proteins and other feed components from the diet are hydrolysed to simpler monomer components and then fermented by rumen microbes to volatile fatty acids (VFAs), CO<sub>2</sub> and H<sub>2</sub>. Methanogenic archaea (methanogens) use H<sub>2</sub>, CO<sub>2</sub> and to some extent other intermediate products of fermentation (see below) as substrates for producing methane that is their sole mechanism to obtain energy for growth. The energy obtained from feed is used by microbes for biomass and heat production (heat of fermentation), with fermentation intermediates and end products becoming less complex and with less energy value with each fermentation reaction. There are transfers of metabolites from microbial producers to consumers, with methanogens, which are able to grow by using reduced molecules (H<sub>2</sub> and other electron carriers) produced by other rumen microbes, found at the bottom of this microbial GIT trophic chain (Morgavi et al., 2010; Mizrahi et al., 2021). Methanogens thus occupy a special niche in the ecosystem as the vast majority of members of the microbiota cannot grow on these substrates. Also, the physicochemical conditions in the GIT of ruminants, particularly in the rumen in terms of temperature, pH, rate of passage and substrates (feeds) favour the utilisation of H<sub>2</sub> for the production of methane by methanogenic archaea.

The functions of different microbial members and their relationships including with methanogens have been reviewed by others (e.g. Saengkerdsut & Ricke, 2014; Huws et al., 2018; Mizrahi et al., 2021) and that is not the aim of this review. Instead, we highlight some particularities of methanogens and methanogenesis that influence methane production in the rumen

and potentially the relationship with energy conservation. Methanogenesis can proceed from the reduction of CO<sub>2</sub> using H<sub>2</sub> as the electron donor, from methyl-group reduction and from acetate (Liu & Whitman, 2008). Of these three known pathways, the hydrogenotrophic reduction of CO<sub>2</sub> predominates in the rumen whereas production from acetate via the acetoclastic pathway is minor (Morgavi et al., 2010). In contrast, the methylotrophic pathway once thought minor is of considerable importance in the rumen (Poulsen et al., 2013). Functionally important methyl-reducing methanogens in the rumen are obligate hydrogenotrophs that belong to different lineages: Methanobacteriales *Methanosphaera stadtmanae*, Methanosarcinales *Methanimicrococcus blatticola*, and the more recently described Methanomassiliicoccales. All of them exhibit adaptations appropriate to the GIT environment where methyl compounds and hydrogen are readily available (Li et al., 2016; Thomas et al., 2021; 2022). Hydrogenotrophic methyl reduction uses a single H<sub>2</sub> molecule to produce one molecule of CH<sub>4</sub> as opposed to four for reducing CO<sub>2</sub>, resulting in a threshold of dissolved H<sub>2</sub> (<0.1 Pa) that is up to 100 folds lower than hydrogenotrophs CO<sub>2</sub>-reducers (Feldewert et al., 2020). The availability of methyl substrates may limit the activity of methylotrophic methanogens rather than H<sub>2</sub> (Feldewert et al., 2020). In the rumen, H<sub>2</sub> concentration varies depending on diet and feeding time (Janssen, 2010). The variation in the rumen environment associated with feed intake throughout the day could alternatively favour the thermodynamics of energy production between CO<sub>2</sub>-reducing and methyl-reducing methanogens and may be a reason for why they are both present in the rumen.

## Electron flows in the gastrointestinal tract ecosystem

Microbes in the GIT harvest energy from feeds through the transfer of electrons from oxidised to reduced compounds. In the

anaerobic rumen ecosystem, fermentation releases metabolites such as VFAs that are absorbed and used by the host animal whereas the production of  $H_2$  and other reactions transferring metabolic H are essential for the oxidation of reduced cofactors. These processes take place inside cells and also by interacting microbial guilds that release or incorporate metabolic hydrogen, thus having different ecological niches in the ecosystem. In methanogenic ecosystems such as the rumen,  $H_2$  and formate produced by bacteria, protozoa and fungi are the main electron shuttles (Janssen, 2010). Methane at the other end of the spectrum is the main electron sink. Methanogens utilise a relatively small number of simple substrates to produce methane, with methanogenesis being their only sole source of ATP for growth (Thauer et al., 2008); thus, methanogens need other microbes for the supply of  $H_2$  and  $CO_2$ , the main substrates for methanogenesis in the rumen.

Dihydrogen is central in the flow of electrons, and its production and utilisation are linked to methane production in the rumen (Janssen, 2010; Greening et al., 2019; Leahy et al., 2022). The concentration of  $H_2$  in the rumen also affects the production of VFA and the microbiota (Janssen, 2010; Xie et al., 2022). It is estimated that most (50–80%) of the  $H_2$  dissolved in rumen contents is converted to methane, 20–30% is used in the production of propionate and the minor VFA valerate –which are the only two VFA requiring net incorporation of metabolic hydrogen in their production pathways– and the remaining is incorporated in the microbial biomass (Czerkawski, 1986; Mills et al., 2001). These general calculations and derived models are useful to estimate methane emissions from ruminants under uniform fermentation conditions (Mills, 2008) but presently cannot entirely explain the fate of metabolic hydrogen when methanogenesis is inhibited. Dihydrogen produced inside cells diffuses through the cell cytoplasmic membrane, and its diffusion rate is negatively correlated to the  $H_2$  concentration in rumen fluid. This maintains an equilibrated gradient between the cell and its environment (Guyader, 2015). The concentration of dissolved  $H_2$  in the rumen liquid was assumed to be at equilibrium with the gaseous  $H_2$  concentrations, but Wang et al. (2016) showed that  $H_2$  in the liquid phase is supersaturated. A consequence is that, except for those microbes relying on hydrogenotrophy, greater  $H_2$  concentration in the liquid decreases the thermodynamic feasibility of microbial metabolic reactions for obtaining energy (less favourable Gibbs energy changes) in the rumen (Wang et al., 2016). Although important, adjusted calculations using this greater concentration of dissolved  $H_2$  lessen only marginally the difference between expected and observed energy balance. Notwithstanding, obtaining information on dissolved  $H_2$  concentrations under various dietary conditions and throughout the day will improve model predictions (Ungerfeld, 2020).

In the rumen, the energy contained in feeds is partly transformed into ATP that is partly used for growth and maintenance functions. Mixed rumen microbes use about 1/3–2/3 of the energy obtained as ATP for microbial growth, whereas the rest is used for maintenance and other functions (Hackmann & Firkins, 2015) (Fig. 1C). Methanogens represent only 1–3% of the microbial biomass in the rumen, and therefore, their contribution to microbial protein available to the host animal is minor. In the same way, their role in energy losses due to maintenance, energy spilling and the synthesis of storage carbohydrates (Hackmann & Firkins, 2015) would be thought to be minor (see below).

#### Energy spilling, storage of energy and maintenance in methanogens

In essence, energy-spilling mechanisms cause microbial cells to spend ATP in non-growth functions, rather than preventing ATP generation. In that regard, chemical inhibitors of methane production that act through inhibiting the methanogenesis pathway itself (e.g., methane halogenated analogues, 3-nitrooxypropanol, or 2-

bromoethanesulfonate) prevent methane formation in the methanogen cells, and therefore prevent the generation of electrochemical gradients resulting in ATP generation. In principle, compounds with this type of action would therefore not be expected to induce energy spilling in methanogens. In contrast, inhibitors might have an indirect action as energy spilling has been shown in methanogens when excess  $H_2$ /formate is present in the environment (Costa et al., 2013). This is the situation when methanogenesis is inhibited and the growth of methanogens is retarded (Janssen, 2010). It was proposed that a futile cycle consuming ATP or membrane potential is used for competing with other  $H_2$ /formate utilisers or for improved syntrophic associations with other microbial partners (Costa et al., 2013).

There is scarce information on the type and amount of energy reserves in rumen methanogens. Methanogens in general contain enzymes for the production of polyphosphate, whereas glycogen can also be produced by some genera (Wang et al., 2019). Polyphosphate might have a role in methanogens adaptation to stresses from the environment in addition to its role as energy reserve and P source (Orell et al., 2012).

Methanogens are autotrophs that use end products of fermentation with little energetic value for other microbes upstream in the trophic chain. Methanogens collect only 0.5–2 moles of ATP per mole methane with most, up to 99% of the  $H_2$ ,  $CO_2$ , and other substrates used being released back as methane with little substrate retained for growth (Buan, 2018). This implies that a proportionally small number of rumen methanogens produce a sizable amount of methane gas that is emitted into the atmosphere by each ruminant.

#### Is energy conserved when methane is not produced?

We refer here to the inhibition of methanogenesis caused by specific inhibitors that directly target methanogens but do not directly affect other rumen microorganisms when tested in pure cultures. It is recognised thought that specific inhibition may have an indirect effect on the rumen microbiome via modifications of electron flow. Inhibition of rumen methanogenesis through the use of chemical inhibitors does not entail direct changes in the provision of nutrients to the animal or to rumen microbes via diet as the dietary content of nutrients is unaltered when chemical inhibitors of methanogenesis are used. Thus, any changes in the holobiont, other than less methane production, are indirect effects of the inhibition of rumen methanogenesis, and cannot be ascribed to diet changes.

A common effect of inhibiting methanogenesis is the increase in  $H_2$  emissions (Czerkawski, 1986; Ungerfeld et al., 2022), a gas that, similarly to methane, is not metabolised by mammalian cells although it has been shown to have a positive effect in humans and animal models of disease (Xie et al., 2022). It is tempting to hypothesise that energy losses as  $H_2$  simply replace energy losses as methane but this is not supported by meta-analyses that found that losses are non-linear and variable, and in general moderate. For example, at 80% methanogenesis inhibition with respect to control treatments, energy losses in  $H_2$  expelled were on average equal to 11% [CI95 = 4.5, 17%] of the energy saved from methane not formed (Ungerfeld, 2018; Ungerfeld et al., 2022). More studies with measurements of  $H_2$  are necessary to better assess the factors underlying the variation in  $H_2$  emissions caused by inhibiting rumen methanogenesis. It is noted though that methods reported in the literature for quantifying  $H_2$  emissions in ruminants have different sensitivities and accuracies (van Lingen et al., 2019a).

Production of  $H_2$  is a common characteristic of rumen microbes that is regulated by substrate availability as well as physicochemical and thermodynamic conditions (Janssen, 2010; Greening et al.,



2019). In the rumen, there are hydrogenotrophic microbes other than methanogens (Jeyanathan et al., 2014; Greening et al., 2019) that can potentially fill the niche left by less active methanogens. Notwithstanding,  $H_2$  emissions have remained relatively high in long-term studies of methanogenesis inhibition that have provided time for adaptation and the development of other hydrogenotrophic microbial populations (Ungerfeld, 2020).

The same as methane,  $H_2$  in the rumen headspace is mostly eliminated through eructation but the rate and potential amount of  $H_2$  both dissolved and in the headspace that is absorbed from the rumen and other parts of the GIT of ruminants is not known. This information is necessary for a better account of energy transactions. In human subjects, up to 65% of produced  $H_2$  in the colon is absorbed and eliminated in breath (Christl et al., 1992). Note that the use of  $H_2$  in water or inhaled, modified the metabolome and intestinal microbiota of healthy rats (Xie et al., 2022). In the study of Xie et al., a decrease in DMI was also reported. Interestingly, methanogenesis inhibitors reduce voluntary intake in ruminants (Ungerfeld, 2018; Kim et al., 2020). Some difficulties associated with the measurement of  $H_2$  should also be considered. Measurement of dissolved  $H_2$  cannot capture the microenvironments present in biofilms and aggregated microbial consortia so important for the interspecies transfer (Stams & Plugge, 2009). Specialised equipment should be used and dedicated studies should be performed to obtain this information within the rumen environment (e.g., microscale measurement of hydrogen using microelectrodes Ebert & Brune, 1997; Cai et al., 2020). Also,  $H_2$  is a small molecule that can penetrate and diffuse out of materials used for storage and measurements. Experimental procedures should minimise this potential technical bias.

As stated above, the heat produced by biological processes is the largest energy loss in ruminants. About half of the total heat production in sheep comes from the metabolism of nutrients absorbed from the rumen (Torrent & Johnson, 1996) and it has been shown that diets that promote greater production of propionate decrease the temperature in the rumen and rectum of ruminants (Cho et al., 2014) but there is no information on whether inhibition of methanogenesis affects microbial heat production. For microbial metabolism, specific calorimetric studies similar to those of Hackmann et al. (2013) for quantifying the effect of excess carbohydrate should be performed. For heat production at the whole animal level, the data available are not extensive and not conclusive with reported increases and decreases in heat production when methane was inhibited by different additives (van Zijderveld et al., 2010; Shinkai et al., 2012). In his meta-analysis, Ungerfeld (2018) summarised eight existing studies, including 21 treatments, reporting the effects of inhibiting rumen methanogenesis on ruminant heat production, finding no evidence that inhibiting methane production in the rumen altered animal heat production. It is noted that heat production in those studies was estimated based on respiratory gas exchange, i.e. indirect calorimetry, and constant values (Brouwer, 1965). The Brouwer equation includes the production of methane but it remains to be verified whether it is valid when methane is specifically inhibited. The equation also includes adjustments for  $H_2$  production but its use is limited in the literature (Reynolds, 2000) and there is the caveat of the correct measurement mentioned above. Ideally, direct calorimetry should be used to validate that estimates made from respirometric indirect calorimetry are correct (Kaiyala & Ramsay, 2011) when methanogenesis is inhibited.

Microbial biomass is more reduced than the substrate and might be an additional electron sink for unaccounted metabolic hydrogen when methanogenesis is inhibited (Ungerfeld, 2015). Inhibiting rumen methanogenesis in vitro has resulted in increased production of microbial protein in some (Ungerfeld et al., 2007; Guo et al., 2009) but not all (Ungerfeld et al., 2019) studies, with variable results in others (Guyader et al., 2017). Redirection of

metabolic H towards the synthesis of microbial amino acids when methanogenesis is inhibited may depend on the availability of energy and carbon (Ungerfeld et al., 2020).

#### *Electron sinks – the case of nitrate*

Rumen microbes can convert nitrate to ammonia. If nitrate is given to animals as a feed supplement, the conversion into ammonia outcompetes methanogenesis because of the more favourable thermodynamic conditions. Nitrate, although not a methanogenesis inhibitor, is considered because, when used as prescribed, it does not change the nutritional composition of the diet. Nitrate can also be used to replace urea, a non-protein nitrogen compound, in diets deficient in degradable nitrogen such as those based on corn silage, sugar cane, sugar beet, molasses or cassava (Leng, 2008). Nitrate effectively reduces methane emissions by 15% or more without altering the health of the animal, and ammonia, the final product of nitrate conversion, is used by microbes to produce proteins utilisable by the host ruminant. Yet, there is no concomitant improvement in animal performance (Lee & Beauchemin, 2014; Guyader et al., 2015; Arndt et al., 2022). A possible reason is that the control diets used in comparative studies were isonitrogenous and did not limit the amount of rumen nitrogen for microbial protein synthesis (van Zijderveld et al., 2011). On the other hand, the supplementation of nitrate to a nitrogen-deficient diet improved animal performance, digestibility, and VFA concentration, although it also increased total methane production due to increased DMI and digestibility (Nguyen et al., 2016). Feeding nitrates also induces increased dissolved  $H_2$  concentration in the rumen fluid (Guyader, 2015) and gaseous losses as  $H_2$  (van Zijderveld et al., 2011; Lee & Beauchemin, 2014) which explain some of the energy loss and contrast with the assumed mode of action of nitrate. The theoretical effect of nitrate as an electron sink for  $H_2$  is calculated stoichiometrically, and thus,  $H_2$  emissions should not increase. Electron capturing is probably the most important mode of action for decreasing methane production, but the pharmacokinetics and biological activity of nitrate and nitrite also play a role that has not been fully quantified (Guyader et al., 2016a). Nitrate in the rumen is quickly converted to nitrite where it accumulates as conversion to ammonia is slower (Wang et al., 1961; Iwamoto et al., 1999). Nitrate and nitrite are rapidly absorbed in the rumen, abomasum and intestines and although a proportion of nitrate can re-enter the GIT and be reduced to ammonia by microbial activity, up to 40% of nitrate and nitrite accumulate in muscle and skins (Villar et al., 2021) although at levels that do not pose a risk for consumers (Doreau et al., 2018). Also, nitrate can be converted in mammalian cells through the nitrate-nitrite-nitric oxide pathway to form nitric oxide and other beneficial bioactive nitrogen oxides (Lundberg et al., 2008) that is another route of the utilisation of absorbed nitrate. On the other hand, nitrite is toxic to methanogens at low doses (Iwamoto et al., 2002). This dual mode of action (electron competition with methanogenesis and direct toxicity of nitrite to methanogens) explains why nitrate supplementation causes the accumulation of  $H_2$ . Also, there is an unavoidable increase in methaemoglobin in ruminants fed nitrate that, although below what is considered clinically toxic (Lee & Beauchemin, 2014; Guyader et al., 2016b) may still affect energy metabolism.

#### *Closing knowledge gaps and further roads for exploration*

Mathematical modelling is a way to fill knowledge gaps about the fate of hydrogen, methane and VFA at normal and methane inhibition conditions. For that purpose, rumen models should be improved to consider major aspects such as thermodynamic control on fermentation fluxes and enhanced description of the rumen

**Table 2**

Recommended information required for a better account of energy transactions and dihydrogen flows when enteric methanogenesis is inhibited in ruminants.

Knowledge gap
<ul style="list-style-type: none"> <li>Independent quantification of fermentation heat and metabolic heat for detecting changes in rumen fermentations that can be of nutritional relevance</li> <li>Validation by direct calorimetry of the estimates made by respirometric indirect calorimetry</li> <li>Assess possible changes in energy partitioning in the rumen that may affect microbial production of nutrients for the host animal, e.g. volatile fatty acids and proteins</li> <li>Improved rumen models incorporating thermodynamics and microbial information for estimating production and flows of dihydrogen and volatile fatty acids</li> <li>Fate and possible biological role of absorbed dihydrogen</li> </ul>

microbiota. Thermodynamic control operates dynamically depending on the actual rumen thermodynamic conditions (e.g. partial H<sub>2</sub> pressure) and drives the fermentation profile. Recent model developments have incorporated thermodynamic regulation by NADH/NAD control (van Lingen et al., 2019b) and H<sub>2</sub> control (Muñoz-Tamayo et al., 2021) on the rumen fermentation pattern. These models have been applied to study methanogenesis inhibition by 3-nitrooxypropanol and nitrate *in vivo* (van Lingen et al., 2021) and by *Asparagopsis taxiformis* *in vitro* (Muñoz-Tamayo et al., 2021). Incorporating thermodynamic control and enhancing the representation of the rumen microbiota in rumen models will help to better predict and describe VFA formation and hydrogen and energy balance, in particular under methanogenesis inhibition conditions. Indeed, when methane is inhibited, the observed excess of H<sub>2</sub> does not account for the decrease in methane production (Kelly et al., 2022). Recovery of metabolic hydrogen has been observed to decrease when methanogenesis was inhibited under *in vitro* batch and continuous conditions (Ungerfeld, 2015), indicating that mathematical models should account for other H<sub>2</sub> sinks. Another critical aspect that hampers the calculation of hydrogen recovery *in vivo* is the fact that most of the studies report data on VFA concentration on a single sample point. The VFA concentration at a given time is the result of the balance between production, absorption and passage rates. Single time points are insufficient to determine production rates and thus to calculate an accurate mass balance. It is common to perform quantitative evaluations of rumen responses assuming that the system operates at steady-state (Kass et al., 2022). However, the steady-state assumption underestimates the fluctuating nature of the rumen environment. Indeed, using a theoretical modelling exercise (Muñoz-Tamayo et al., 2019), we have shown that the ecology of rumen methanogens is shaped not only by the specific microbial kinetic properties of the methanogens but also by non-linear behaviours related to spatial and temporal variations taking place in the rumen. Models and experiments can complement each other to better characterise the rumen system dynamics and thus deepen our understanding of rumen function. This can be done by using for example optimal experiment design techniques allowing to define experimental conditions and sampling times with high informative content for the characterisation of key processes such as VFA production and methanogenesis inhibition rates. The knowledge gained on rumen temporal dynamics can be used further to inform on the nature and timing of interventions to reduce methane emissions (Wang et al., 2015).

Despite the absence of consistent positive effects on production, changes in plasma and milk metabolites that are associated with a better energy balance have been observed in lactating dairy cows fed a specific methane inhibitor as compared to a control group (Yanibada et al., 2020; 2021). These changes although subtle were overall congruent, showing in treated cows increased concentration of energetic compounds and amino acids, decreased concentration of NEFA and alterations in metabolites pointing to low lipolysis status (Yanibada et al., 2020). Similar results were reported for naturally low-emission beef cattle (Kim et al., 2022). The extent and importance of these changes on the long-term energetic metabolism of ruminants when enteric methane is inhibited

merits to be further explored. Other aspects that require more investigation are the feasibility to increase the use of H<sub>2</sub> from non-methanogens that could be achieved by the use of hydrogen acceptors (prebiotics) (Martinez-Fernandez et al., 2017), direct-fed microbials (Jeyanathan et al., 2014) or both. Also, dairy cows and sheep harbouring microbiomes with greater capacity to obtain energy from feeds (Shabat et al., 2016; Patil et al., 2018) could bring new insight on the relationship between methane production and energy metabolism under various feeding conditions.

### Final remarks

Decreasing enteric methane production in ruminants does not result in an improved energy balance as the potential gains in net energy that could be used for production are theoretically and empirically of low biological significance with the inhibition rates of 20–30% commonly reported in the literature. We have identified knowledge gaps (Table 2) in both the calculation of energy flows at the animal scale that could improve the accountability of energy transactions when methanogenesis is inhibited and also, at the microbial scale, the production, pharmacokinetics and utilisation of H<sub>2</sub> and other fermentation products that can affect microbial and animal functions.

### Supplementary material

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Not applicable.

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No data generated for this review article.

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All authors contributed to the conceptualisation of the work, writing sections, reviewing, editing the original draft and approval of the final version.

## Declaration of interest

The authors declare that they have no competing interests.

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