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Design, implementation and interpretation of *in vitro* batch culture experiments to assess enteric methane mitigation in ruminants – a review

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Highlights:

- There are many technical factors that may influence the results obtained from using the *in vitro* gas production (IVGP) technique to assess the effect of different nutritional strategies on methane production.
- The factors include: i) donor animal species and number of animal used, ii) diet fed to donor animals, iii) collection and processing of rumen fluid as inoculum, iv) choice of substrate and incubation buffer, v) incubation procedures and CH_4 measurements and vi) headspace gas composition

Guidelines are provided to interpret results obtained from in vitro methods before assessing mitigation strategies *in vivo*

Abstract

In vitro fermentation techniques (IVFT) have been widely used to evaluate the nutritive value of feeds for ruminants and in the last decade to assess the effect of different nutritional strategies on methane production. However, many technical factors may influence the results obtained. The present review has been prepared by the 'Global Network' FACCE-JPI international research consortium to provide a critical evaluation of the main factors that need to be considered when designing, conducting and interpreting IVFT experiments investigate nutritional strategies to mitigate methane (CH_4) emission from ruminants. Given the increasing and wide-scale use of IVFT techniques, there is a need to critically review reports in the literature and establish what criteria are essential to the establishment and implementation of *in vitro* techniques. Key aspects considered include: i) donor animal species and number of animal used, ii) diet fed to donor animals, iii) collection and processing of rumen fluid as inoculum, iv) choice of substrate and incubation buffer, v) incubation procedures and CH_4 measurements, vi) headspace gas composition and vii) comparability of *in vitro* and *in vivo* measurements. Based on an evaluation of experimental evidence, a set of technical recommendations are presented that allowing the harmonization of IVFT laboratory methods employed with IVFT, of and procedures for feed evaluation, assessment of rumen function and CH_4 production.

Abbreviations: DM, dry matter; DMI, dry matter intake; IVFT, *in vitro* fermentation technique; NDF, neutral detergent fibre; OM, organic matter; VFA, volatile fatty acids.

Keywords: feed evaluation, *in vitro* gas production, methane, rumen, mitigation, microbial inoculum

1. Introduction

In vitro fermentation techniques (IVFT) that involve incubations of substrates with rumen fluid have been used extensively to evaluate the nutritive value of ruminant feeds. Measurements based on IVFT complement standard laboratory analysis of chemical composition and therefore offer a rapid and less expensive alternative to the determination of nutrient digestibility *in vivo* (Rymer et al., 2005). Also, application of the IVFT to reduce the use of experimental animals represents an advantage when large number of treatments needs to be tested. More recently, IVFT techniques have been used to assess the potential of diet, dietary ingredients and modifiers of rumen fermentation to decrease methane (CH_4) emissions from ruminant livestock (Bodas et al., 2008; Durmic et al. 2010). For many research groups with limited resources, the use of *in vitro* tools is often the only option available for investigating potential agents for CH_4 mitigation. Depending on the research question, *in vitro* studies can be valuable for screening and informing on the suitability for further evaluation *in vivo*. However, a positive outcome *in vitro* does not guarantee that the same treatment will have a similar effect *in vivo*. In some cases, IVFT results for feed evaluation and CH_4 mitigation can be misleading when the inherent characteristics of a batch culture system are not carefully considered (reviewed by Dijkstra et al., 2005). Furthermore, the goals, experimental design, results and conclusions of *in vitro* experiments require cautious and considered interpretation. Often the findings from *in vitro* studies have little relevance to commercial conditions, simply because in the amounts tested, an additive would be too expensive for use on-farm, efficacy cannot be confirmed *in vivo* or have detrimental effect on animal health and function.

The FACCE-JPI 'Global Network' project is an international initiative that intends, among other goals, to develop reliable and robust guidelines for generating and

evaluating data from *in vitro* and *in vivo* experiments examining the potential to mitigate greenhouse gas (GHG) emissions from ruminant livestock systems. The present review is one output from the project that provides a critical evaluation of the key points for consideration when planning and performing *in vitro* studies and guidance for end users in the interpretation of experimental data from IVFT experiments. Major emphasis is placed on factors that influence microbial activity within *in vitro* systems typically used to assess CH_4 production, and how these can be balanced or accounted for, rather than simply providing a contrast and comparison of reports in the scientific literature. It is not intended to provide an in-depth and comprehensive description of different *in vitro* systems available, but rather provide an appraisal of key aspects that are central to undertaking robust, representative and reproducible *in vitro* experiments.

2. History and use of *in vitro* batch culture

Early *in vitro* studies focused on endpoint measurements such as the extent of substrate degradation (Tilley and Terry, 1963). In the 1970's, researchers recognized that measurement of fermentation gases in combination with dietary chemical composition could be used to estimate both feed metabolisable energy content and ruminal organic matter degradability. Czerkawski and Breckenridge (1975) developed a system that involved recording the direct displacement of a piston by gases produced during the fermentation of feeds by rumen fluid in a glass syringe. This was the basis of the 'Hohenheim Gas Test' developed by Menke et al. (1979, Table 1). The 'syringe technique' was originally developed to determine end-point fermentation of feeds after 24 h of incubation. Blümmel and Ørskov (1993) modified the technique by incubating syringes in a water bath rather than a rotating incubator. By recording gas production at

more frequent intervals, the kinetics of fermentation could also be determined.

Wilkins (1974) described a different approach to measure fermentation kinetics *in vitro*, whereby fermentation took place in a sealed vessel containing rumen fluid, buffer and substrate, and a pressure transducer was used to measure gas accumulation in the vessel headspace. In the simplest setup of this system, headspace pressure is measured manually, as described by Theodorou et al. (1994, Table 1), while gas samples are collected for the analysis of CO_2, CH_4 and/or H_2 concentrations when gas pressure is released (Tekippe et al., 2013). In the 1990's the first automated pressure based systems were developed (Pell and Schofield, 1993, Table 1), providing real-time measurements of gas accumulation allowing for a better understanding of the kinetics of fermentation for a range of substrates (Groot et al., 1996). During the development of these systems, it became increasingly clear that increased pressure within the fermentation container could affect fermentation end-products (Jouany and Lassalas, 2002) and the rate and extent of fermentation (Tagliapietra et al., 2010). More advanced systems periodically release and collect the gas via a solenoid valve (Cone et al., 1996; Davies et al., 2000, Table 1) thereby avoiding the build-up of pressure. Even in automated systems, analysis of gas composition (e.g. CH_4) typically requires manual injection of sample gases into a gas analyzer (Martínez et al., 2010; Pellikaan et al., 2011).

Cornou et al. (2013) described the results of a ring-test evaluating the use of a wireless system for automated gas release developed by Ankom (Ankom Technology, Macedon, NY, USA). This system is being used in various laboratories, but still relies on manual gas sampling and analysis.

More recently Muetzel et al. (2014) developed an automated gas measurement system by which gas production is monitored in real-time via pressure sensors and the

proportion of CH_4 and H_2 in the vented fermentation gases is measured automatically by gas chromatography. The main difference with previous automated systems (Cornou et al., 2013) is that fermentation gases are collected and analysed by a computer-controlled gas chromatograph, rather than being released into the air once a threshold pressure is reached.

In vitro gas production systems have been used extensively for rapid screening of chemical substances, plant species, plant extracts and dietary ingredients on CH_4 emissions from rumen fermentation. Such experimental approaches have allowed the mode of action of a range of chemicals (Busquet et al., 2005; Bodas et al., 2008; Garcia-Gonzalez et al., 2008; Durmic et al., 2010) and dietary substrates (Patra and Yu, 2013; Hatew et al., 2015) to be investigated. Use of IVFT offers the opportunity to evaluate a broad spectrum of chemical agents alone or in a number of combinations over a wide range of concentrations (e.g., Busquet et al., 2005; Garcia-Gonzalez et al., 2008). However, this technique does not generate reliable information for agents that are only effective for decreasing CH_4 emissions over an extended period (Castro-Montoya et al., 2015). Furthermore, results from screening studies (Bodas et al., 2008; Durmic et al., 2010) are often inconclusive and may be conflicting due to variation in dosage, chemical structure of the test substance or compound, diet, combination of treatments applied, adaptation of rumen microbes or the form in which an agent is introduced into the system (Cardozo et al., 2004, 2005). Substantial decreases of CH_4 production *in vitro* (Tan et al., 2011) have been reported, but in several cases these have been accompanied by adverse effects on feed degradation, with the implication that a similar effect may occur *in vivo* which would compromise diet digestibility and animal performance.

Please insert Table 1 around here

3. Aspects to consider

3.1. Donor animal species and animal numbers

Different animal species (sheep, goats, cattle and buffaloes) may vary in their response to the same CH_4 mitigation strategy. The obvious recommendation is to use the same species as donors of rumen fluid for *in vitro* incubations as the intended target species. However, this is not always possible, and due to cost small ruminants are often used as donors of rumen contents, even when cattle are the target species. One key question is, therefore, whether sheep or goats can be used as suitable surrogates for cattle for the study of CH_4 production *in vitro*.

Bueno et al. (1999) compared the microbial biomass in bovine and ovine rumen fluid and, although no intake data were reported, the inocula were adjusted to provide the same microbial biomass. It was concluded that rumen fluid from sheep could replace that from cattle or vice versa as rumen inoculum and that the two sources were comparable under tropical feeding conditions. Cone et al. (2002) reported a comparison of rumen samples collected in the same way from sheep and cattle maintained under similar conditions. Incubations of 22 different feeds were performed. A close association was observed ($r = 0.98$) for gas production at 24 (ranging from 100 to 325 ml/g OM incubated) and 48 (ranging from 150 to 350 ml/g OM) h for incubations with rumen fluid from cows and sheep. However, the relationship based on the rate of gas production (ml/g OM/h) was weaker ($r = 0.79$). Calabro et al (2005) compared rumen fluid from buffalo and sheep as a source of inoculum and observed higher fermentation rates and extent of degradation during incubations with rumen fluid from sheep. Differences in fermentation kinetics were greater when fibre-rich substrates were tested, such as straw and hay, but negligible for barley grain.

Muetzel et al. (2014) compared rumen fluid from cattle (Holstein × Jersey cows) and sheep using a newly developed automated *in vitro* system and reported that total gas production is unaffected by donor animal species. Prior to rumen sampling, cattle and sheep had been adapted to a medium quality hay diet fed to meet maintenance energy requirements for 14 days. However, proportions of CH_4 in vented fermentation gases were lower during incubations with rumen fluid from sheep than cattle (150 vs. 158 ml/L, P=0.003) associated with a lower proportion of acetate and a higher proportion of propionate. There is no evidence of differences in methanogen communities in sheep and cattle (Jeyanathan et al., 2011), which suggests that the differences are driven by H_2 production from the bacterial or protozoal communities. No interaction between animal species (sheep vs. cattle) and the type of substrate incubated (chicory, lucerne, ryegrass, straw and white clover) was observed (Muetzel et al., 2014). Bueno et al. (2015) compared *in vitro* CH_4 production using rumen fluid from taurine dairy cattle (*Bos taurus* taurus), zebu beef cattle (*Bos taurus* indicus), water buffaloes (*Bubalus bubalis*), sheep (*Ovis aries*) and goats (*Capra hircus*) fed similar diets while testing the effect of condensed tannins from an Acacia extract. Rumen fluid from cattle resulted in higher CH_4 per unit of degraded organic matter (OM) formation than rumen fluid from small ruminants.

Although microbiota of ruminant species housed in close contact and fed a similar diet may be of a similar composition, the microbial ecology of rumen samples between sheep and goats, for example, may vary due to differences in dentition, eating and ruminating behaviour, digestive tract physiology and ruminal retention time (Ammar et al., 2004). For this reason, collection of inoculum from animals of the same target species fed a diet containing the same feedstuffs would be recommended. Differences due to feeding behaviour and diet composition can to some extent be overcome by the

collection of rumen samples before morning feeding, when the effect of diet composition on rumen metabolites or microbiota are likely to be minimized (Martinez et al., 2010) (see section 3.3).

There remains some uncertainty on the number of animals that need to be sampled to provide a representative sample of rumen inocula. Several studies using different ruminant species (e.g., Pinares-Patiño et al., 2003; Waghorn et al., 2006; Yan et al., 2006) have reported that CH_4 emissions per unit dry matter intake vary between individual animals. Such variation has been associated, among other factors, with differences in the rumen microbiome associated with between-animal variation in passage rates, rumen volume and morphology, eating behaviour, etc. (Kittelman et al., 2014). Martínez et al. (2010) observed consistent differences in CH_4 production and H_2 recovery *in vitro* during incubations with rumen liquor collected from 6 different sheep fed the same diet. Such differences can only be explained by differences in microbial populations or activities in the starting inocula or variation in the survival or activity of microbes over the incubation *period.* To reduce the effects of unusual rumen inocula, it has been suggested that, normally, at least 3 animal sources should be used to provide a representative source of rumen inoculum (Editorial, Animal Feed Science and Technology, 2012). However, this might be difficult when large ruminants are used. Another issue to consider is the number of independent incubation runs to be conducted in different days, which should be at least 3. Unfortunately, there is no work available assessing in statistical terms the minimum number of donor animals needed to provide a representative source of rumen inoculum.

RECOMMENDATION: Where possible the target animal species should be used as the donor of rumen fluid. Sampling before feeding is advantageous for minimizing diet by

animal interactions. We recommend using 3 or more (optimum) or 2 (minimum) animals as donors of rumen inoculum and at least 3 independent incubation runs.

3.2. Donor animal diet

Diet composition and nutrient intake are major factors affecting both microbial populations in the rumen and microbial activity of rumen inoculum (Mould et al., 2005). Compared with ruminants fed high-concentrate diets, a greater proportion of fibrolytic bacteria and methanogenic archaea can be expected in rumen fluid collected from animals on high-forage diets (Demeyer and Fievez, 2000). However, the extent to which concentrate feeds affect rumen digestion and microbial populations may depend on the source and proportion of concentrate ingredients in the diet, as well as forage quality (Dijkstra, 1994).

Martínez et al. (2010) assessed the effect of feeding sheep diets differing in forage:concentrate ratios (F:C; 70:30 vs 30:70) and forage source (alfalfa hay vs grass hay) on rumen fermentation and CH_4 production. *In vitro* CH_4 production (per g of incubated DM) was increased by decreases in the F:C ratio of diets fed to donor animals or when Lucerne hay was replaced by grass hay. Differences in the F:C ratio altered pH and the activity of certain glycoside hydrolases (carboxymethylcellulase, xylanase and amylase) in rumen fluid. Forage type also influenced NH_3 content and carboxymethylcellulase activity in rumen fluid. These results suggest that feeding donor animals a diet similar to the substrate to be incubated *in vitro* may be advantageous.

Of particular importance is the observation that some mitigation strategies have been evaluated using rumen fluid from animals fed poor quality feeds. This raises the question of whether outcomes on the efficacy of mitigation agents under these circumstances can be considered reliable when donor animals have not been fed a diet

of similar characteristics as that offered to target animals. The effects of differences in diet composition fed to donor animals may be minimized by obtaining rumen fluid immediately before feeding. Huntington and Givens (1998) obtained fluid from cows fed either a silage:barley diet (80:20) or a barley straw diet. Although microbial activity of rumen fluid collected on the straw diet was lower than the silage:barley diet, this did not alter the gas production profile, which was attributed to sampling prior to morning feeding.

The diet fed to donor animals also needs to be considered when testing additives as CH_4 mitigation agents. Mateos et al. (2013) reported that the effect of garlic oil and cinnamaldehyde on *in vitro* fermentation and CH_4 production varied depending on whether the donor animals were fed a typical dairy diet (alfalfa hay:concentrate 50:50) or a fattening diet (barley straw: concentrate 15:85). Observations from several *in vitro* studies suggest that the effects of essential oils on rumen function are pH-dependent, and this also appears to be true for garlic oil and some of its components (Cardozo et al. 2005; Kamel et al. 2008). Cardozo et al. (2005) found that garlic oil had a more pronounced impact on rumen volatile fatty acids (VFA) profile at low compared with high rumen pH (5.5 versus 7.0), an effect explained by differences in the status of the active molecules (i.e. dissociated or un-dissociated) possibly mediated by changes in rumen pH. However, batch cultures are usually highly buffered systems allowing ruminal microorganisms to grow for a prolonged period despite the accumulation of fermentation end-products. In such cases, factors other than pH (e.g. microbial composition) may explain differences in the efficacy of additives. Hatew et al. (2015) provided further evidence on the importance of diet fed to the donor animal. Experiments involved the incubation of the same substrate (grass silage or beet pulp) with rumen inoculum obtained from donor cows fed on diets that differed in starch

source (native vs. gelatinized maize grain) and starch level (270 vs 530 g/kg concentrate DM). A higher level of starch and gelatinized rather than native maize were found to lower gas and CH_4 production after 24-h incubations.

Level of feed intake is also an important consideration, given that a higher DM intake (DMI) lowers retention time in the rumen, decreasing the amount of time available for feed degradation of feeds and hence ruminal digestibility (Clauss et al., 2007). Rumen pH, proteolytic and cellulolytic activities are thought to be influenced by the level of DMI which can in turn influence growth rates and the metabolic activity of inoculum used in *in vitro* systems. Increasing feeding frequency will generally lower diurnal variation in rumen fermentation parameters. For example, in lactating cows increasing feeding frequency from two to six times-daily was found to decrease post-feeding variation in rumen pH, osmolality, VFA and NH_3 concentrations (Le Liboux and Peyraud, 1999).

The period of adaptation to a given diet by the donor animal probably needs to be revisited. It is common to collect rumen fluid from animals fed a diet for 2 weeks. However, there are indications that the methanogenic archaeal population requires an adaptation period of around 30 days after a change in diet (Williams et al., 2009). Also Monteils et al., (2012) reported that protozoa counts in the rumen needed 25 days to stabilize after different dietary changes. Given the lower generation time that protozoa and archaea have as compared to bacteria (Dehority, 2003), further research is required to assess the effect of dietary treatments on the adaptation time needed for methanogenesis tests.

RECOMMENDATION: The diet fed to donor animals should be similar in composition to the substrate incubated in vitro. Care should be taken to ensure sufficient buffering capacity when investigating diets or dietary ingredients or additives that promote

differences in rumen pH (see section 3.4), in particular when samples are not taken immediately before feeding. It is recommended that donor animals are fed a diet at a restricted level of feeding as frequent meals to ensure constancy of diet composition and digestion, minimize variation in feed intake and avoid diurnal variation in rumen fermentation.

3.3. Rumen fluid sampling: time, location and processing

In all *in vitro* fermentation systems it is essential to create an environment, which, for any set of parameters, mimics the fermentation in a specific section of the gastro-intestinal tract *in vivo* (e.g. reticulo-rumen or caecum). Therefore, the inoculum should be representative of that environment with respect to both the composition and abundance of the microbial population. For *in vitro* systems to be robust (i.e., reproducible over time and representative of conditions *in vivo*), the inoculum must meet certain criteria. Making a valid assessment of whether a given study has met these criteria may be problematic, as often-essential information is not reported. Given the precision of gas release kinetic techniques relative to degradability at a set end-point over extended periods, variations in inoculum characteristics due to host animal effects, nutrition and sampling time, as well as sample preparation and inoculation, can have substantial cumulative effects on *in vitro* fermentation. It seems pertinent, not only to permit comparison between studies, but also to limit potential errors, to have a set of accepted guidelines and standard procedures for preparing inoculum for measuring CH_4 *in vitro*, as proposed for animal studies *in vivo*. Such guidelines should include host animal management, sampling techniques (time, location, alternatives) and inoculum preparation.

Sampling time:

Diurnal changes of the rumen microbiome, both in terms of abundance and metabolic activity have been documented. Concentrations of viable microbial populations in the rumen typically decrease 4 h post-feeding, due to dilution with feed, water and saliva, and peaks at 6-12 h post feeding (depending on diet and level of feed intake) (Leedle et al., 1982; Dehority, 2003). Furthermore, microbial abundances were found to decline during the degradation of available nutrients. Cone et al. (1996) observed that the rate of fermentation was highest when rumen fluid was collected after morning feeding, although rumen sampling time had no effect on the total gas production. Menke and Steingass (1988) stated that sampling rumen contents just before feeding lowered variation in composition and activity of the inoculum and minimized the influence of diet fed to donor animals. However, Payne et al. (2002) observed that the total gas production from both starch and ground straw were less variable between replicate bottles and between weeks of collection when rumen fluid inoculum was collected either 4 or 8 h post feeding, compared with samples collected just before, or 2 h after feeding. Presumably, the activity of the inoculum is determined just as much by time of rumen sampling relative to feeding as by feeding pattern and eating time. Furthermore, microbial diversity and activity is at its lowest before feeding and while this reduces the variability, it may not reflect the 'true' effect that the feed/additive may have on much diverse population and their activities present after feeding. This deserves further research in the future.

RECOMMENDATION: There is no general recommendation on the ideal rumen sampling time as it depends on the objectives of a specific experiment. Given the difficulty of minimizing diurnal variation, rumen fluid samples are best collected

immediately before feeding, based on a consistent protocol for dietary access by donor animals across experiments. When a series of studies are conducted over time, feeding and sampling procedures should be kept as identical as possible.

Sampling procedure: Rumen digesta is comprised of different fractions (large and small particulate matter and liquid). Ample evidence exists on the different abundance and diversity of bacteria associated with the liquid and solid ruminal contents (Pei et al., 2010). The pH and VFA concentration varies between different sections of the reticulo-rumen in cattle (Bryant 1964). De Visser et al. (1993) indicated that rumen VFA concentrations were about 20% higher for the entire evacuated rumen content compared with calculations based on samples of rumen fluid collected in a standardized manner. Storm and Kristensen (2010) indicated differences of 0.4 to 0.6 pH units and of 40 to 50 mM VFA between the central and ventral regions of the rumen, with the lowest pH and highest VFA concentrations in the medial rumen. Rumen fermentation parameters have also been found to differ between samples collected at different locations in the rumen (Shen et al., 2012), implying possible differences in microbial abundance and activity within the rumen.

Rumen cannulation is considered the reference method allowing the collection of representative samples of rumen digesta from donor animals (Komarek, 1981; Kristensen et al., 2010). Access to surgically-modified animals is not universal, and therefore less invasive techniques, such as oral stomach probing, have been used as an alternative. In the relatively few studies that have compared sampling through the rumen cannula or by stomach probing, differences in fermentation profile and microbiota have been reported in some (e.g., Geishauser and Gitzel, 1996; Duffieldet al., 2004), but not all cases (e.g., Lodge-Ivey et al., 2009; Shen et al., 2012; Terré et al.,

2013). Part of the discrepancy between studies may reflect differences in the procedures used to avoid salivary dilution and contamination, the type of samples collected and rumen sampling site. Stomach probing results in the collection of samples containing a high proportion of liquid, whereas sampling via a rumen cannula allows both solid and liquid digesta fractions to be obtained. Differences in the methods used to collect rumen samples are of greater relevance when treatments are not expected to have the same effect on microbial populations attached to solids or inhabiting the liquid phase (Martínez et al., 2010). The study of Shen et al. (2012), attributed the differences between samples collected via cannula or stomach tube to rumen sampling site, as a consequence of the probe not being inserted to a depth sufficient to reach the ventral sac. Accurate probe insertion to a desired location within the rumen is extremely challenging in small ruminants. In a recent study, Ramos-Morales et al. (2014) found that stomach-tubing in sheep and goats detected the same differences in rumen fermentation due to species, diet or sampling time as sampling via the cannula. However, certain differences were more readily detected in rumen cannula samples, while substantial differences in the bacterial community structure were detected between the sampling methods.

Faeces has also been used as an alternative source of inoculum to rumen fluid (El Shaer et al., 1987). Cultures of ruminal or faecal microorganisms appear to result in similar fermentation processes (El-Meadaway et al., 1998). However, fewer microorganisms in faecal inocula may result in lower degradation capacity and decreased gas production (Cone et al., 2002; Vàradyovà, et al., 2005), a longer lag phase and a slower rate of degradation at the outset (Mauricio et al., 2001). Furthermore, for poor-quality forages, there is only a weak relationship between gas production during incubations with inocula sourced from faeces and rumen fluid (El-

Meadaway et al., 1998; Varadyova et al., 2005). Dhanoa et al. (2004) proposed a method allowing mathematical adjustments to convert or translate the degradation profiles produced by faecal inoculum to correspond with ruminal fluid, but this requires application of different prediction equations for each group of feeds incubated.

Rumen contents collected post-morten at abattoir can also be used as an alternative to rumen fluid (Mould et al., 2005). Several IVFT have been performed using rumen fluid collected from slaughtered cattle, sheep, buffalo and dromedary (Haddi et al., 2003; Salem, 2005). To date, there are no reports directly comparing the use of rumen fluid from slaughtered animals with oral or rumen sampling in the same animal. Such an approach requires sampling of rumen contents soon after slaughter, as well as the same criteria for other sources of inocula being met. While the intake and diet composition of slaughtered animals are not known, access to entire rumen contents allows the collection of representative samples that can also be used to inform on nutrient supply. If sampling of rumen cannulated animals is not possible, collection of rumen content from slaughtered animals may prove a viable alternative.

RECOMMENDATION: To be as representative of the rumen environment as possible, samples of ruminal contents for the preparation of in vitro inoculum need to be collected from several locations. This is more feasible in large ruminants and requires the collection of rumen contents from animals fitted with rumen cannula following a clearly defined and standardized sampling protocol. Stomach tubing or faecal inoculae may serve as an alternative for ranking purposes, but quantitative data using these alternatives may differ from sampling of rumen contents in cannulated animals. Stomach tubing should be performed by well-trained persons to minimize salivary dilution.

Preservation of inoculum: Anaerobiosis is essential to culture rumen microorganisms, methanogenic archaea, in particular (Joblin, 2005). Certain experiments may require rumen inoculum to be stored until culturing *in vitro*. Short-term storage (<1 h), for example during transport from donor animals to the IVFT laboratory, should exclude exposure to air. It is equally important that any increase in headspace pressure does not cause CO_2 to go into solution, thus lowering pH. Excessive fermentation due to extended storage at 39°C should be restricted to prevent any microbial group from becoming dominant and modifying the composition of the original inoculum.

The influence of storage time and temperature on neutral detergent fibre (NDF) degradation by rumen microorganisms has been investigated (Robinson et al., 1999). Studies involved the use of the ANKOM end-point system to examine the effect of delaying inoculum storage at 39ºC by up to 6.5 h, or up to 48 h after storage over a range of temperatures from – 22 and 39 °C. No apparent effect on 48 h end-point degradation of medium-term (6.5 h) storage compared with long-term storage (48 h) was identified. Authors concluded that no storage method, irrespective of temperature, would maintain rumen inoculum activity for up to 48 h that support normal fermentation *in vitro*. Subsequent works (Cone et al., 2000; Hervás et al., 2005; Prates et al., 2010) presented data on the effect of using rumen fluid directly or stored anaerobically at 39°C for increments of up to 24 h or at –24°C for 1, 3, 10, 40 or 76 days before use. In general, gas production in terms of kinetics and cumulative yields, decreased as storage period increased. Final gas volumes were similar when rumen fluid was stored up to 4-6 h compared with no prior storage. Gas production rates were lower for inocula stored for 8 or 24 h, while gas production was considerably decreased by extended storage at -20ºC of more than 10 days. Microbial activity was lowered by

freezing, with the decrease being substrate-dependent with the degradation of pure starch and cellulose being less affected compared with lucerne hay and barley straw. Freezing in liquid nitrogen is preferred over storage at -20ºC (Prates et al., 2010) and thawing of small volumes (approximately 20 ml) at 39ºC for 2 min. The time taken for freezing and thawing appears to be as equally important as storage temperature. Overall, studies suggest that preservation of rumen fluid at 0ºC for up to 6 h offered a practical alternative, where necessary, to freshly collected inocula. Protozoa are lost after freezing which could have an impact on the fermentation. There are, however, no reports documenting the effect of different preservation methods on methanogenic activity.

RECOMMENDATION: Fresh rumen fluid maintained under anaerobic conditions at 39ºC should be inoculated into in vitro vessels as soon as possible, ideally within 1 h post collection. When this is not possible, rumen fluid can be preserved at 0-4 ºC for up to 6 h or frozen in liquid nitrogen following addition of a cryo-protectant (i.e. 15% glycerol) for longer periods for use as inoculum. In either case, implementation of standardized procedures to avoid undesirable variation in microbial activity is highly recommended.

Preparation of inoculum prior to incubation: Method of preparation also influences the microbial activity of rumen inoculum. The rumen microbiome consists of three sub-populations of microbes: those in the fluid phase, adherent to the particulate phase (further divided into loosely- and firmly-associated with the feed particles) (Cheng et al., 1993) or attached to the epithelium (Sadet et al., 2007). The latter tends to be primarily involved in the release of ammonia from urea absorbed across the rumen

epithelium and, as such, has only a minor role in feed degradation, and for this reason does not need to be sampled (Mueller et al., 1984). Fluid and particulate associated bacterial populations (Kim et al., 2004) and methanogenic archaea (Shin et al., 2004) differ in growth characteristics and in the activities of most enzymes (Moharrery and Das, 2001). Microorganisms in rumen liquid (20 to 30% of total microbes) including free-living bacteria and bacteria detached from solid substrate, have little direct involvement in structural carbohydrate digestion (Miron et al., 2001). Microbes attached to feed particles predominate (70 to 80% of microbial matter and microbial ATP production) and play a key role in feed particle digestion in the rumen (Miron et al., 2001; Trabalza-Marinucci et al., 2006). Consequently, once samples of whole rumen contents have been collected, the problem arises of how to effectively detach the microorganisms associated with feed particles. Failure to do so will result in a high proportion remaining to be attached after filtration, while the use of multiple layers of cheesecloth, muslin or surgical gauze leads to different microbial fractions being retained and inoculated *in vitro*. For example, use of a cloth with 50 µm pores would substantially lower the number of large protozoa (which range from to 15–250 µm in size, Dehority, 2003) in inocula. Williams and Coleman (1991) reported that most protozoa are retained using a 100 µm pore size filter. Therefore, if total protozoa are to be included in the microbial culture, a 250 µm pore size cloth should be used. Furthermore, some of the physical methods used to detach particle-associated microbes (e.g., a stomacher or maceration of rumen contents in a food processor) may also cause cell damage. Rymer et al. (1999) examined four methods of inoculum preparation: strained, blended fluid, "stomacher" or strained plus blended residues. No consistent treatment effect was observed, apart from blending which tended to decrease substrate degradation. Authors concluded that there was little advantage from blending inoculum,

particularly in light of the risk of exposing microorganisms to oxygen. Even though homogenizing rumen fluid may increase numbers of particle-associated bacteria in the inoculum, Pell and Schofield (1993) excluded this step on the grounds that it i) introduced an extra procedure into the laboratory protocol, ii) increased the risk of exposing rumen microorganisms to oxygen, iii) increased the quantity of gas released from the blanks, and iv) had no obvious effect on the results of IVFT.

A number of techniques that have been proposed for detaching microorganisms from rumen feed particles that involve various combinations of chemical and physical treatments and h yield removal rates of between 20 and 80% (Hristov et al., 1999; Ranilla et al., 2001). While the use of these techniques is essential when collecting microbial biomass to determine their composition to accurately assess passage of nutrients of microbial origin to the intestine, it is not clear whether a standard protocol for microbial detachment should be applied for *in vitro* methods. In recent work, Soto et al. (2013) studied the development of the microbiota in different *in vitro* rumen simulation systems inoculated with intact or filtered rumen fluid from goats. Incubation of filtered rumen fluid fraction in batch culture resulted in lower microbial diversity compared with non-filtered rumen fluid inoculum. Substantial growth of fibrolytic bacteria and methanogenic archaea over the first 24 h partially compensated for low numbers in the inoculum due to filtering.

RECOMMENDATION: Filtration of rumen fluid using the same pore size across incubation runs is a straightforward method for preparing inoculum suitable for in vitro experiments. A larger pore size results in greater numbers of small particulate associated bacteria and protozoa. A pore size of 250 μm is recommended. Use of multiple layers of cheesecloth is not recommended due to inconsistencies in pore size.

3.4. Substrate and incubation buffer

Substrate:

Provided donor animals are fed the same or a similar diet as that tested *in vitro*, there remains an uncertainty of the choice of substrate to be used in the evaluation of additives on CH_4 production *in vitro*. The composition of diets fermented *in vitro* determines the production of dissolved H_2 that serves as a substrate for methanogens. Most *in vitro* studies have tested additives in incubations with a single substrate, but there are reports on the effects of additives using different fermentation substrates in a single experiment. Machmüller et al. (2001) investigated effects of medium chain fatty acids in incubations containing high or low amounts of fiber. The efficacy of monensin has been examined using corn meal or timothy hay (Russell and Strobel, 1988) and corn meal or soyabean hulls (Pellikaan et al., 2011) as substrates. In certain instances a substrate by additive interaction has been observed. Pellikaan et al. (2011) reported a complete inhibition of CH_4 production during the first 30 h of incubation, irrespective of substrate composition (soyhulls or maize), with bromoethanesulphonate (BES) and cinnamaldehyde. However, CH_4 production from soybean hulls with BES or cinnamaldehyde was 65% lower compared with soyhulls without additive after 72 h, suggesting an influence on adaptation, whereas no CH_4 was produced after 72 h production when maize was incubated with these additives. Subsequent studies covering a range of plant extracts and fatty acids (Castro-Montoya et al., 2012; Klevenhusen et al., 2012; O'Brien et al., 2013) have provided further evidence that responses of CH_4 to a given additive differs depending on the feed substrate incubated. For example, addition of fatty acids were found more effective in lowering CH_4 during incubations containing higher proportions of concentrate ingredients, an effect attributed to greater

protonation of fatty acids at a lower pH (e.g. Zhou et al. 2015). While specific experiments have provided examples of substrate by additive interactions, drawing firm conclusions on the magnitude of these effects remains challenging. Nevertheless, there is a need for end users to recognize that specific characteristics of incubated substrates impact on the outcomes of IVFT and be aware of a possible mismatch between the diet of the donor animal and incubated substrate.

Implementing a standardized protocol for preparing substrates to be incubated is also critical in allowing for between IVFT comparisons (Rymer et al., 2005). The most critical issue appears the methods used to dry fresh material, such as grass. Comparisons of freeze-drying with oven drying at 60 or 105ºC are often contradictory. There are reports on the effect of feed processing on CH_4 production. Nevertheless, *a priori* freeze-drying is the method of choice for minimizing cell damage that potentially alters the dynamics of microbial attachment, substrate degradation and altering bioactive compounds (Rymer et al., 2005).

With regards to particle size, using cereals as substrates resulted in particle size having little effect on *in vitro* fermentation, provided that the grain kernel had been cut (Lowman et al., 2002), although some evidence to contradict this argument exists with maize (Rymer et al., 2005). With fibrous and more slowly degraded feeds, fermentation rate increases as particle size decreases (Lowman et al., 2002) and it seems likely that this is a consequence of increased surface area as a result of grinding, thereby allowing better microbial access. The most common procedure is to mill the substrate through a 1 mm screen and store under dry, cool and dark conditions in sealed containers prior to use. Adoption of a standardized approach to sample preparation may enable comparison between independently produced *in vitro* fermentation data of different feeds.

RECOMMENDATION: It is recommended that a range of substrates that reflect the types of feeds used in commercial production systems are used in the initial screening of new additives, unless the objective of an in vitro experiment requires a predefined substrate. Freeze-drying is preferred to oven drying for the drying of high moisture substrates.

Incubation medium and rumen fluid:medium ratio (RF:M)

There is considerable variation in the composition of the medium used for *in vitro* studies reported in the literature. It is important to make a distinction between the term 'medium' (i.e., a solution containing a number of components including buffering agents, trace elements, true protein and reducing agents) and 'buffer' (Williams, 1998). The types of buffers used in IVFT and the implications on fermentation has been comprehensively reviewed (Rymer et al., 2005). A medium with a high buffering capacity, when used in IVFT, may be disadvantageous because it creates conditions that are not representative of the rumen *in vivo*. This is particularly important when assessing the effectiveness of CH_4 mitigations strategies that may rely on a decrease in rumen pH that include an inhibition of fibrolytic bacteria and/or methanogens (Argyle and Baldwin, 1988; Van Kessel and Russell, 1996; Navarro-Villa et al., 2011). Furthermore, through the prevention of a sharp decline in pH, a highly buffered medium may increase acetic:propionic acid ratios more than would otherwise occur *in vivo*, which impacts on the availability of H_2 for CH_4 production (Lana et al., 1998).

The effect of the incubation medium and rumen fluid to medium ratio (RF:M) on IVFT has been investigated (Rymer et al., 1999; Pell and Schofield, 1993; Cone et al., 2000). Such studies have demonstrated that increases in the proportion of rumen fluid is associated with a decrease in lag phase and a higher rate of gas production,

while the effect on total gas production varies. Navarro-Villa et al. (2011) investigated the effect of variable RF:M ratios (1:2, 1:4, and 1:6) in IVFT involving the incubation of different amounts (0.3, 0.5, and 0.7 g) of three contrasting feeds (barley grain, grass silage and barley straw) . The results indicated that CH_4 per unit of DM degraded was a more appropriate unit for expressing *in vitro* CH_4 output than CH_4 per unit of DM incubated. Incubation of 0.3 g dried milled feed in 50 ml of *in vitro* culture containing 1:2 RF:M was an acceptable combination for 24 h incubations allowing for a decline in pH declined and maximizing the difference in CH_4 output between substrates.

For a given substrate, pH in the incubation vessel should ideally mimic that in the rumen, i.e. between 6.0-7.0 for forage based diets and 5.5- 6.0 for concentrate based diets. Patra and Yu (2013) evaluated IVFT containing different bicarbonate concentrations (80, 100, and 120 mM) in buffer. Results indicated that bicarbonate concentrations above 80 mM should be avoided to minimize non-microbial CO_2 production associated with changes in pH. A recent comparison of two buffers commonly used in IVFT (McDoughall's (McDoughal, 1948) and Mould's (Mould et al., 2005) buffer indicated that buffer composition had no effect on total gas production (Muetzel et al., 2014). These findings are in direct contrast to earlier reports that a higher phosphate concentration decreased gas production over a 9 h fermentation period (Mould et al., 2005). The differences the latter authors reported, however, were about 4% and decreasing over incubation time. A trend towards higher gas production for McDoughall buffer compared with Mould's buffer may be related to a higher carbonate concentration (Muetzel et al., 2014). The buffer composition had no effect on CH_4 production or on the percentage of CH_4 released or on VFA production. However, the proportions of major VFA were altered; molar proportion of acetate was higher and that of propionate was lower for incubations with Mould´s buffer compared with

McDougall´s buffer. It is possible that differences in the phosphate to carbonate ratio and associated changes in pH may be responsible (Broudiscou et al., 1999). Based on the work of Kohn and Dunlap (2008) a recent report demonstrated that by adjusting the concentration of buffer bicarbonate, pH can be reasonably well controlled at specific target pH (6.50, 6.25, 6.00, 5.75 and 5.50) during 12 h incubations (Amanzougarene et al., 2015). What is less clear is whether different antimethanogenic additives perform similarly in IVFT over a range of pH.

RECOMMENDATION: Available data do not allow for recommendations on an ideal RF:M. A ratio of 1:2 appears to generate the most reliable results for 24 h incubations. However, this ratio and the amount of substrate incubated needs to be considered on the basis of the frequency of gas sampling and the duration of the incubation depending on the research objectives. Bicarbonate concentration in buffer may influence methanogenesis. To minimize non-metabolic CO_2 production use of buffers containing bicarbonate concentrations above 80 mM should be avoided. Furthermore, adjusting the concentration of bicarbonate in the buffer or the RF:M ratio offers the possibility of setting a target pH according to the substrate incubated.

3.5. Incubation procedure and CH_4 measurements

In vitro gas production systems are typically conducted over intervals of between 16 to 72 h. As such, IVFT do not mimic important physiological processes *in vivo* such as ruminal digesta turnover. Removal of soluble particles in the liquid medium may have adverse effects on microbial fermentation, by decreasing the amount of soluble substrate available for microbial growth or may conversely stimulate activity (Roger et al., 1990). Soto et al. (2013) reported that the numbers of all quantified microorganisms

(total bacteria, protozoa, methanogens, fungi, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*) declined sharply during 24 h to 72 h of incubation. This is likely due to the exhaustion of fermentable substrate and the accumulation of fermentation end products. Different substrates (soluble carbohydrates, starch, pectins, cellulose, hemicellulose and protein) that are fermented can be degraded at different rates also have a variable contribution to CH_4 production (Bannink et al., 2006). In cows, mean retention time of NDF components in the reticulorumen is around 28 h (using external markers, Schwarm et al., 2015) or 28 to 60 h (using intrinsic stable isotope labelling techniques, Warner et al., 2014), being longer than the standard *in vitro* incubation time of 24 h. Retention time in the reticulorumen is related to particle size (Schwarm et al. 2008). It can be argued that NDF fermentation during *in vitro* incubations probably approaches a plateau after 24 h given that relatively small feed particles (ca. 1 mm) are usually incubated.

Some IVFT have tested the effects of different substrates on CH_4 production based on the collection of a single gas sample after 24 h (García-González et al., 2008). Such an approach may result in gas pressure in the headspace exceeding a given threshold (48 kPa) and consequently an impairment of microbial activity (Rymer et al., 2005; Taglapietra et al., 2010). Several protocols, such as those described by Theodorou et al., 1994, Cone et al., 1996 and Davies et al., 2000, stipulate that headspace gases being released at pre-determined intervals or when a pre-set threshold of pressure is reached (Muetzel et al., 2014). Venting the gas produced requires that CH_4 concentration is measured simultaneously, given that different CH_4 concentrations can be expected depending on substrate. Although the most common method to measure CH_4 concentration on gas samples collected from incubation vessels is by using gas chromatography, as for *in vivo* measurements, other techniques are available and have

been used such as infrared methane analysis (Goel et al., 2008, Cobellis et al., 2015) and absorption of CO_2 (Fievez et al., 2005).

For successful IVFT, it is essential that experimental treatments be randomly allocated to bottle positions. The order in which bottles are inoculated also needs to be randomized across bottle positions and treatments. Randomization serves to minimize possible confounding effects of bottle position (or water baths used), treatments and timing of inoculation. Such an approach is analogous to the random allocation of treatments to animals for *in vivo* experiments. It is also important to consider establishing incubations with a single, identical source of medium, and a single, identical source of substrate. Finally, independently of the number of independent incubation runs performed, different replicates (bottles) of each treatment need to be included. Three bottles minimum is sensible as it allows possible outliers to be identified.

RECOMMENDATION: The duration of the incubation should be adjusted based on the composition and physical properties of substrate incubated that determines the frequency of gas sampling required for measuring CH_4 production. Gas composition should be determined at the same time gas pressure is vented. It is highly desirable that the timing of inoculation and the allocation of treatments with respect to bottle position are randomized as much as possible. It is recommended that 3 bottles be used per unique treatment in each incubation run.

3.6. Headspace gas composition

It is well established that H_2 concentration can affect the thermodynamics of fermentation and the growth rate of hydrogenotrophic methanogens in the rumen

(Janssen, 2010). Hydrogen produced in the rumen is present in two forms, as dissolved H_2 and as H_2 gas, but methanogens only utilize dissolved H_2 (Wang et al., 2014). Reports on the influence of headspace gas composition on *in vitro* gas production and rumen fermentation are scarce. In one study, Patra and Yu (2013) noted that initial CO_2 headspace, but not N_2 headspace, was positively correlated with CH_4 production after fermentation. This prompted the hypothesize that headspace gas composition, CO_2 in particular, which is in exchange with H_2CO_3/HCO_3^- in the medium, depending on concentration, acid-base balance and gas pressure, may affect fermentation characteristics and gas production in ruminal *in vitro* cultures. A range of initial headspace composition of *in vitro* cultures has been reported, including 100% CO_2 (Anderson et al., 2003; Weimer et al., 2005), 100% N_2 (Hoover et al., 1976) and a mixture of gases typical of an anaerobic chamber (85% N_2, 10% H_2, and 5% CO_2; Zhou et al., 2011; Patra et al., 2012). Patra and Yu (2013) investigated the effects of three different headspace gases ($N_2 + CO_2 + H_2$ in the ratio of 90:5:5, 100 % CO_2, and 100 % N_2) and the interaction with type of substrate (alfalfa hay or alfalfa hay and concentrate) and media bicarbonate concentration on gas and CH_4 production. Methane production was much higher when CO_2 was present in the headspace. It is conceivable that equilibrium is established between CO_2 dissolved in the inoculum and CO_2 in headspace gas (Alford, 1976), such that a higher concentration of CO_2 in the headspace would result in a greater concentration of dissolved CO_2 in the media. Higher CH_4 production corresponding to CO_2 in the headspace may be explained by an immediate and greater availability of CO_2 in the inoculum that serves as the electron acceptor for the primary hydrogenotrophic methanogenesis pathway. An increase in dissolved CO_2 may also promote growth and activity of methanogens. Even though CH_4 production in the study of Patra and Yu (2013) was greater when the headspace contained CO_2 rather

than N_2, total or net gas production was lower for the former compared with the latter. Further investigations are required to understand the impact of a mixture of CO_2 and N_2 that best mimics rumen gas composition *in vivo*.

RECOMMENDATION: Owing to the effect of headspace gas composition on gas production, including CH_4 production, it is recommended that all future studies should both consider this as an influencing factor and report headspace gas composition. Rumen fluid should be flushed continuously, and once added into the medium, continued to be flushed for at least 10 min before incubation is initiated. Following the addition of inoculum into the bottle, the headspace should be flushed continuously until the bottle is sealed.

Please insert Table 2 around here

3.7. *In vitro* versus *in vivo*

Numerous studies have examined the influence of antimethanogenic compounds on CH_4 production *in vitro*, but few have undertaken a simultaneous evaluation *in vivo* and *in vitro*. Direct comparison of effects *in vitro* and *in vivo* would allow a better interpretation of IVFT data and inform on the treatments suitable for further evaluation *in vivo*. When addressing inconsistences between results from *in vitro* and *in vivo* studies it is worthwhile considering:

i) The accuracy of *in vitro* systems to predict the CH_4 production of a given diet per unit feed intake or digested matter

ii) The ability to simulate the direction of changes (not absolute values) in CH_4 production when anti-methanogenic agents are tested relative to an appropriate control.

i) Flachowsky and Lebzien (2012), using data from Moss and Givens (1997) reported a poor relationship (r^2=0.264) in CH_4 production obtained in vivo and in vitro methods. Bhatta et al. (2007) compared IVFT measurements of CH_4 production with the SF_6-technique across a range of diets. Methane production (ml/g DM) estimated from 48 h *in vitro* gas production was higher than measurements *in vivo* for all diets. Of particular note is that the average of CH_4 production at 24 h and 48 h was closely correlated with values based on SF_6 (R^2=0.78, 5 diets and 4 animals used) (Table 2). Blümmel et al. (2005) conducted a study to compare feed intake, digestibility and CH_4 production by open-circuit respiration measurements in sheep fed 15 untreated, sodium hydroxide (NaOH) treated and anhydrous ammonia (NH_3) treated wheat, barley and oat straws also evaluated using IVFT. Total daily CH_4 production, calculated from *in vitro* fermentation characteristics (i.e., true degradability, SCFA ratio and efficiency of microbial production) and OM intake were found to be closely correlated with CH_4 emissions (L/d) measured in respiration chambers ($y = 2.5 + 0.86x$, $R^2 = 0.89$, $P < 0.001$, 15 diets and 4 animals used; y=CH_4 production in vivo; x=CH_4 predicted *in vitro*). Intake of OM measured *in vivo* OM intake was also used to calculate CH_4 production *in vitro*. As such OM intake was common to both the independent and the dependent variable, which could explain the close association between *in vitro* and *in vivo* measurements. It is important to point that Blümmel et al. (2005) and Bhatta et al. (2007) did not use the same animals from the *in vivo* trials as donor of rumen fluid for the *in vitro* incubations.

More recently, Hatew et al. (2015) reported a study comparing measurements of CH_4 production *in vitro* and *in vivo*, and those were conducted simultaneously (animals adapted to the same substrate as incubated in bottles were used as a source of rumen inocula for 72 h *in vitro* incubations). Measurements of CH_4 production for 24 h *in vitro* (expressed per unit of OM incubated) were found to be moderately correlated (R^2 = 0.54; *P* = 0.04, 4 diets and 16 animals used) with *in vivo* CH_4 production (when expressed per unit of estimated rumen-fermentable OM) across a range of diets differing in source and amount of starch in dietary concentrates. However, no association was found when *in vivo* CH_4 production was expressed per unit of ingested OM (R^2 = 0.04; P = 0.88). More research is needed using a wider range of diets representing different production systems.

ii) Few direct comparisons of antimethanogenic compounds *in vitro* and *in vivo* are available (Table 2). Martínez-Fernández et al. (2013) compared the effectiveness of bromchloromethane and propyl propane thiosulfinate to inhibit CH_4 production *in vitro* and *in vivo*. Even though both compounds were found to decrease CH_4 production *in vitro* by as much as 90% per unit of DM intake responses in goats were much lower (-33%), although measurements *in vivo* and *in vitro* were not made simultaneously. Two newly developed molecules (ethyl-3-nitrooxy propionate, ENP, and 3-nitrooxypropanol, 3NOP) have also been evaluated *in vitro* and *in vivo* (Martinez-Fernandez et al., 2014). Both compounds were given to sheep at two different doses of 50 and 500 mg/animal per day, corresponding to around 10 and 100 mg/L of rumen content or concentrations of 68 and 681 μM, respectively. Administration of 500 mg/d of h 3NOP at decreased CH_4 production by -29% on d 14, which was much lower than a value of -95%, determined *in vitro* (Martínez-Fernández et al., 2014). Similar differences in responses to Japanese horseradish oil have been reported between *in vitro* and *in vivo* studies

(Mohammed et al., 2004). Much larger decreases in CH_4 production were observed *in vitro* (-89%) than *in vivo* (-18.7%, Table 2), findings that are in agreement a recent meta-analysis (Hristov et al. 2012). Such discrepancies in the effectiveness of test compounds when given in similar doses may be explained by a combination of several factors: (1) test compounds used are typically administered in 1-2 pulses via the ruminal cannula that often coincide with feeding times, and as a consequence not be rapidly and well mixed with rumen contents; (2) differences in the degradation rate of the active compounds *in vitro* and *in vivo*; (3) decrease in microbial density and changes in bacterial community structure of rumen contents during processing as inoculum for *in vitro* studies associated with the exposure of microorganisms to oxygen and the removal of solids during filtration (Soto et al., 2012); (4) potential washout of these compounds from the rumen or absorption through the rumen wall and (5) adaptation of the rumen microbial ecosystem to the tested compound *in vivo* that is not emulated by inoculated microbiota *in vitro*.

A different scenario as described above has been recently reported by Castro-Montoya et al. (2015): a blend of essential oil was effective reducing daily emissions of methane in dairy cattle and emissions relative to body weight in beef cattle, interestingly, these effects were not observed *in vitro* regardless of the technique used to replicate *in vivo* results (IVFT or consecutive batch culture). This might be due to differences in the mode of action of the essential oils *in vitro* and *in vivo*, which merits attention for future research.

RECOMMENDATIONS:

In vitro CH_4 production is more closely correlated with in vivo CH_4 production across a range of feeding regimes when in vivo CH_4 emissions are expressed per unit of

degraded material rather than per unit of material ingested, although the ratio of substrate (feed) to volume of rumen fluid needs to be considered. The ability of IVFT to reliably predict effects in vivo is affected by adaptation of the rumen inoculum to the substrate tested in vitro. IVFT offers a valuable tool for the study and screening of anti-methanogenic additives before testing in vivo. It is recommended that in vitro data are confirmed in vivo before conclusions on the effectiveness of feed ingredients or additives for lowering CH_4 production are drawn given that inhibition potential is often overestimated in vitro.

Please insert Table 3 around here

3.8. Units to express CH_4 production

In vitro CH_4 production is usually expressed as ml (or g or mmol) of CH_4/g of substrate incubated (Bodas et al., 2008), ml (or g or mmol) of CH_4/g of substrate degraded (García-González et al., 2008), or ml of CH_4/ml of gas produced (Goel et al., 2008). As rumen fermentation modifiers may affect substrate degradation in a dose dependent manner (Russell and Strobel, 1988), effects on CH_4 production are better expressed per unit of substrate degraded, rather than per unit of substrate incubated. For example, Navarro-Villa et al. (2011) highlighted the importance of measuring substrate degradability over the incubation period based on the observation that the ranking of CH_4 production potential of incubated feeds (barley grain, grass silage, barley straw) differed depending on whether CH_4 was expressed per unit DM incubated or DM degraded. Methane output/g DM incubated (CH_4/DMi) was in the order barley grain > grass silage > barley straw, whereas when CH_4 output was expressed per g DM disappeared (CH_4/DMd) the ranking was barley straw > grass silage > barley grain. This

last observation is consistent among all quantities of feed, and ratios of rumen fluid to buffer combinations, and concurs with findings from *in vivo* studies, which compared low and high concentrate diets (Yan et al., 2000).

To calculate the volume of CH_4 produced per unit of substrate degraded, net values of gas and CH_4 production, as well as net amount of substrate degraded, are needed. These are obtained by blanks (i.e., flasks without substrate which contain only inoculum and medium), which are necessary to correct for gas, CH_4 and fermentation of residual OM in the inoculum (Rymer et al., 2005; Navarro-Villa et al., 2011). The CH_4/DMd values assume that all DM which disappeared is digested, and that no undigested soluble or particulate DM passed through the sintered glass crucible when the post-incubation DM residue was being isolated. Total gas or VFA produced is not as precise as substrate degraded but a good, simple proxy when degradation of substrate can not be determined.

RECOMMENDATIONS:

Regardless of the duration of the incubation in vitro, the amount of CH_4 produced should ideally be expressed relative to the amount of substrate degraded, rather than the amount of substrate incubated. If not possible, it is recommended to be expressed relative to total gas or VFA produced.

4. Conclusion

There is no standard protocol for assessing enteric methane mitigation in ruminants using *in vitro* gas production technique, as conditions need to be adjusted according to the research question. However, numerous technical issues relating to donor animals, microbial inoculum and general procedures need to be considered (Table 3) to ensure

the objectives of experiments can be properly fulfilled. This would allow harmonization of laboratory methods, better interpretation of results and facilitate inter-studies comparisons. Results from *in vitro* gas production technique studies need to be carefully interpreted before assessing mitigation strategies *in vivo.*

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Table 1.

Typical *in vitro* fermentation techniques (IVFT) used to quantify CH_4 (and CO_2 and H_2) production (adapted from Rymer et al., 2005).

In vitro system	Device and volume	Inoculum	Inoculum collection time	Incubation volume, ml	Inoculum: medium ratio	Buffer reference	Duration of incubation, h	Dietary substrate, mg	Gas release/collected	Pressure control	Gas sampling and analysis (GC)
Menke et al. (1979)	Syringes kept in rotor	Liquid phase	Before feeding	30	1:3	Menke et al. (1979)	24	200-300	Manual/End-point sampling	Yes, moveable glass piston	Manual
Theodorou et al. (1994)	Bottle in incubator	Liquid and solid phases	Before feeding	60	1:5	Theodorou (1993)	24-72	500-1000	Manual/End-point sampling	No, pressure increases	Manual
Mauricio et al. (1999)	Bottle in incubator	Liquid phase	Before feeding	100	1:10	Theodorou (1993)	n.s.	1000	Manual/ End-point sampling	No, pressure increases	Manual
Pell and Schofield (1993)	Bottle and stirrer kept in incubator	Liquid phase	2 h after feeding	10	1:5	Goering and Van Soest (1970)	n.s.	100	Manual/End-point sampling	No, pressure increases	Manual, CH_4 estimated by difference to CO_2
Cone et al. (1996)	Bottle in shaking water bath	Liquid phase	2 h after feeding	100	1:3	Steingass (1983)	48	400-500	Automated/ Fixed pressure	Yes	Manual
Davies et al. (1998)	Bottle in incubator	Liquid and solid phases	Before feeding	100	1:10	Theodorou (1993)	n.s.	1000	Automated/ Fixed pressure	Yes	n.s.
Cornou et al. (2012)	Bottle in incubator	Liquid phase	n.s.	60	1:3	Menke and Steigass (1988)	72	500	Automated/ Fixed pressure	Yes	Manual
Muetzel et al. (2014)	Bottle in incubator	Liquid phase	n.s.	60	1:5	Mould et al. (2005)	48	600	Automated/ Automated	Yes	Automated GC

GC, gas chromatograph; n.s., not specified

Table 2.

Comparison of results based on *in vitro* gas production and studies *in vivo* evaluating the effect of a) diets or b) feed additives on methane production

Diets	**Animal species /Intake level**	**Adaptation period**	**IVFT used**	**CH_4 *in vivo***	**R^2, P value**	**Reference**
Straw ± NH_3/NaOH	Sheep / Maintenance	10 days	Menke et al. (1979) [3]	Open circuit respiration chamber, g/kg OM	0.89, *P*<0.0001	Blümmel et al. (2005)
AH, CS+SBM, RG+SBM, RS+SBM, SG+SBM[1]	Cattle / Maintenance	12 days	Menke and Steingass (1988)	SF6 ml/kg DMI	0.98, *P*<0.0001	Bhatta et al. (2007)
GS:CO 60:40[2]	Cattle / 95 % voluntary DMI	12 days	Cone et al. (1996)	Open circuit respiration chamber g/kg OMI (a) or OMFR (b)	0.04, *P*= 0.878 (a) 0.54, *P*= 0.040 (b)	Hatew et al. (2015)

Additive[4]	**Dose *in vitro* // Inclusion rate *in vivo***	**Animal species**	**Adaptation period**	**IVFT used/ incubation time**	**CH_4 *in vivo***	**Change in CH_4 (%, L/kg DMI) *in vitro* // *In vivo***	**Reference**
Horseradish oil	0.17, 0.85 and 1.7 g/L // 20 g, kg DM	Cattle	14 days	Russel and Martin 1984/6 h	Headhood collection chamber	-18, -89 // -18.6	Mohamed et al. (2004)
PTS	100, 320 µl/L // 50, 100, 200 mg/L rumen content	Goats	7 days	Theodorou et al. (1994)/24 h	Open circuit respiration chamber	-28, -96 // -13, -18	Martínez-Fernández et al. (2013)
BCM	100, 320 µl/L // 50, 100, 160 mg/L rumen content	Goats	7 days	Theodorou et al. (1994)/24 h	Open circuit respiration chamber	-94, -96 // -34, - 45	Martínez-Fernández et al. (2013)
E3NP	25, 50 µM // 100 mg/d	Sheep	30 days	Theodorou et al. (1994)/24 h	Open circuit respiration chamber	-90, - 96 // -21	Martínez-Fernández et al. (2014)
3NOP	33, 66 µm // 100 mg/d	Sheep	30 days	Theodorou et al. (1994), 24 h	Open circuit respiration chamber	-99, -99 // -24	Martínez-Fernández et al. (2014)
Blend essential oils	0.25, 0.5, 1, 2, 5, 15, 30 mg/L // 0.2 g/day	Cattle	42 days	Theodorou et al. (1994), 24 h, 72 h	Open circuit respiration chamber	0 // - 15 (dairy cattle), 0 (beef cattle)	Castro-Montoya et al. (2015)

[1] AH=alfalfa hay, CS= corn silage, SBM=soyabean meal, RS=rice straw, SG=sudan grass; [2]GS= grass silage + 4 types of concentrate containing low or high amounts of slowly or rapidly degraded sources of starch; [3]CH_4 estimated from fermentation end products. [4]PTS: propyl thyosulfanite, BCM: brochloromethane, E3NP: ethyl-3-nitrooxy propionate, 3NPOP: and 3-nitrooxypropanol, 3NOP

Table 3.

Summary of technical recommendations on the use of *in vitro* gas production methods for measuring methane production

Aspect	Recommendation
Donor animals	
Species	Donor animals should be the same as target species
Numbers	3 or more animals where possible, minimum 2 animals.
Replications	Minimum of 3 independent incubation runs
Diet	Donor animals should be fed the same diet as incubated or of similar nutrient composition
Adaptation	Collect inoculum from animals that have been adapted to treatment for at least 2 weeks
Microbial inoculum	
Collection	Collection before feeding unless the experimental design requires a different sampling regimen.
Preservation	Ideally maintain inocula under anaerobic conditions at 39ºC and use within 1 h. Preservation at 0ºC up to 6 h or after freezing in liquid N are viable alternatives.
Processing	Filter through 250 μm pore size cloth.
Procedure	
Substrate incubated	Use a range of feeds for additives screening. Include a blank substrates to control for intra and inter variability and as a reference for comparisons between laboratories.
Buffer composition	No single buffer is recommended. For all incubations adjust bicarbonate concentration to maintain a target culture pH culture. Concentrations should not exceed 80 mM.
Inoculum:medium ratio	Adjust depending on the duration of incubation and frequency of gas sampling.
Duration of incubation	Adjusted according to the composition and particle size of substrate based on retention time in the rumen *in vivo*.

Gas measurements	Determine CH_4 and CO_2 when gas pressure is vented.
Units	Preferably express data relative to substrate (DM, OM, NDF) degradation. If this is not possible, data should be expressed relative to total gas, CO_2 or VFA production.
Mandatory technical details to be reported in peer-reviewed publications	Numbers of donor animals used to obtain inocula. Composition of diet fed to donor animal and the time on diet. Inoculum collection time relative to feeding. Headspace gas composition. Pore size of filter used to strain rumen fluid. Buffer and media composition. Rumen inoculum storage conditions, both temperature and the time from collection to inoculation. Ratio of medium to rumen inoculum. Substrate processing procedures, frequency of gas sampling and CH_4 measurements