Early life interventions and possible mechanisms affecting methane emissions from ruminants. A summary of key findings from the literature.

There was a party inside; closure of the bloat shed in about 1977; a lot of digesta had been exchanged over a few decades

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KEY FINDINGS

- Early life intervention has been advocated as an approach to influence the assembly of the rumen microbiome, especially in the context of methane mitigation. To date there has been limited evidence of success or understanding of the underlying biology. Several research groups have active programmes in this area.
- Individual ruminants do differ in their methane yield (g/kg dry matter intake) and this is heritable.
    - The extent of the difference between individuals is diet-dependent.
- The causes of individual variation are poorly defined but include non-microbial characteristics, such as mastication and digesta residence time/rate of passage as affected by rumen capacity and intake.
    - These characteristics may affect the likelihood of a successful intervention, but have not been studied.
- Studies in ruminants have shown that microbial interventions in adults do not persist, except for the introduction of *Synergistes jonesii* which detoxifies mimosine from *Leucaena*.
- Studies in ruminants and other species suggest intervention is best undertaken in early life.
    - However, optimal procedures are unknown, because the host/microbiome relationship is poorly understood.
- One of approximately 8 trials (in 5 publications) investigating early-life interventions has demonstrated a defensible reduction in methane yield following the cessation of treatment (see Table 1).
    - Dietary change, including provision of lipids, and inoculation with rumen digesta were largely unsuccessful, post-treatment.
- Only when dams and progeny were both treated (with bromochloromethane; BCM) did the reduction in methane yield persist beyond the treatment period. However long-term persistence in methane yield was not measured.
    - BCM is not an appropriate chemical to use and we suggest 3-NOP (3-nitrooxypropanol) be evaluated because it has a similar mode of action.
    - The reduction in methane emissions needs to be monitored over the animal's life and in their progeny.
- The reason that treatment of dam and progeny was successful, whereas treatment of only the progeny was not, is unknown.
    - A suggestion is that it is essential to 'overwhelm' the environment to lessen the chances of inoculation from external sources.
- A successful mitigation must be accompanied by ongoing research to understand the mechanisms of action and to ensure animal health and production is not compromised.

We should realise that attempts to alter evolution in a couple of decades may not be easy.

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INTRODUCTION

Although the focus of this report concerns methane, information is also presented concerning attempts to alter the rumen or gastro-intestinal microbiome for other purposes. This is because of any information dealing a manipulation of the microbiome will provide an insight into the methodology and the likelihood of success for mitigating methane. An early example is the extensive research concerning the aetiology of legume bloat (which is affected by the rumen microflora) which showed relationships between the host animals' physiology, the microflora and susceptibility to bloat (Clarke and Reid, 1974), which has a heritability of 0.19±0.04 (Morris et al., 1997).

The possibility of manipulating the rumen fermentation has long attracted interest from microbiologists and animal scientists, but has proved to be a challenging prospect. Although there is a general similarity of bacteria and especially methanogens across all ruminant animals (Henderson et al., 2015), the rumen contains a diverse population of microorganisms described as being both redundant and resilient (Weimer, 2015). Redundant in that there is considerable overlap of functions between different microbes (Seshadri et al., 2018), and resilient in that the microbial population is relatively resistant to disruption. These characteristics help ensure that the rumen provides a stable microbial environment but as a result attempts to alter rumen functions such as improving fibre degradation or reducing methane production have had limited success at least in adult animals. Consequently, several authors have proposed manipulation of the rumen microbiome in early life as a strategy to program rumen function towards particular goals (Yáñez-Ruiz et al., 2015, Bickhart and Weimer, 2018), because this is the most sensitive window for interventions to have a long term effect, although analyses often lack the predictive power necessary to associate particular microbial profiles with rumen metabolic activity. Understanding of the bases of the host/microbial specificity and the mechanisms for microbiome establishment in the new born may provide opportunities to influence methanogenesis and feed utilisation efficiency.

Gut development after birth

New born ruminants have a physically (Figure 1) and metabolically underdeveloped rumen (fore-stomach) which means they are naturally dependent on milk during their early stages of life, and gradually transition and adapt to solid feed during which time the rumen develops (Khan et al., 2016). The gastro-intestinal tract changes anatomically as the animal ages, with the rumen accounting for an increasing portion of the gastrointestinal tract (GIT), but the relative size of the rumen is also dependent upon consumption of roughages (Thivend et al., 1980). Structural characteristics of the organ may be important when either sampling or inoculating young ruminants. The changes in structure (growth and papillae development), enabling microbial colonisation, fermentation and absorption of metabolites has been described in detail by Yáñez-Ruiz et al., 2015. Strategies used to facilitate rapid rumen development in calves have recently been reviewed (Diao et al., 2019).

Microbial colonisation

Microbial communities in the GIT of young animals are essential for physiological and anatomical development, and for the digestion of fibrous material. The ruminant gastrointestinal tract is usually assumed to be sterile before birth although the presence of a diverse low-abundance microbiota in the newborn rectal meconium and mucosa has been reported (Alipour et al., 2018). After birth the GI tract is then rapidly colonized (Bryant et al., 1958, Fonty et al., 1987) via transmission of microbes from the mother and from the surrounding environment. Colonization is reported to occur sequentially (Li et al., 2012, Jami et al., 2013, Rey et al., 2014, Dill-McFarland et al., 2017, O'Hara et al., 2020) but strict anaerobes characteristic of the mature rumen are already present 1-2 days after birth.

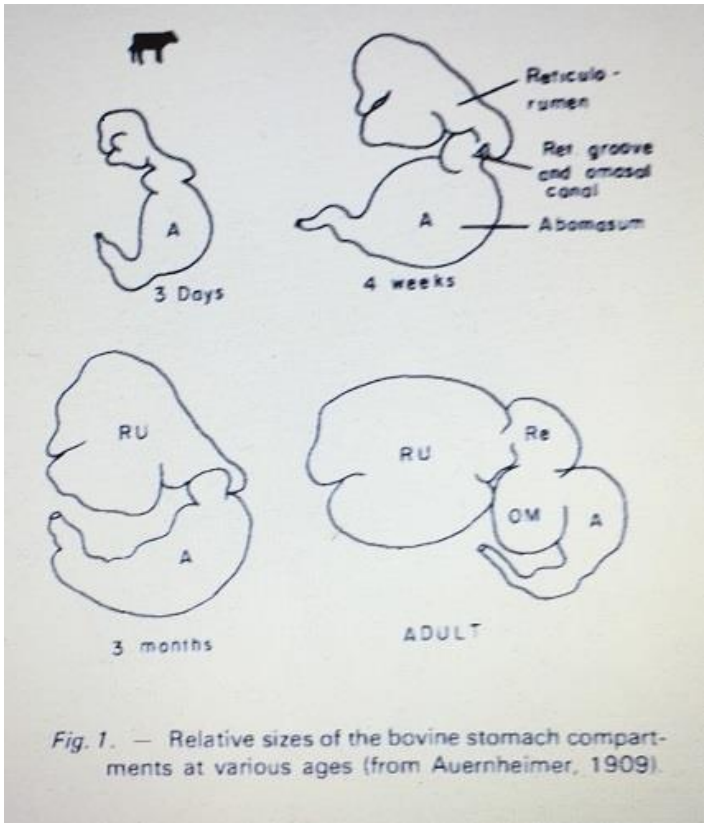

Fig. 1. — Relative sizes of the bovine stomach compartments at various ages (from Auernheimer, 1909).

Figure 1 illustrates the very considerable change in the size of the structures comprising the ruminant stomach. At birth the vestigial reticulorumen has not developed papillae, that are characteristic of a ruminant consuming solid feed, which may impact upon the presence vs. colonisation of microflora.

This example is based on a calf from birth to adult and was taken from Ruckebusch et al., 1983

Early colonizers include facultative anaerobes, such as *Escherichia coli*, from the bacterial phylum Proteobacteria and bifidobacteria adapted to growth on milk oligosaccharides. While members of the genus *Bifidobacterium* can be readily cultured from young ruminant sources (Kelly et al., 2016), knowledge of their apparent abundance in the microbiome of the young ruminant has been limited as they are not detected by the commonly used primers. From weaning the bacterial population becomes more like that of the mature rumen and is dominated by members of the phyla Bacteroidetes and Firmicutes.

Methanogenic archaea also colonize the GI tract soon after birth (Fonty et al., 1987; Morvan et al., 1994) and reach concentrations equivalent to those found in adult animals by three weeks. Several studies have assessed the methanogen population found in young animals (Skillman et al., 2004, Guzman et al., 2015, Friedman et al., 2017, Li et al., 2019), and members of the family Methanobacteriales (*Methanobrevibacter*, *Methanosphaera* and

Methanobacterium) generally predominate, although other methanogens including members of the family Methanomassiliicoccales have also been detected (Li et al., 2019).

Of the 501 microbial strains whose genomes were sequenced in the Hungate1000 collection, only 24 (7 from the rumen including one methanogen, and 17 from faeces) are recorded as isolates from young animals. Several bacteria isolated from the rumen of young animals (*Kandleria vitulina*, *Sharpea azabuensis*, *Streptococcus equinus* and *Ruminococcus flavefaciens*) have comparable gene complements to strains of the same species from adult animals. However, little is currently known of the microbial-host interactions that occur during a ruminants' early life and whether these interactions persist or influence the lifetime performance and health of the animal. The microbial community is affected by both the composition and intake of solid food (e.g. Dill-McFarland et al., 2019) and the mechanisms associated with initial and ongoing colonisation must be elucidated to enable their manipulation.

RUMEN MICROBIOME ANALYSIS.

Microbiomes are defined as assemblages of interacting microorganisms and are now recognised as being crucial to the functioning of ecosystems. The symbiotic relationship between ruminants and a complex microbiome consisting of bacteria, archaea, fungi, protozoa, and viruses enables the animal host to use the lignocellulose component of plant material as an energy source. The resulting microbial fermentation produces short-chain fatty acids that are used by the animal for growth, while the microbial cells become the main source of amino acids and protein. In recent years microbiome analysis has become commonplace, with major progress in sequencing approaches coupled with the development of sophisticated statistical analysis pipelines focussing on the composition of the microbiome, its metabolic capability, the key metabolites produced, and host-microbe interactions.

There have been numerous published studies describing the rumen microbiome and reporting differences observed under various conditions. A variety of DNA isolation methods and sequencing approaches have been used. However, DNA-based determination of microbial composition from 16S rRNA relative abundance, and function from metagenome analysis is insufficient and does not necessarily paint a true picture of the functionally active microbial population. The upshot of this is that considerable caution is needed in interpreting these studies.

The structure and function of microbiomes depends on which members of the community are alive or dead. While there is no information for the rumen, studies of the human gut (Ben-Amor et al., 2005, Bellali et al., 2019) indicate that live cells constitute 49-62% of the bacterial population, with dead (26-35%) and injured cells (10-19%) also present. Therefore, some organisms characterised as abundant from metagenome data may be dead or inactive and consequently show little gene expression. As demonstrated by Shi et al., (2014) the organisms actually responsible for important microbiome functions, such as methane production, are only identifiable when samples are analysed at the transcriptional level. It has

been suggested that large-scale sequence-based studies of microbial communities should include viability assays, but at present this is technologically difficult (Emerson et al., 2017).

Genome sequencing of microbial isolates (Seshadri et al., 2018), complemented recently by the assembly of microbial genomes from metagenomics data (MAGS, Stewart et al., 2019) means that there is now good coverage of the bacteria and archaea found in the rumen. However, there is still little evidence for which microbes are performing particular roles within the rumen (Bickhart and Weimer, 2018). Recent work has identified the bacteria most likely to produce the hydrogen (Greening et al., 2019) and methyl compounds (Kelly et al., 2019) used as substrates for methanogenesis. The availability of characterised pure cultures enable experimental investigation of the rumen microbiota, validation of hypotheses made with sequence-based analyses, and phenotypic characterisation of its constituent microbes. Nevertheless, identification of key microorganisms is only the beginning and if rumen microbiome research is to move from the descriptive to the intervention stage well designed culture-based and multi-omic approaches will be required to effect the transition from statistical correlations to causal relationships (Denman et al., 2018, Neville et al., 2018, Newbold and Ramos-Morales, 2020).

OPTIONS FOR INTERVENTION AND MODE OF ACTION IN EARLY-LIFE.

Manipulating rumen fermentation, for lowering incidence of bloat, increasing efficiency of feed utilisation and recently, the mitigation of methane, has long attracted interest from microbiologists and animal scientists. However, this has proved to be challenging and changes have usually been short-term. Microbiome science has enabled extensive investigations of this concept with regard to the human gut microbiota and its role in regulating multiple aspects of human growth, nutrition and health (Robertson et al., 2019). The main conclusion from this body or work is that the early life period is the critical time to focus on mechanistic research and interventions.

Individual animals have an association with their microbiome, but neither the development (from birth) nor the processes responsible for maintaining this relationship, are understood. Important factors that will affect the outcome of manipulation will include characteristics of the gastro-intestinal tract anatomy, its function (e.g. digesta residence time), as well as saliva (a source of immune factors), and all are complicated by challenges in determining the active microbial population. DNA analyses quantify populations (dead or alive), distinct from activity, and it is essential this be understood. Understanding host-microbiota interactions during early life growth will help future targeted interventions.

Furthermore, many compounds are able to reduce methanogenesis but these must be given with feed and are impractical under New Zealand farming systems. Successful mitigants must not be hazardous, but be persistent, and animal health, efficiency and productivity must not be compromised.

Several trials involving early life intervention are summarised in Table 1; these are inevitably complicated and this report includes a range of examples and explanations to aid interpretation.

Diet

Changing diet from colostrum and milk to solid feed is associated with the anatomical (Figure 1) and morphological changes to the ruminant stomach. Morphological changes include development of papillae and capacity for absorption and tissue metabolism of volatile fatty acids (VFA). In addition, salivary secretion increases and motility is essential for eructation, mixing and controlled passage to the omasum and beyond (Wyburn, 1980; Waghorn and Reid, 1983).

Factors that influence establishment of microbes include proximity to other ruminants, diet, bedding soil and water (Nagaraja, 2016), but the relationships between these factors and the microflora are not straightforward. For example, cellulolytic bacteria and methanogens are present in the rumen 3 days after birth, and fungi less than a week later (Nagaraja 2016), despite a total absence of ingested solids. Only the establishment of ciliated protozoa in the young calf requires some form of contact with faunated ruminants, and calves that were isolated from mature ruminants usually remain defaunated.

Early colonisation was also reported by Dias et al. (2017), who compared calves given either milk or milk + calf starter concentrate, from day 8 after birth. Whilst the concentrate promoted diverse bacterial taxa (able to utilise concentrate), the calves raised on milk showed a diminished diversity as they aged (to 63 days). Dias et al (2017) concluded that both diet and age simultaneously drive changes in the structure and abundance of bacterial communities in the developing rumen, and diet was the primary driver of the methanogens. Although anaerobic fungi were not affected by either diet or calf age, there is good evidence to support manipulation of the rumen microbiota by dietary intervention before weaning.

This conclusion was supported by Dill-McFarland et al. (2019) who fed pre-weaned calves either a concentrate-based calf starter or maize silage, or a 50:50 mixture of the two (composition ranged 7-23% crude protein and 11-39% NDF). The diet did have significant effects on total microbial communities at weaning (8 weeks), but much of the difference had disappeared by 1 year of age. Nevertheless, there remained some calf-diet-driven differences in the microbiota as adults, but these dissimilarities did not correlate with growth or milk production. Methane was not reported.

Although the findings Dill-McFarland et al. (2019) and others support those of Dias et al. (2017), the post-weaning factors may have a greater influence on communities in the adult, and lessen differences evident in young stock. The Global Rumen Census project (Henderson et al., 2015) showed that while a core microbiome of bacteria and archaea dominated in nearly all samples, differences in microbial communities were mainly diet-related. Dietary approaches to modulate rumen methane production, have been reviewed (Stanton et al., 2019, Beauchemin et al., 2020).

Table 1. Summary of early life dietary interventions in sheep (and a single goat trial‡) on CH_4 emissions, rumen and other parameters during and after the intervention. Derived from Debruyne, 2019; more details in Appendix 1 and the text regarding BCM treatment.

Treatment	Dose	Duration of treatment	Effects on CH_4 & rumen fermentation	Effects on other parameters during and after treatment	Ref.
Diet Hay vs. 40:60 hay:concentrate	Ad lib	Lamb: birth until 16 weeks; wean @ 8 weeks	@16 wk: **CH_4 yield** ↓14%; No effect 4 mo after end of treat. Similar fermentation pattern.	Rumen bacterial & methanogen communities differ @16 wk. Rumen bacterial differ 4 mo after end of dietary treat.	1
BCM Bromochloromethane ‡**Goats**	Doe and kid 3 mg/kg BW	Doe: first 2 mo after birth. Kid: birth to 3 mo	**CH_4 yield**↓ 52% - 59% in treated kids at weaning and 3 mo after treatment ↓28% when Doe and Kid were given BCM; 15% ↑daily gain. Altered A/P ratios.	Modified methanogen communities at weaning (does & kids), some differences @ 4 mo after weaning	2, 3, 4
Lipid Coconut oil (CO) vs. rumen-protected fat (RPF) vs. RPF + Rumen inoculum from CO or RPF donors	4% of DMI. lamb inoculation (20 or 40 mL)	Ewe: 1 mo pre-lambing until weaning. Lamb: birth to 3 mo; inoc. weekly until 8 wk	Lambs: **CH_4**↓ 9%, CO diet; Lambs and ewes: no effect on VFA production or pH. Minor effects of inoculation (high dose; CO donors).	CO ↓DMI and protozoa in ewes and lambs. Lipids had a greater effect on the bacterial community than inoculation with rumen fluid from donor ewes on different diets	5, 6
Linseed oil	Ewe: 1.7 g/kg BW Lamb: 1.2 g/kg BW	Ewe: until weaning Lamb: birth until weaning or until 16 wk	**CH_4** not measured. 16 wk; no effect on fermentation in lambs	Linseed oil affected rumen bacterial and methanogen communities; methanogen did not persist	7
linseed oil + garlic oil	1.6 mL/kg BW* 3 µL/kg BW°	Lamb: birth until 10 wk of age, and from 16 until 20 wk (re treatment)	At 10 wk; **CH_4**↓23% (µmol/mL); no diff @ 14 wks @ 20 wks CH_4↓14%. At 20 wks ↓A/P	No effect on archaeal and protozoal communities; minor effects on bacterial community	8

References: **1**. Yáñez-Ruiz et al., 2010; **2**. Abecia et al., 2013; **3**. Abecia et al., 2014; **4**. Abecia et al., 2012; **5**. De Barbieri et al., 2015 b; **6**. De Barbieri et al., 2015 a; **7**. Lyons et al., 2017; **8**. Saro et al., 2018.

Research summarised in the Thesis of Debruyne (2019) aimed to reduce methane emissions in ruminants by feeding supplements supposedly able to reduce methane, in early life, and having the effects persisting to the adult. The supplements included long-chain poly-unsaturated fatty acids (extruded linseed; Nutex68), medium chain length fatty acids from coconut, marine micro-algae (DHA-Gold) and 'essential oil blend' marketed as Agolin Ruminant.

Feeding linseed and the essential oils to calves from birth until 4 months of age improved feed efficiency and daily gain but neither affected methane production during or after supplementation ceased. Goats were used to evaluate medium chain fatty acids and also the micro-algae, but methane was not measured in these trials. However, the medium chain fatty acids had a negative impact upon kid goat growth rate and also negative impacts on the morphology of rumen papillae, which remained evident 4 months after treatment was discontinued. In contrast, the micro-algae were not detrimental to daily gain, but it did affect papillae morphology and this did appear to increase methanogenesis, *in vitro*. All supplements reduced rumen protozoal populations.

The main finding was that treating young animals did not reduce methane. The lack of effect is possibly because methanogen numbers in the young were too low and less active than in older animals. This finding was contrary to expectation that methane production would be reduced more effectively by treating young animals than older (post weaning) animals. An assessment of published literature (page 170 in the thesis) suggested early life exposure to CH_4-reducing supplements may desensitize the rumen microbes to the inhibitory effects of the supplement later in life.

Biological (Probiotics/Direct-fed microbials etc)

A variety of microbial strains (bacteria, fungi and yeasts) have been tested as feed additives to improve ruminant production (Weimer, 2015, Stanton et al., 2019). In general, it has been difficult to demonstrate a mode of action and inoculated strains fail to establish. The exception (see below) is the successful inoculation of a *Synergistes jonesii* culture which enables animals to detoxify mimosine found in the tropical legume *Leucaena leucocephala* (Hammond, 1995). In this example there is strong selective pressure for the introduced microbe to colonize a vacant ecological niche. Inoculation of lambs prior to weaning with rumen fluid from adult animals resulted in changes in the rumen microbiome (De Barbieri et al., 2015b, Yu et al., 2020), but these diminished after weaning and did not result in improved animal performance (De Barbieri et al., 2015a, 2015b).

Chemical (Inhibitors, ionophores etc)

The use of chemical feed additives to improve production efficiency and reduce ruminant methane emissions has been widely studied and recently reviewed (Hristov et al., 2013, Ungerfeld, 2018, Stanton et al., 2019, Beauchemin et al., 2020). Compounds trialled included seaweeds, monensin, alternative hydrogen acceptors (nitrate) and phytochemicals such as tannins. Many compounds that affect methanogenesis *in vitro* do not persist *in vivo*, because of microbial degradation of the inhibitor, or adaption of the rumen ecology to render the

treatment ineffective. Some compounds can be detrimental to animal health, and if absorbed (e.g. long chain fatty acids) may affect the composition of milk or meat. The most common approach has been to use compounds that directly inhibit methanogenesis, with most inhibitors being analogues of methane or methyl-coenzyme M (Henderson et al., 2018). Some such as bromochloromethane (BCM) or chloroform are known to be toxic, but others (particularly 3-nitrooxypropanol, 3-NOP) have shown promising results (Beauchemin et al., 2020). An important consideration is the difficulty in applying these methane mitigation approaches to grazing ruminants, but they may have a role in modification of the microflora of young ruminants.

Ruminant methane production is easily inhibited with small daily doses of halogenated methane analogues, of which BCM (CH_2BrCl) appears to be the most effective (Johnson et al., 1972). Methanogenesis was reduced by 60% with BCM given twice daily to steers over several weeks (Tomkins et al., 2009) and by over 30% with goats (Abecia et al., 2012), which increased milk production by 36% without affecting composition. Bromochloromethane has an ozone-depleting effect and is banned from commercial use in many countries.

Chloroform (1.5 ml; $CHCl_3$) was given once daily to cattle fed a lucerne silage-based diet, and dramatically lowered both methane emissions (to about 10% of pre-treatment values) and methanogen populations (especially *Methanobrevibacter* species; Knight et al., 2011). As the treatment progressed, methane emissions began to increase slowly, and after 42 days were about 40% of pre-treatment values, suggesting an adaptation was taking place.

A more promising mitigant is 3-nitrooxypropanol (3-NOP) which has no negative effects on animals or production, but reduced methane yields by 30% from high producing dairy cows when applied to feed at 40-80 mg/kg DM. The 3NOP inhibits the MCR enzyme (as with halogenated methane analogues), is non-toxic, commercially available, and the effects appear to persist as long as it is incorporated in the feed.

Ionophores, such as sodium monensin (Rumensin) are effective in altering rumen function and improving feed conversion efficiency when the diet contains a high proportion of grain, but effects on methane yields are variable. Monensin did not reduce methane yields from cattle fed pasture with grain (Grainger et al., 2010), pasture as a sole diet (Waghorn et al., 2008) or a silage-based diet (McGinn et al., 2004); it cannot be considered a reliable means for methane mitigation in cattle. Long-term monensin supplementation had little effect on the quantity or diversity of methanogens in the rumens of lactating dairy cattle (Hook et al., 2009) and Vyas et al (2018) reported few interactions between MON and NOP given to beef finishing cattle, suggesting the effects of the 2 compounds were independent.

MICROBIAL MANIPULATION IN RUMINANTS

Attempts to alter the rumen microbial community are not new, and examples are given below of the very extensive research undertaken to understand and reduce legume bloat and also the only successful (persistent) introduction of a microbial species into ruminants. This history that highlights the futility of attempting to change the microflora in the adult, but also indicates the possibility of change.

Legume bloat research

The first attempts to manipulate the rumen microflora probably involved legume bloat, which is affected by the rumen microflora. Aside from observations published in 1716 (Beddows, 1952), the first publication of bloat research in New Zealand was by Johns (1954). This was at a time when veterinary advice suggested emptying the rumen for the exchange of contents was likely to endanger the cow (C.S.W. Reid, pers. comm). However, the advice was incorrect, and there were a further 47 publications in the 'Bloat in Cattle' series, the last being Waghorn and Shelton, (1994). There has been a substantial research output from bloat studies in New Zealand and overseas, especially from Canada (e.g. Majak et al., 2003).

Bloat was a very significant problem for farmers, and the research in New Zealand and elsewhere investigated relationships between cow physiology, the rumen microflora and susceptibility to bloat (Clarke and Reid, 1974). The linkage between the host animal and its influence over the microflora in its rumino-reticulum (RR) was demonstrated by the exchange of rumen contents between cows that were 'susceptible' and 'resistant' to bloat, and resulted in a short term (24-48 h) reversal their bloat susceptibility (Clarke and Reid, 1970). The heritability of the trait was recognised (Reid et al., 1972) and later estimated to be 0.19 (Morris et al., 1997).

The extensive review of bloat in cattle by Clarke and Reid (1974) covered the period 1959-1974 and included over 400 citations. Detailed consideration was given to salivation and salivary composition, but no clear relationship to bloat susceptibility was evident for this or other animal parameters evaluated (rumen anatomy, motility and eructation efficiency, fluid outflow, gas production, microbial population, protozoal populations, rumen pH degradation of nitrogenous components of the diet). More recently Majak et al (1986) suggested cattle susceptible to bloat had a lower salivation rate and a longer rumen fluid retention time (half-life 12-17 h) than cattle that were not susceptible to bloat (half-life 8h), but these differences were diet dependent.

Bloat research investigated a wide range of variables affecting susceptibility. Clearly the microbiome is central to bloat susceptibility and although this is influenced by the host, the primary regulatory factors remain elusive. The early research would have benefitted greatly from the gene-based identification and quantification of the microflora, developed in the last 2-3 decades; bloat research is reputed to be a physiologist's graveyard; best avoided.

Leucaena and mimosine

The only truly successful introduction of a bacteria into the rumen, which persisted and was transferred between cattle, and to their calves (Pratchett et al., 1991) is that of *Synergistes jonesii*. Briefly, Australian cattle survived, but performed very poorly when fed the tree legume *Leucaena leucocephala*, whereas goats and cattle in Hawaii were able to graze on this forage and perform well. The Leucaena contains mimosine, which is a toxic non-protein amino acid chemically similar to tyrosine, and it is degraded in the rumen to, 3-hydroxy-4(1H)-pyridone (3,4-dihydroxypyridine; 3,4-DHP). Mimosine is a depilatory agent and 3,4-DHP is a potent goitrogen. In the 1980s, Australian researchers successfully introduced 3,4-

DHP degrading ruminal bacteria from a Hawaiian goat into goats and cattle in Australia (Hammond 1995). Ruminal inoculation with either ruminal contents from adapted animals (e.g. oral dose of 600 ml rumen fluid; Pratchett et al., 1991), enriched cultures of 3,4-DHP-degrading ruminal bacteria, and pure cultures of S. jonesii have all been used successfully to establish ruminal populations that are capable of degrading 3,4-DHP and preventing leucaena toxicosis.

What is interesting about these bacteria (several strains of 3,4-DHP degrading ruminal bacteria have been characterized) is that they have remained in the rumen for periods of 10 months when cattle had no access to forage containing mimosine (R. Jones pers. comm). More important cattle without mimosine degrading bacteria (Controls) were inoculated within 12 weeks by another herd that grazed at least 100 m away, but had been weighed in the same yards 4 weeks 4 weeks previously (Pratchett et al., 1991). Apparently, this was not the first instance of cross-inoculation and the authors speculate this was due to faeces in the yards that contained the bacteria; once one animal was infected, it infected the others of the Control group. This example demonstrates the capacity of some bacteria to extend their range, very effectively!

In summary, there has been one successful inoculation of ruminants with a bacterial species, and this has been remarkably successful, persisting without access to the substrate that it is able to degrade, and spreading to other cattle, most likely from faeces that have been voided in a hot climate one month previously. We are unaware of other examples of a successful (persistent) inoculation with rumen bacteria.

DIFFERENCES IN RUMEN MICROBIOTA OF ANIMALS FED THE SAME DIET

Individual animals fed the same diet at the same intake do differ in methane yield (g CH_4/kg dry matter intake) and these differences are heritable, despite methane originating from the rumen microbiome. The causes of this variation have been only partially defined and likely include both microbial and animal (e.g. rate of passage through the rumen; below) factors (Pinares-Patiño et al., 2013, Shi et al., 2014, Xiang et al., 2018, Malmuthuge et al., 2019, Wallace et al., 2019), although the two are largely independent (Difford et al., 2018). Several studies have used statistical approaches to identify animal and microbial biomarkers associated with methane emissions in attempts to quantify the heritability of methane production (Auffret et al., 2017, Jonker at al., 2019, Ramayo-Caldas et al., 2020, Saborío-Montero et al., 2020).

FACTORS AFFECTING RUMEN MICROBIOTA AND METHANOGENESIS

Feed intake

One factor that should be considered when evaluating divergence between individuals in methane yield, is that divergence appears to be greatest when intakes are high, in both sheep (Pinares-Patiño, et al., 2003a) and Cattle (Pinares-Patiño et al., 2007). In addition, methane

yields decrease as intakes increase in sheep (Hammond et al., 2013) whilst the situation with cattle is less certain (see citations in Hammond et al., 2014; Charmley et al., 2016). Hence, it is important that intakes are similar (e.g. expressed in terms of metabolic body weight) when evaluating treatment comparisons.

Animal physiology,

Reports of close host-microbiome relationships, and especially relationships associated with divergent methane yields, demonstrates the animal contribution to its microbiome. There is also a developing interest in associations between the ruminant microbiome and the health and productivity of the host animal. Some developments have been drawn from rodent and human studies; the increase caesarean deliveries parallels an upward trend in autoimmune diseases and allergies, and changes in the microbiome of the babies (see Cammack et al., 2018 for details). Also, as with cattle, family members tend to have a microflora that is more similar, than to non-family members; ruminant researchers need to be aware of developments in other species.

Research associated with the causes of bloat, and differences between cattle that were susceptible and resistant to bloat, suggested saliva production and rumen liquid outflow (Majak et al, 1986) and also salivary proteins (Clark and Reid, 1974) influenced susceptibility. Although theories concerning salivary proteins were popular in some research groups, analyses by Jones et al. (1986) failed to find clear relationships with secretory tissues and bloat susceptibility. Unfortunately, the extensive research concerning legume bloat and animal characteristics has not yielded helpful insights concerning factors likely to affect the rumen microbiome.

One reason that an understanding of factors responsible for the host-microbe relationship is important, is that manipulations maybe more successful in animals that are physiologically most appropriate. For example, some individuals may be more (or less) susceptible to alterations of the rumen environment, perhaps indicated by the 'strength' of the host/microbial relationship and affected by their physiology, immunology and responses to dietary and other changes. Weimer et al. (2010) reported that only some cows returned to their previous bacterial community composition, following an exchange of digesta. Similarly, some treatments may facilitate the creation of a low methane microbiome, and hopefully this would be passed through the generations.

The rumen digesta pool size and methanogenesis

Pinares-Patiño et al. (2003b) showed in sheep, that methane yield was negatively correlated with and rumen particle outflow rate (r= - 0.75; P=0.01), buffering capacity of the rumen (r = -0.72; P=0.02) and positively correlated with digestibility of cellulose (r=0.66; P=0.04). Sheep with longer rumen retention times had larger rumen fills, higher fibre digestibility and higher methane yields. On the basis of these data it may be expected that sheep with small rumens will require a more rapid rate of passage to maintain intake, and this will result in lower digestibility as well as lower methane yields.

These findings have been confirmed by Goopy (2014), where sheep with a low methane yield (20.8 vs 23.5 g/kg DM for the high emitters) had a smaller rumen volume (P=0.05) and a shorter retention time of both particles (P=0.01) and liquid (P=0.001). Smuts et al. (1995) have reported differences in rumen retention times within breeds of sheep and Goopy (2014) points out that selection resulting in a smaller rumen may have important consequences for nutritional physiology. Sheep with a small rumen will have lower intakes of poor-medium quality feeds, so production will be substantially reduced and emissions/unit of product increased.

A similar conclusion can be drawn from a comparison of US Holstein cows bought to New Zealand, and New Zealand Friesians, when grazing grass (Robertson and Waghorn, 2002). The Holsteins had substantially lower methane yields (g/kg DMI) than Friesians at day 60 of lactation (15.1 vs 18.0) and day 150 (19.9 vs. 22.4) but not at day 240 (23.4 vs. 23.8). In a separate comparison between the two strains of cattle fed roughage and at a similar stage of lactation (Waghorn 2002), rumen digesta weight (% of bodyweight) was 12-17% for Holsteins and 17-22% for Friesians. These differences suggest a faster passage of feed through the rumen of the Holsteins, which may account for the lower methane yield. The Holsteins had been adapted to pasture, but bred for a silage and grain-based diet; pasture feeding resulted in an empty rate of 62% vs. 7% in the Friesians (Kolver et al., 2002).

Any selection to mitigate methane that affects changes to animal anatomy and physiology is ill advised, because of potential problems with production on farm. There may be other differences between animals divergent for methane yield and these must be evaluated carefully before distributing the selection to farmers or attempting to alter the microbiome.

Weaning management,

As mentioned above, Dias et al., (2017) showed calves raised on a milk only diet had a less diverse microflora in the developing rumen than calves with access to concentrate as well as milk. Their study monitored the calves until 63 days of age, whereas Dill-McFarland et al. (2019) who fed pre-weaned calves either a concentrate-based calf starter or maize silage, or a 50:50 mixture of the two found the differences at 8 weeks had disappeared by 1 year of age. This observation, along with those of Abecia et al. (2013) where cessation of bromochloromethane given to kid goats from birth to 3 months resulted an increase in methane emissions, similar to control animals suggests manipulations before weaning to influence the rumen microflora may not be as effective as anticipated (Yáñez-Ruiz et al. 2015). Their review did discuss the establishment of acetogens in lambs, but this required a sterile environment, and practical options to influence bacterial and archaeal colonisation were not demonstrated.

Diet,

Preweaning diets do affect the microflora establishing in the developing rumen, but differences disappear postweaning. After weaning, effects of diet on the microbiome, and methane yield, have been reported extensively and reviewed, for example from a biochemical/ microbiological aspect (McAllister et al., 1996), in terms of additives,

especially lipids (Grainger and Beauchemin, 2011), on farm (Martine et al., 2010; Pacheco et al., 2014) and in a comprehensive review by Hristov et al (2014).

Rather than repeating parts of this literature, it may be more informative to compare sheep selected for divergent emissions when fed contrasting diets. Pinares-patino et al., 2011 shows the emission from sheep fed the Pasture diet was much higher (P <0.001) than from the 40:60 forage:concentrate Pellet diet (23.2 ± 0.62 vs. 8.6 ± 0.68 g/kg DMI). There was no ranking × diet interaction effect on methane yield, but the difference in methane yield between High and Low emission ranking sheep was much higher on the pellet diet (69%) than on the Pasture diet (13%; Table 2). This shows that animals that do differ in methane yield (for whatever reason) maintain that ranking across contrasting diets, over time, but with substantial variation in the extent of the differences. Successful selection/manipulation of young animals should extend to a variety of diets, but it must be noted that the differences in the low- and high-ranking selection lines (Table 2) were associated with rumen physiological function, and this cannot be achieved by manipulation in early life.

Table 2. Effect of methane emission ranking of sheep as either Low or High, when fed either a Pasture or Pellet diet on methane yield, feed dry matter digestibility and rumen retention times of digesta particulate and solute phases. From Pinares et al., 2011.

	Low ranking		High ranking		Probability	
	Pasture	Pellet	Pasture	Pellet	Rank	Diet
Methane yield (g/kg DMI)	24.6 ± 0.9	6.4 ± 0.9	21.7 ± 0.8	10.8 ± 1.0	<0.001	<0.001
DM Digestibility (%)	59.5 ± 1.9	58.1 ± 2.0	62.9 ± 2.1	63.4 ± 2.2	0.05	0.81
Rumen solids retention time; h	27.0 ± 1.8	36.4 ± 1.7	32.9 ± 1.8	37.3 ± 2.1	0.17	0.001
Rumen liquid retention time; h	13.1 ± 0.7	14.1 ± 0.7	16.0 ± 0.7	15.0 ± 0.8	0.04	0.92

Data are mean ± standard error

Antibiotic administration,

Antibiotics were used in early studies of legume bloat (Clarke and Reid, 1974 list over 20 publications evaluating their use for controlling legume bloat,) but development of antibiotic resistance was curtailing their use nearly 50 years ago. Although anti-microbials such as monensin or lasalocid have been used for legume and feedlot bloat control, and to limit acidosis in cattle during transition to a high grain diet (virginiamycin), or prevent liver abscesses (tylosin) there is a trend toward their reduction (especially in Europe) because of increasing antibiotic resistance. Studies have even shown an 80% increase in methane emissions from manure, due to antibiotics in manure affecting changes in dung beetle microflora (Hammer et al., 2016). This cascade of effects illustrates an unexpected outcome of antibiotic use, and it would be best to limit their use as much as possible, even when attempting to manipulate the rumen microbiome.

Immunology

Unlike monogastric digestive systems, the reticulo-rumen is non-glandular, and the organ has a keratinised stratified squamous epithelium. This limits options for provision of antibodies and compounds able to affect rumen microflora, to salivary secretions. The ruminant secretes copious saliva, especially when roughages are consumed, and

manipulations have increased immunoglobulin G titres in ruminants (below; *vaccinations*). Human studies are increasingly focusing on salivary provision of antibodies, either derived from serum or produced *in situe* (e.g. Brandtzaeg, 2007; Ponzio and Saunders, 2017) but the extent to which the ruminant will respond to manipulations is not known. This is especially true of attempts to manipulate the young animal, which has limited saliva production soon after birth. However, the reasons why kangaroos produce very little methane are indicated below (and Appendix 2) because this highlights the extent of the challenge for ongoing mitigation of methane.

Klieve et al. (2012) found that many kangaroos did not have archaea in their digestive system, and in others the numbers were 10 to 1000-fold lower than sheep, and Gulino et al. (2013) suggested the presence of acetogens. More recently, Leng (2018) evaluated a broad literature to develop a theory that may explain the near-absence of methanogens in macropods (Kangaroos and Wallabies), which have about one quarter of the methane yield of ruminants.

The basis for his theory is that gases are less able to escape from tubular organs (intestine) compared to the rumen, and the host uses its immune capability to manipulate and reduce gas producing microflora, for its own protection. This enables a predominance of acetogens in kangaroos and wallabies because methanogens are suppressed. He postulates that the differences in gas production is associated with microgels containing immune factors formed in the parietal blind sac of the macropod foregut (and in the caecum of ruminants and horses) and this may inoculate proto-biofilms before they attach to particles and develop into mature biofilms. He speculates that when protoboifilms detach from the mucosa they contain host immune secretions that may include antimicrobial peptides (AMPs), immunoglobulins (IGs), innate lymphoid cells (ILCs) and mucin and these either prevent establishment of methanogens or kill them, enabling establishment of acetogens as the major hydrogenotrophs. This theory may have implications for attempts to lower methane yields from ruminants and also emphasise the futility of attempts to establish acetogens in ruminants. More details are provided in Appendix 1.

Vaccinations

Vaccination is an attractive option for methane mitigation because, if successful, it would require little effort from farmers, few vaccinations would be required and they could be given to ruminants in all farming environments, including animals in hilly terrain that have little human contact. In their 2015 review, Yáñez-Ruiz et al. suggest that little is known about the mechanisms involved in the 'tolerance' to the first colonizers of the rumen. This is because the lining of the reticulorumen is stratified squamous epithelium, and very different from the mucosal lining of the intestines. The rumen can respond to microflora but saliva may be the prime route for antibody delivery. Little is known about this, or epithelial responses in young animals. They concluded that 'Identifying the key immune elements at molecular level involved in early life colonization (with special attention to the rumen epimural population) may help to understand the host animal response, and the extent of persistency of effects in adult life'.

There have been examples of successful vaccination for methane reduction, but these are few in number. Wright et al (2004) reported a substantial concentration of antibodies in both plasm and saliva in mature sheep fed poor quality pasture, but only after a second vaccination (153 days after the first), and this resulted in a 7.7% reduction in methane yield (P=0.051) compared to Control sheep. This study was followed with a vaccine based on 5 methanogens (representing 52% of rumen methanogens - from 16S rRNA gene libraries) given to sheep (Williams et al., 2009). Each vaccination (on days 0, 28 and 103) increased immunoglobulin G titres in plasma, saliva and rumen fluid and, although not statistically significant, the methane yield after the second and third vaccinations were 20% and 18% higher, respectively, than those for Control animals. Methanogen numbers were not affected by the vaccine.

Leahy et al. (2010) have sequenced the genome of *Methanobrevibacter ruminantium* and identified specific genes for targeting in vaccine development or for chemogenomic inhibition. This has assisted in the identification of surface membrane-associated proteins that are conserved across a range of methanogen species, and should enable identification of appropriate antigens with a view to antibody delivery in saliva (Wedlock et al., 2013). However, a vaccine has not reduced methane yield in sheep (Subharat et al., 2016, Leahy et al., 2019) or in a separate program with goats (Zhang et al., 2015), despite an intensive immune response in both saliva and plasma. There is a surprising paucity of publications concerning methane vaccines, although there are patent applications (e.g. Altermann et al., 2017).

EXAMPLES OF GASTROINTESTINAL TRACT INOCULATION.

When is the best time of life to inoculate?

It is clear from experiments involving exchange of rumen contents (as in bloat trials, and Weimer et al 2010, 2017; below) that once the host/microflora relationship is established, it is resistant (impossible) to change, except by diet. The only exception appears to be that of *Synergistes jonesii*, which is capable of degrading a breakdown product of mimosine (3,4-DHP), and it established and remained in cattle with no contact with the host carriers.

Therefore, inoculation must be undertaken in young animals, when the host/microbial relationship is developing. The question is, how young should that be, for how long and how frequently should they be inoculated, how much inoculum and also what form should the inoculum take? And are there other things to be aware of, and will it work?

There are questions and few answers to the optimal timing of inoculation of young animals, but there may be risks. For example, Debruyne (2019) summarised trials where early life exposure to methane inhibitors appeared to lessen responses to the same compound when the animal was older. She suggested that prenatal or early life exposure to methane-reducing supplements may desensitize the rumen microbes (or the animal's immune system) to the inhibitory effects of the supplement later in life, which is not a desirable result.

However, another easily tested option would be to treat the pregnant dam with a methane inhibitor in an endeavour to influence the foetus. This concept is based on human studies showing relationships between prenatal nutrition and foetal growth and birth outcomes including subsequent risk of diseases, such as type 2-diabetes and cardiovascular disease (Robertson et al., 2019). Outcomes concerning caesarean delivery increasing the risk of obesity, asthma, allergies and immune deficiencies in humans have been linked to the types of microbial colonisation; more pathogens than commensals (Debruyne 2019).

Have there been successes, in what species?

Other than daily administration of biological or chemical inhibitors or selection of individuals with divergent methane yields (probably associated with digesta residence time in the rumen), diet is the main option for altering the rumen microflora and methane emissions. Exceptions have been one instance of vaccination (Wright et al., 2004), the success with *Synergistes jonesii* preventing Leucaena toxicity, and use of bromochloromethane with kid goats and their dams (Abecia et al. 2013). These very few successes are confirmed by the review of host-microbe interactions, by Bickhart and Weimer (2017) who concluded that probiotics or dosing with common rumen microbes or whole rumen contents from unrelated cows have only resulted in temporary changes in microbial profile or production.

Exchange of rumen contents

Studies by Weimer and others (Weimer et al., 2010; 2017) involving the exchange of rumen contents has provided insight into the importance of the animal-regulated microbiome. In their first trial, rumen contents of cows fed the same diet, but with divergent rumen pH and VFA concentrations were exchanged (Weimer et al., 2010) and these parameters returned to pre-exchange values for individual cows within 1 day, but the bacterial community composition took longer ~ 14 days to return to pre-exchange characteristics. In some instances, the community did not return to pre-exchange characteristics after 2 months, but differences between cows remained.

Weimer et al. (2017) exchanged rumen contents of lactating dairy cows with a high milk production efficiency (HE) with those having a lower milk production efficiency (LE). The HE cows had higher total ruminal VFA concentrations, higher molar percentages of propionate and valerate, and lower molar percentages of acetate and butyrate than did LE cows. As with the previous trial, cow differences were re-established, along with rumen pH, 1-day after the contents were exchanged, but most cows adopted the efficiency of the donor cow for seven days. The solids-associated bacterial community composition returned to pre-exchange values after about 10 days, whilst the liquid bacterial community took longer.

The data of Weimer et al., 2010 and 2017 support the concept established several decades previously, that the host plays a role in regulation of the rumen microflora. Although details of the rumen environment are described in more detail in recent trials, the mechanisms remain elusive, except that the rapid re-establishment of pH and VFA concentration do suggest a role of both salivation and rumen residence time in overall function.

In Summary, experiments involving near total exchange of the rumen contents between animals have shown that the rumen microbial community is capable of rapidly returning to that present before the exchange (Weimer, 2015, Zhou et al., 2018), suggesting that the host animal has a strong effect on the rumen microbial population. Studies have also shown that there is little day-to-day or seasonal variation in the microbiome of dairy cows (Noel, et al., 2017, Skarlupka et al., 2019). The mechanisms by which the host might control the rumen microbial population remain unknown. One aspect that has not been investigated in any detail is the role of saliva in shaping the rumen microbiome. Ruminants secrete large volumes of saliva but there is limited information on the interaction between salivary proteins and rumen bacteria (Zhang et al., 2019). Seshadri et al., (2018) noted that the presence of animal glycan-degrading enzymes in rumen ruminal *Prevotella* sp. which may allow them to utilize the carbohydrate component of salivary N-linked glycoproteins as a carbon source, and help explain their abundance in the rumen microbiome

Direct fed microbials

This topic, in relation to methane mitigation has been reviewed by Jeyanathan et al. (2014), exploring options for increased proportions of propionate, alternative hydrogen sinks and capability of acetogens to compete in the rumen.

There have been numerous studies describing the use of direct fed microbials (DFMs) in ruminant animals, and a range of products, usually based on yeasts or lactic acid bacteria (LAB), are commercially available. The use of yeasts or LAB to reduce methane production in ruminants has been reviewed (Darabighane et al., 2019, Doyle et al., 2019). Yeasts are not considered to have a significant effect on methane production. With LAB the effect is strain dependent and it is not understood whether the culture or its metabolites affect the methanogens themselves, or whether the affect is against the other rumen microbes that produce substrates necessary for methanogenesis. LAB can reduce methane production effectively *in vitro* but results *in vivo* have been variable (Jeyanathan et al., 2016, 2019). If appropriate LAB cultures can be identified, and proven to be effective in vivo then a range of delivery options are available. The possibility of using bacteriocin-producing strains of LAB as a methane mitigation option is also under investigation in the METHLAB project (Doyle et al., 2019). There are reports on the use of *Bifidobacterium* strains as probiotics, which highlight their influence on immune function and their ability to reduce the incidence of diarrhoea in young calves (Santillo et al., 2012, Signorini et al., 2012). However, there is no research investigating the role of ruminant-derived bifidobacteria on methane production.

SPECIFIC EXAMPLES RELATING TO METHANE REDUCTION

Methane mitigation in animals through manipulation of the microbiome, without ongoing chemical, diet or other treatment has been rare. In nearly all cases the animal reverts to its previous emissions (methane yield) once the treatment is stopped.

The possibility of early life intervention impacting ruminant methane production has gained support from the study reported by Abecia et al (2013, 2014). This used 16 goats giving birth to twin kids. Eight does were treated (D+) with the inhibitor bromochloromethane after

giving birth and over 2 months while the other eight were untreated (D-). One kid per doe in both groups was treated with bromochloromethane (k+) for 3 months while the other was untreated (k-). The BCM reduced methane by 52-59% at the end of the treatment period (3 months of age). More interesting was that 3 months later, methane was 33% lower (and daily gain higher) in lambs which received BCM and their dams had also received BCM, than the other treatment groups. Kids receiving BCM, with does who did not receive BCM had similar emissions to controls.

Sequencing of rumen samples collected from kids at weaning showed a modified archaeal community composition but those changes did not persist. Subsequent work extended the analysis to the bacterial community and the rumen metabolome Abecia et al (2018), with the conclusion highlighting the differences observed before and after weaning. However, the sequencing approaches used do not provide an indication of which organisms are functionally active. Nevertheless, only when the does also deceived BCM did the effect persist over 3 months without further treatment. The trial was terminated 3 months after BCM treatment ceased, so the longevity of the change is unreported but personal communication (Harry Clarke) with David Yáñez-Ruiz suggested that the treatment effect was diminishing over time.

A single study examining the effect of the inhibitor 3-NOP on early life methane production in 18 female calves (10 treated with 3-NOP and 8 controls) has been presented as a conference abstract (Meale et al., 2019). It is stated that the effect persisted after the treatment period, but methane yield was not provided and this work has yet to published following a peer review. Consequently, this abstract does not provide defensible information and avoids data that could indicate the outcome of the trial (methane yield, daily gain, diet), although measures of rumen parameters suggest minimal change.

In another study (Saro et al. 2018; Table 1) a combination of linseed oil and garlic essential oil was administered to lambs from birth until 10 weeks old, and again from 16 to 20 weeks. Methane emissions were reduced and the methanogen community was altered during the treatment, but no differences were observed 1 month after the treatment ended. Debruyne (2019) similarly showed that early life supplementation with linseed oil or an essential oil blend had no effect on *in vivo* methane emissions.

ANY TRICKS OR IDEAS?

Nanobubbles

In terms of odd ideas, one that should be considered is the use of oxygen nanobubbles, firstly to test them; they may make existing microflora weaker and enable a more effective intervention.

An example of their effectiveness was a 3.2-fold reduction in methane emissions from wastewater by treating it with oxygen nanobubbles (Shi et al., 2018). Water with oxygen nanobubbles also increased plant and fish growth (Ebina et a., 2013) and is used to target cancer tumours for treatment (Bhandari et al., 2017).

Very briefly, the average diameter of a nano bubble is 80 nm, providing only 40 ml oxygen/L (10^{12}/bubbles/L) and they stay in water for 3 weeks. The effect of nanobubbles on a range of scenarios is a consequence of both their surface charge (zeta potential) as well as the gas. The equipment to produce them is not expensive; The main concern is that the general microbiome may be affected excessively, but the archaea may be more susceptible. The concept requires evaluation and could be useful, providing the treatment is not detrimental to fermentation and digestion.

Other

Although options for long-term modification of the rumen microbiome for lowering methane emissions whilst maintaining production, are limited, any aspects of intervention need to be planned carefully. For example, should inoculations be carried out before or after feeding, and with a drench or a stomach tube? These decisions may impact upon the likelihood that material will reach the reticulum, or rumen, rather than be diverted to the abomasum via reflex oesophageal groove closure.

The answer will depend on objectives and age of animal, but there is a considerable risk of oesophageal groove closure when suckling (preweaning) animals are drenched. Details of copper and sodium salts eliciting groove closure in sheep and cattle (respectively), are discussed in Waghorn and Shelton, (1994), as well as a consideration of outflow from the reticulum in a 'full' animal; these factors may affect experimental outcomes.

CONCLUSION

Ruminants maintain intakes and production when methanogenesis is suppressed, so efforts to reduce methane emissions will benefit the environment without detriment to the animal or its productivity. Individual ruminants may differ in their microbial community composition when fed the same diet, and the communities change with dietary change; a return to the original diet will result in the same community composition in some animals, whilst others are less well controlled. Measurement of composition remains imprecise and composition is not necessarily indicative of activity.

This review has highlighted the host/microbiome relationship, but equally a lack of understanding of host factors that determine their microflora and also enable the host to re-establish their microflora following a perturbation (e.g. through dietary change, sickness, antibiotic treatment or exchange of rumen contents). Even intervention preweaning rarely has a lasting impact on the microflora or methane production. The sources of inoculum may be dominated by the dam, but are varied and new born animals removed from their dam at birth still become fully inoculated. For this reason, any opportunity that has a defensible basis for achieving a change to the microflora to reduce methane, should be undertaken. A suggestion is given for evaluation, and the concept is to provide an over whelming change and to minimise other sources of microbes (by treating dams as well as progeny).

It is noted that several other research groups have active programmes specifically targeting the use of methane inhibitors as an early life intervention approach, and consideration should

be given to interaction/collaboration with these groups. An example is a PhD project funded from the METH-ABATE project in Ireland. This advocates "animal trials and *in vitro* studies to understand the life-time effect of early-life microbial programming in sheep. In particular, the project will focus on studying anti-methanogenic compounds that will reduce the environmental impact of ruminants". It is assumed that further work exploring the use of 3-NOP as an early life anti-methanogen intervention is currently in progress by groups working in partnership with DSM (https://www.dsm.com/content/dam/dsm/corporate/en_US/documents/summary-scientific-papers-3nop-booklet.pdf).

Recommendations for methodology applicable to ruminants

This review has highlighted the lack of persistent changes in the microbiome and methanogenesis when probiotics and chemical inhibitors have been used in young and in mature ruminants. This is supported by the research of Abecia et al (2013); when they treated the new-borne kids with bromochloromethane (BCM) for 3 months it did not result in an ongoing reduction in methanogenesis.

However, treating both the doe and the new born kid with BCM resulted in a change in both the microflora and methane production. It is almost as if the young animal has to be overwhelmed with treatment, and other influences (e.g. ongoing inoculation from the doe) excluded, for the change to remain, albeit at reduced levels, post treatment. The treatment of calves with the methane inhibitor 3-NOP has also been reported to result in a persistent effect (Meale et al., 2019), although this has yet to be confirmed.

Because bromochloromethane is environmentally unacceptable, the treatment of young and adult sheep could be undertaken with 3-NOP, which is commercially available and appears not to have detrimental effects on either the animals or the environment. The extent to which mitigation persists has yet to be determined, along with changes bought about by the treatment.

The limitations of these previous studies is that there is no indication of which microbes are transcriptionally active, and thus no biological information is provided on how the inhibitors are achieving their effect. Consequently, it is imperative that future research moves beyond simple measurement of microbial relative abundance and concentrates on the organisms that are functionally active, either producing methane or the substrates used for methanogenesis. Such work is technically more demanding but will provide valuable insights into the biology of the developing rumen.

The use of bacterial strains as direct-fed microbials/probiotics capable of achieving a methane mitigation effect is also worthy of further investigation. A neglected aspect is an understanding of the microbes that colonize from birth and any future studies should also include the isolation of cultures from neonatal animals with the aim of assembling a biobank of strains suitable for testing as future direct-fed microbials.

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APPENDIX 1. Overview of early life dietary interventions in sheep (and a single goat trial‡) and the effects on rumen fermentation pattern (VFA concentrations), CH^4 emissions and other parameters during and after the intervention. Only 1 study tested a genuine prenatal dietary treatment [De Barbieri et al., 2015a, b]. From Debruyne, S. 2019.

Supplement/ treatment	Dose	Duration of treatment	Effects on rumen fermentation and CH4 production during and after treatment	Effects on other parameters during and after treatment	Reference
Hay (H) or concentrate + hay (60:40) (C	Ad lib	Lamb: birth until 8 wk after weaning (16 wk)	16 wk: different CH4 emissions (C diet: -14% L/kg DMI) but similar fermentation pattern. No differences 4 mo after end of different dietary treatments	16 wk: Rumen bacterial and methanogen communities differ. Rumen bacterial communities still differ 4 mo after end of different dietary treatments	1
‡**Goats** Bromochloromethane	Doe: 3 mg/kg BW Kid: 3 mg/kg BW	Doe (with 1 kid): first 2 mo after birth of kid Kid: birth - 3 mo of age	Altered fermentation pattern in K+[A] (A↓, P↑, B↑, V↑) leading to A/P↓ in D+K+[B]. CH4↓ (-52% and 59% in K+ within D+ and D- group resp.). CH4↓ (-28%) and P↑ (+15%) compared to D-K-[3], persisted only in D+K+ until 3 mo after treatment	Modified rumen methanogen communities at W (both does and K+ kids), not entirely persisting until 4 mo after W (only some less abundant groups). Higher daily gain in D+K+	2, 3, 4
Coconut oil (CO) or rumen-protected fat (RPF) + inoculation with water or with rumen fluid from donors fed CO or RPF	Ewe: diet with 4% supplement (ad libitum) Lamb: same diet as ewe and inoculation (20 mL or 40 mL)	Ewe (with 1 lamb): from 1 mo prelambing until weaning of lamb Lamb: birth – 3 mo of age (diet), weekly until 8 wk of age (inoculation)	Ewes: no effect on VFA production or pH. Lambs: diet no effect on VFA production or pH. CH4↓ (-9%, CO diet). Inoculation altered rumen fermentation but only higher B (Inocul.CO) remained after inoculation was ceased.	Ewes: no effect on BW or milk production. DMI↓ and protozoa↓ (CO diet). Lambs: DMI↓, protozoa↓ and reticulorumen weight↑ (CO diet). Diet affected the bacterial community more and longer compared to inoculation with rumen fluid from donor ewes on different diets	5, 6

Linseed oil	Ewe: 1.7 g/kg BW[D] Lamb: 1.2 g/kg BW[E]	Ewe (with kids): until weaning of lamb Lamb: birth until weaning (L-P) or birth until 10 wk after weaning (L;16 wk)	16 wk: similar fermentation pattern in lambs (no CH4 measured).	Similar feed intake and daily gain. Rumen bacterial and methanogen community differed during treatment, only bacterial community remained different after treatment.	7
Mix of linseed oil* and garlic essential oil°	1.6 mL/kg BW* 3 µL/kg BW°	Lamb: birth until 10 wk of age, and again from 16 until 20 wk of age (re-/cross treatment	First treatment period: CH4↓ (-23%, µmol/mL), no difference in tVFA production or VFA proportions. One mo after first treatment period: no difference in CH4 nor VFA. After second treatment period: CH4↓ (-14%, g/d or g/kg DMI), tVFA↓ (-23%), altered VFA proportions leading to A/P↓ in treated compared to control lambs. No interaction effect of retreatment.	Similar daily gain throughout trial. Rumen bacterial community differed during first treatment, 1 mo after first treatment had ceased and after retreatment. Little or no effect on archaeal and protozoal communities	8

[A]K+: kids treated postnatally; [B] D+K+: kids treated both pre- and postnatally; [C] D-K-: kids never treated.

[D]Ewes received 3 kg/d of concentrates (40 g/kg linseed oil content) at parturition. The concentrate dose was lowered throughout the pre-weaning period until 1.5 kg/d. No BW of ewes was measured at parturition. Reported dose is assuming a BW of 70 kg.

[E]Lambs were eating more than 550 g/d of the concentrate at approx. 6 weeks of age (19 kg BW), suggesting a minimum intake of linseed oil of 1.2 g/kg BW at the end of the treatment period.

References: **1**. Yáñez-Ruiz et al., 2010; **2**. Abecia et al., 2013; **3**. Abecia et al., 2014; **4**. Abecia et al., 2012; **5**. De Barbieri et al., 2015 b; **6**. De Barbieri et al., 2015 a; **7**. Lyons et al., 2017; **8**. Saro et al., 2018.

APPENDIX 2

Klieve et al. (2012) found that many kangaroos did not have archaea in their digestive system, and in others the numbers were 10 to 1000-fold lower than sheep., and Gulino et al. (2013) suggested the presence of acetogens. More recently, Leng (2018) evaluated a broad literature to develop a theory that may explain the near-absence of methanogens in macropods (Kangaroos and Wallabies), which have about one quarter of the methane yield of ruminants. He lists recent reviews concerning the microbial ecosystem and its relationship with host immune system, for their mutual benefit, and makes the point that this is a two-way process and secretions from the host may account for differences in fermentation and end products of fermentation.

The basis for his theory is that gases are less able to escape from tubular organs (intestine) compared to the rumen, and the host uses its immune capability to manipulate and reduce gas producing microflora, for its own protection. This enables a predominance of acetogens in kangaroos and wallabies because methanogens are suppressed.

Much of the microflora digesting particulate matter (in ruminants and macropods) reside in a biofilm covering particles; in ruminants this originates from saliva during chewing during eating and rumination, whereas macropods (which do not ruminate) also add mucus via their tubular stomach (Leng, 2018). Given the sensitivity of the intestine to gas, a yield of 22 g methane from 1 kg feed dry matter has a volume of 30.8 L, but the hydrogen (H_2) would have a volume of 61.6 L and this could cause discomfort, especially in macropods because they are unable to eructate and I am guessing absorption into the bloodstream is low. It is therefore in the interests of the animal to remove the hydrogen gas and convert it to acetate. Leng (2018) argues that the higher partial pressure of H_2 needed to support homoacetogenesis (compared to methanogens) could be maintained in biofilms via syntrophism. This concept (from Leng, 2018) is summarised in Box 2.

Box 2 Syntrophic growth of microbes; (from Leng, 2018).

Syntrophism is a mutualistic interaction between two or more metabolically different organisms that are linked by the need to maintain an exchange of metabolites at low concentrations, making their overall metabolism feasible. The cooperation between fermentative microorganisms is based, in part, on the transfer of H2, formate, or acetate from fermentative bacteria to methanogens/acetogens, which ensures that the degradation of electron-rich substrates is thermodynamically favourable. Syntrophic metabolism proceeds at very low Gibbs' free energy changes, close to the minimum free energy change needed to conserve energy biologically, i.e. the energy needed to transport one proton across the cytoplasmic membrane (Schink 1997).

Leng (2018) postulates that the differences in gas production between ruminants and macropods is associated with microgels containing immune factors formed in the parietal blind sac of the macropod foregut and in the caecum of ruminants (and horses) and this may inoculate proto-biofilms before they attach to particles and develop into mature biofilms. He speculates that when protoboifilms detach from the mucosa they contain host immune secretions that may include antimicrobial peptides (AMPs), immunoglobulins (IGs), innate lymphoid cells (ILCs) and mucin and these either prevent establishment of methanogens or kill them, enabling establishment of acetogens as the major hydrogenotrophs. This theory may have implications for attempts to lower methane yields from ruminants and also emphasise the futility of attempts to establish acetogens in ruminants. Box 6, provides a summary of the manuscript.

Box 6. The hypotheses proposed to explain differences in H2 utilisation in the forestomach of macropods as compared with ruminants

Gaseous products of fermentation (H2 and methane, CH4) are readily removed by eructation from the rumen and also via flatus from the large bowel in all mammals. However, in the forestomach of macropods and the caecum–colon of the horse, no such 'gas release' mechanism is available. As the volume of gas produced in the latter is large, in comparison to the volume of digesta, the pressure attained could potentially damage the organ. For this reason, these animals have evolved to support acetogenic-type fermentation; this type of fermentation is maintained by immune modulation of the species composition of microbial biofilms and differences in the gut anatomy where fermentative digestion occurs. It is postulated that mucosal cells, in close association with lymphoid tissues in the blind sacs at the proximal end of the haustral structure of the forestomach of the macropod, and the caecum of the horse and ruminants, secrete gel-like proto-biofilms containing immune agents (that suppress or kill methanogenic Archaea). These secretions also contain mucin that may promote the establishment of acetogens as the proto-biofilms develops into a mature fermentative biofilm. These biofilms solubilise the organic matter as they move distally through the forestomach and caecum. The biofilm consortia are determined by these immune secretions that may include antimicrobial peptides (AMPs), immunoglobulins A (IgAs) and possibly innate lymphoid cells (ILCs). The AMPs that lyse the distinct cell envelope of Archaea pave the way for total or partial replacement of methanogens by acetogenic bacteria. The result is that CH4 or H2 production is markedly reduced or eliminated in the caecum–colon of the equids or the foregut of macropods relative to that produced in the rumen

The consideration of the host immune system proposed by Ron Leng offers insights into host-microbiome relationships, but other developments need also to be considered. For example, that colonisation of the new borne may have initiated before birth, with evidence of the placenta having its own microbiome (especially *Escherichia*) and that in the human population, factors such as stress, lifestyle as well as diet and antibiotics can affect the microbiome (Cannock et al., 2018). They have used information from human and rodent studies to explore the host/microbiome relationship and stress the 'need to identify factors that influence rumen microbial colonization, along with continued efforts to characterize and

quantify the populations by taxonomic and functional assignments and better understand the interplay between the host and the microbes, as well as among the microbes themselves'.

I wonder if studies have been undertaken to measure diversity in the microbiome of calves taken from their mothers soon after birth (as in the New Zealand dairy industry) compared to calves raised with their mothers until weaning.