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

The *in vitro* gas production (IVGP) techniques 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 IVGP experiments investigate nutritional strategies to mitigate methane (CH_4) emission from ruminants. Given the increasing and wide-scale use of IVGP 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 IVGP laboratory methods employed with IVGP experiments, of and procedures for feed evaluation, assessment of rumen function and CH_4 production.

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

Abbreviations: DM, dry matter; DMI, dry matter intake; IGVP, *in vitro* gas production; NDF, neutral detergent fibre; OM, organic matter; VFA, volatile fatty acids.

52

53

54 **1. Introduction**

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

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

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

89

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

91 Early *in vitro* studies focused on endpoint measurements such as the extent of substrate
92 degradation (Tilley and Terry, 1963). In the 1970’s, researchers recognized that
93 measurement of fermentation gases in combination with dietary chemical composition
94 could be used to estimate both feed metabolisable energy content and ruminal organic
95 matter degradability. Czerkawski and Breckenridge (1975) developed a system that
96 involved recording the direct displacement of a piston by gases produced during the
97 fermentation of feeds by rumen fluid in a glass syringe. This was the basis of the
98 ‘Hohenheim Gas Test’ developed by Menke et al. (1979, Table 1). The ‘syringe
99 technique’ was originally developed to determine end-point fermentation of feeds after
100 24 h of incubation. Blümmel and Ørskov (1993) modified the technique by incubating
101 syringes in a water bath rather than a rotating incubator. By recording gas production at

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

103 Wilkins (1974) described a different approach to measure fermentation kinetics 104 *in vitro*, whereby fermentation took place in a sealed vessel containing rumen fluid, 105 buffer and substrate, and a pressure transducer was used to measure gas accumulation in 106 the vessel headspace. In the simplest setup of this system, headspace pressure is 107 measured manually, as described by Theodorou et al. (1994, Table 1), while gas 108 samples are collected for the analysis of CO_2, CH_4 and/or H_2 concentrations when gas 109 pressure is released (Tekippe et al., 2012). In the 1990's the first automated pressure 110 based systems were developed (Pell and Schofield, 1993, Table 1), providing real-time 111 measurements of gas accumulation allowing for a better understanding of the kinetics of 112 fermentation for a range of substrates (Groot et al., 1996). During the development of 113 these systems, it became increasingly clear that increased pressure within the 114 fermentation container could affect fermentation end-products (Jouany and Lassalas, 115 2002) and the rate and extent of fermentation (Tagliapietra et al., 2010). More advanced 116 systems periodically release and collect the gas via a solenoid valve (Cone et al., 1996; 117 Davies et al., 2000, Table 1) thereby avoiding the build-up of pressure. Even in 118 automated systems, analysis of gas composition (e.g. CH_4) typically requires manual 119 injection of sample gases into a gas chromatograph (Martínez et al., 2010; Pellikaan et 120 al., 2011).

121 Cornou et al. (2013) described the results of a ring-test evaluating the use of a 122 wireless system for automated gas release developed by Ankom (Ankom Technology, 123 Macedon, NY, USA). This system is being used in various laboratories, but still relies 124 on manual gas sampling and analysis.

125 More recently Muetzel et al. (2014) developed an automated gas measurement 126 system by which gas production is monitored in real-time via pressure sensors and the

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

132 *In vitro* gas production systems have been used extensively for rapid screening
133 of chemical substances, plant species, plant extracts and dietary ingredients on CH_4
134 emissions from rumen fermentation. Such experimental approaches have allowed the
135 mode of action of a range of chemicals (Busquet et al., 2005; Bodas et al., 2008; Garcia-
136 Gonzalez et al., 2008; Durmic et al., 2010) and dietary substrates (Patra and Yu, 2013;
137 Hatew et al., 2015) to be investigated. Use of IVGP offers the opportunity to evaluate a
138 broad spectrum of chemical agents alone or in a number of combinations over a wide
139 range of concentrations (e.g., Busquet et al., 2005; Garcia-Gonzalez et al., 2008b;
140 Falchero et al., 2011; Li et al., 2011; Zamora et al., 2011). However, this technique does
141 not generate reliable information for agents that are only effective for decreasing CH_4
142 emissions over an extended period (Castro-Montoya et al., 2015). Furthermore, results
143 from screening studies (Bodas et al., 2008; Durmic et al., 2010) are often inconclusive
144 and may be conflicting due to variation in dosage, chemical structure of the test
145 substance or compound, diet, combination of treatments applied, adaptation of rumen
146 microbes or the form in which an agent is introduced into the system (Cardozo et al.,
147 2004, 2005; Tager and Krause, 2010). Substantial decreases of CH_4 production *in vitro*
148 (Tan et al., 2011) have been reported, but in several cases these have been accompanied
149 by adverse effects on feed degradation, with the implication that a similar effect may
150 occur in vivo which would compromise diet digestibility and animal performance.
151

152 **Please insert Table 1 here**

153

154 ## 3. Aspects to consider

155 ### 3.1. Donor animal species and animal numbers

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

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

178 Muetzel et al. (2014) compared rumen fluid from cattle (Holstein × Jersey cows)
179 and sheep using a newly developed automated *in vitro* system and reported that total
180 gas production is unaffected by donor animal species. Prior to rumen sampling, cattle
181 and sheep had been adapted to a medium quality hay diet fed to meet maintenance
182 energy requirements for 14 days. However, concentrations of CH_4 were lower during
183 incubations with rumen fluid from sheep than cattle associated with a lower proportion
184 of acetate and a higher proportion of propionate. No interaction between animal species
185 (sheep vs. cattle) and the type of substrate incubated (chicory, lucerne, ryegrass, straw
186 and white clover) was observed (Muetzel et al., 2014). Bueno et al. (2015) compared *in*
187 *vitro* CH_4 production using rumen fluid from taurine dairy cattle (*Bos taurus* taurus),
188 zebu beef cattle (*Bos taurus* indicus), water buffaloes (*Bubalus bubalis*), sheep (*Ovis*
189 *aries*) and goats (*Capra hircus*) fed similar diets while testing the effect of condensed
190 tannins from an Acacia extract. Rumen fluid from cattle resulted in higher CH_4 per unit
191 of degraded organic matter (OM) formation than rumen fluid from small ruminants.
192 Although microbiota of ruminant species housed in close contact and fed a similar diet
193 may be of a similar composition, the microbial ecology of rumen samples between
194 sheep and goats, for example, may vary due to differences in dentition, eating and
195 ruminating behaviour, digestive tract physiology and ruminal retention time (Ammar et
196 al., 2004). For this reason, collection of inoculum from animals of the same target
197 species fed a diet containing the same feedstuffs would be recommended. Differences
198 due to feeding behaviour and diet composition can to some extent be overcome by the
199 collection of rumen samples before morning feeding, when the effect of diet
200 composition on rumen metabolites or microbiota are likely to be minimized (Martinez
201 et al., 2010) (see section 3.3).

202 There remains some uncertainty on the number of animals that need to be
203 sampled to provide a representative sample of rumen inocula. Several studies using
204 different ruminant species (e.g., Pinares-Patiño et al., 2003; Waghorn et al., 2006; Yan
205 et al., 2006) have reported that CH_4 emissions per unit dry matter intake vary between
206 individual animals. Such variation has been associated, among other factors, with
207 differences in the rumen microbiome associated with between-animal variation in
208 passage rates, rumen volume and morphology, eating behaviour, etc. (Kittelman et al.,
209 2014). Martínez et al. (2010) observed consistent differences in CH_4 production and H_2
210 recovery *in vitro* during incubations with rumen liquor collected from 6 different sheep
211 fed the same diet. Such differences can only be explained by differences in microbial
212 populations or activities in the starting inocula or variation in the survival or activity of
213 microbes over the incubation period.
214
215 *RECOMMENDATION: Where possible the target animal species should be used as the*
216 *donor of rumen fluid. Due to between- animal variation, 3 donor animals is considered*
217 *a minimum number to provide a representative source of rumen inoculum. Sampling*
218 *before feeding is advantageous for minimizing diet by animal interactions.*
219
220 **3.2. Donor animal diet**
221 Diet composition and nutrient intake are major factors affecting both microbial
222 populations in the rumen and microbial activity of rumen inoculum (Mould et al.,
223 2005). Compared with ruminants fed high-concentrate diets, a greater proportion of
224 fibrolytic bacteria and methanogenic archaea can be expected in rumen fluid collected
225 from animals on high-forage diets (Demeyer and Fievez, 2000). However, the extent to
226 which concentrate feeds affect rumen digestion and microbial populations may depend

227 on the source and proportion of concentrate ingredients in the diet, as well as forage
228 quality (Dijkstra et al., 1994).

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

238 Of particular importance is the observation that some mitigation strategies have
239 been evaluated using rumen fluid from animals fed poor quality feeds. This raises the
240 question of whether outcomes on the efficacy of mitigation agents under these
241 circumstances can be considered reliable when donor animals have not been fed a diet
242 of similar characteristics as that offered to target animals. The effects of differences in
243 diet composition fed to donor animals may be minimized by obtaining rumen fluid
244 immediately before feeding. Huntington et al. (1998) obtained fluid from cows fed
245 either a silage:barley diet (80:20) or a barley straw diet. Although microbial activity of
246 rumen fluid collected on the straw diet was lower than the silage:barley diet, this did not
247 alter the gas production profile, which was attributed to sampling prior to morning
248 feeding.

249 The diet fed to donor animals also needs to be considered when testing additives
250 as CH_4 mitigation agents. Mateos et al. (2013) reported that the effect of garlic oil and
251 cinnamaldehyde on *in vitro* fermentation and CH_4 production varied depending on

252 whether the donor animals were fed a typical dairy diet (alfalfa hay:concentrate 50:50)
253 or a fattening diet (barley straw: concentrate 15:85). Observations from several *in vitro*
254 studies suggest that the effects of essential oils on rumen function are pH-dependent,
255 and this also appears to be true for garlic oil and some of its components (Cardozo et al.
256 2005; Kamel et al. 2008). Cardozo et al. (2005) found that garlic oil had a more
257 pronounced impact on rumen volatile fatty acids (VFA) profile at low compared with
258 high rumen pH (5.5 versus 7.0), an effect explained by differences in the status of the
259 active molecules (i.e. dissociated or un-dissociated) possibly mediated by changes in
260 rumen pH. However, batch cultures are usually highly buffered systems allowing
261 ruminal microorganisms to grow for a prolonged period despite the accumulation of
262 fermentation end-products. In such cases, factors other than pH (e.g. microbial
263 composition) may explain differences in the efficacy of additives. Hatew et al. (2015)
264 provided further evidence on the importance of diet fed to the donor animal.
265 Experiments involved the incubation of the same substrate (grass silage or beet pulp)
266 with rumen inoculum obtained from donor cows fed on diets that differed in starch
267 source (native vs. gelatinized maize grain) and starch level (270 vs 530 g/kg concentrate
268 DM). A higher level of starch and gelatinized rather than native maize were found to
269 lower gas and CH_4 production after 24-h incubations.

270 Level of feed intake is also an important consideration, given that a higher DM
271 intake (DMI) lowers retention time in the rumen, decreasing the amount of time
272 available for feed degradation of feeds and hence ruminal digestibility (Clauss et al.,
273 2007). Rumen pH, proteolytic and cellulolytic activities are thought to be influenced by
274 the level of DMI which can in turn influence growth rates and the metabolic activity of
275 inoculum used in *in vitro* systems. Increasing feeding frequency will generally lower
276 diurnal variation in rumen fermentation parameters. For example, in lactating cows

277 increasing feeding frequency from two to six times-daily was found to decrease post-
278 feeding variation in rumen pH, osmolality, VFA and NH_3 concentrations (Le Liboux
279 and Peyraud, 1999).

280 The period of adaptation to a given diet by the donor animal probably needs to
281 be revisited. It is common to collect rumen fluid from animals fed a diet for 2 weeks.
282 However, there are indications that the methanogenic archaeal population requires an
283 adaptation period of around 30 days after a change in diet (Williams et al., 2009).

284

285 *RECOMMENDATION: The diet fed to donor animals should be similar in composition*
286 *to the substrate incubated in vitro. Care should be taken to ensure sufficient buffering*
287 *capacity when investigating diets or dietary ingredients or additives that promote*
288 *differences in rumen pH (see section 3.4), in particular when samples are not taken*
289 *immediately before feeding. It is recommended that donor animals are fed a*
290 *standardized diet at a restricted level of feeding as frequent meals to ensure constancy*
291 *of diet composition and digestion, minimize variation in feed intake and avoid diurnal*
292 *variation in rumen fermentation. Samples of rumen fluid should be collected from donor*
293 *animals fed the same diet for at least 4 weeks.*

294

295 **3.3. Rumen fluid sampling: time, location and processing**

296 In all *in vitro* fermentation systems it is essential to create an environment, which, for
297 any set of parameters, mimics the fermentation in a specific section of the gastro-
298 intestinal tract in vivo (e.g. reticulo-rumen or caecum). Therefore, the inoculum should
299 be representative of that environment with respect to both the composition and
300 abundance of the microbial population. For *in vitro* systems to be robust (i.e.,
301 reproducible over time and representative of conditions *in vivo*), the inoculum must

302 meet certain criteria. Making a valid assessment of whether a given study has met these
303 criteria may be problematic, as often-essential information is not reported. Given the
304 precision of gas release kinetic techniques relative to degradability at a set end-point
305 over extended periods, variations in inoculum characteristics due to host animal effects,
306 nutrition and sampling time, as well as sample preparation and inoculation, can have
307 substantial cumulative effects on *in vitro* fermentation. It seems pertinent, not only to
308 permit comparison between studies, but also to limit potential errors, to have a set of
309 accepted guidelines and standard procedures for preparing inoculum for measuring CH_4
310 in vitro, as proposed for animal studies *in vivo* (Hammond et al., unpublished). Such
311 guidelines should include host animal management, sampling techniques (time,
312 location, alternatives) and inoculum preparation.
313
314 Sampling time:
315 Diurnal changes of the rumen microbiome, both in terms of abundance and metabolic
316 activity have been documented. Concentrations of viable microbial populations in the
317 rumen typically decrease 4 h post-feeding, due to dilution with feed, water and saliva,
318 and peaks at 6-12 h post feeding (depending on diet and level of feed intake) (Leedle et
319 al., 1982; Dehority, 2003). Furthermore, microbial abundances were found to decline
320 during the degradation of available nutrients. Cone et al. (1996) observed that the rate
321 of fermentation was highest when rumen fluid was collected after morning feeding,
322 although rumen sampling time had no effect on the total gas production. Menke and
323 Steingass (1988) stated that sampling rumen contents just before feeding lowered
324 variation in composition and activity of the inoculum and minimized the influence of
325 diet fed to donor animals. However, Payne et al. (2002) observed that the total gas
326 production from both starch and ground straw were less variable between replicate

327 bottles and between weeks of collection when rumen fluid inoculum was collected
328 either 4 or 8 h post feeding, compared with samples collected just before, or 2 h after
329 feeding. Presumably, the activity of the inoculum is determined just as much by time of
330 rumen sampling relative to feeding as by feeding pattern and eating time.
331
332 *RECOMMENDATION: There is no general recommendation on the ideal rumen*
333 *sampling time as it depends on the objectives of a specific experiment. Given the*
334 *difficulty of minimizing diurnal variation, rumen fluid samples are best collected*
335 *immediately before feeding, based on a consistent protocol for dietary access by donor*
336 *animals across experiments. When a series of studies are conducted over time, feeding*
337 *and sampling procedures should be kept as identical as possible.*
338
339 Sampling location within the rumen: Rumen digesta is comprised of different fractions
340 (large and small particulate matter and liquid). Ample evidence exists on the different
341 abundance and diversity of bacteria associated with the liquid and solid ruminal
342 contents (Pei et al., 2010). The pH and VFA concentration varies between different
343 sections of the reticulo-rumen in cattle (Bryant 1964). De Visser et al. (1993) indicated
344 that rumen VFA concentrations were about 20% higher for the entire evacuated rumen
345 content compared with calculations based on samples of rumen fluid collected in a
346 standardized manner. Storm and Kristensen (2010) indicated differences of 0.4 to 0.6
347 pH units and of 40 to 50 mM VFA between the central and ventral regions of the
348 rumen, with the lowest pH and highest VFA concentrations in the medial rumen.
349 Rumen fermentation parameters have also been found to differ between samples
350 collected at different locations in the rumen (Shen et al., 2012), implying possible
351 differences in microbial abundance and activity within the rumen.

352 Rumen cannulation is considered the reference method allowing the collection of
353 representative samples of rumen digesta from donor animals (Komarek, 1981;
354 Kristensen et al., 2010). Access to surgically-modified animals is not universal, and
355 therefore less invasive techniques, such as oral stomach probing, have been used as an
356 alternative. In the relatively few studies that have compared sampling through the
357 rumen cannula or by stomach probing, differences in fermentation profile and
358 microbiota have been reported in some (e.g., Geishauser and Gitzel, 1996; Duffieldet
359 al., 2004), but not all cases (e.g., Lodge-Ivey et al., 2009; Shen et al., 2012; Terré et al.,
360 2013). Part of the discrepancy between studies may reflect differences in the procedures
361 used to avoid salivary dilution and contamination, the type of samples collected and
362 rumen sampling site. Stomach probing results in the collection of samples containing a
363 high proportion of liquid, whereas sampling via a rumen cannula allows both solid and
364 liquid digesta fractions to be obtained. Differences in the methods used to collect rumen
365 samples are of greater relevance when treatments are not expected to have the same
366 effect on microbial populations attached to solids or inhabiting the liquid phase
367 (Martínez et al., 2010). The study of Shen et al. (2012), attributed the differences
368 between samples collected via cannula or stomach tube to rumen sampling site, as a
369 consequence of the probe not being inserted to a depth sufficient to reach the ventral
370 sac. Accurate probe insertion to a desired location within the rumen is extremely
371 challenging in small ruminants. In a recent study, Ramos-Morales et al. (2014) found
372 that stomach-tubing in sheep and goats detected the same differences in rumen
373 fermentation due to species, diet or sampling time as sampling via the cannula.
374 However, certain differences were more readily detected in rumen cannula samples,
375 while substantial differences in the bacterial community structure were detected
376 between the sampling methods.

377 Faeces has also been used as an alternative source of inoculum to rumen fluid
378 (El Shaer et al., 1987). Cultures of ruminal or faecal microorganisms appear to result in
379 similar fermentation processes (El-Meadaway et al., 1998). However, fewer
380 microorganisms in faecal inocula may result in lower degradation capacity and
381 decreased gas production (Cone et al., 2002; Vàradyovà, et al., 2005), a longer lag
382 phase and a slower rate of degradation at the outset (Mauricio et al., 2001).
383 Furthermore, for poor-quality forages, there is only a weak relationship between gas
384 production during incubations with inocula sourced from faeces and rumen fluid (El-
385 Meadaway et al., 1998; Varadyova et al., 2005). Dhanoa et al. (2004) proposed a
386 method allowing mathematical adjustments to convert or translate the degradation
387 profiles produced by faecal inoculum to correspond with ruminal fluid, but this requires
388 application of different prediction equations for each group of feeds incubated.
389 Rumen contents collected post-morten at abbatoir can also be used as an
390 alternative to rumen fluid (Mould et al., 2005). Several IVGP experiments have been
391 performed using rumen fluid collected from slaughtered cattle, sheep, buffalo and
392 dromedary (Haddi et al., 2003; Salem, 2005). To date, there are no reports directly
393 comparing the use of rumen fluid from slaughtered animals with oral or rumen sampling
394 in the same animal. Such an approach requires sampling of rumen contents soon after
395 slaughter, as well as the same criteria for other sources of inocula being met. While the
396 intake and diet composition of slaughtered animals are not known, access to entire
397 rumen contents allows the collection of representative samples that can also be used to
398 inform on nutrient supply. If sampling of rumen cannulated animals is not possible,
399 collection of rumen content from slaughtered animals may prove a viable alternative.
400

401 *RECOMMENDATION: To be as representative of the rumen environment as possible,*
402 *samples of ruminal contents for the preparation of in vitro inoculum need to be*
403 *collected from several locations. This is more feasible in large ruminants and requires*
404 *the collection of rumen contents from animals fitted with rumen cannula following a*
405 *clearly defined and standardized sampling protocol. Stomach tubing or faecal inoculae*
406 *may serve as an alternative for ranking purposes, but quantitative data using these*
407 *alternatives may differ from sampling of rumen contents in cannulated animals.*
408 *Stomach tubing should be performed by well-trained persons to minimize salivary*
409 *dilution.*
410

411 Preservation of inoculum: Anaerobiosis is essential to culture rumen microorganisms,
412 methanogenic archaea, in particular (Joblin, 2005). Certain experiments may require
413 rumen inoculum to be stored until culturing *in vitro*. Short-term storage (<1 h), for
414 example during transport from donor animals to the IVGP laboratory, should exclude
415 exposure to air. It is equally important that any increase in headspace pressure does not
416 cause CO_2 to go into solution, thus lowering pH. Excessive fermentation due to
417 extended storage at 39°C should be restricted to prevent any microbial group from
418 becoming dominant and modifying the composition of the original inoculum.
419 The influence of storage time and temperature on neutral detergent fibre (NDF)
420 degradation by rumen microorganisms has been investigated (Robinson et al., 1999).
421 Studies involved the use of the ANKOM end-point system to examine the effect of
422 delaying inoculum storage at 39ºC by up to 6.5 h, or up to 48 h after storage over a
423 range of temperatures from − 22 and 39 °C. No apparent effect on 48 h end-point
424 degradation of medium-term (6.5 h) storage compared with long-term storage (48 h)
425 was identified. Authors concluded that no storage method, irrespective of temperature,

426 would maintain rumen inoculum activity for up to 48 h that support normal
427 fermentation *in vitro*. Subsequent works (Cone et al., 2000; Hervás et al., 2005; Prates
428 et al., 2010) presented data on the effect of using rumen fluid directly or stored
429 anaerobically at 39°C for increments of up to 24 h or at –24°C for 1, 3, 10, 40 or 76
430 days before use. In general, gas production in terms of kinetics and cumulative yields,
431 decreased as storage period increased. Final gas volumes were similar when rumen fluid
432 was stored up to 4-6 h compared with no prior storage. Gas production rates were lower
433 for inocula stored for 8 or 24 h, while gas production was considerably decreased by
434 extended storage at -20ºC of more than 10 days. Microbial activity was lowered by
435 freezing, with the decrease being substrate-dependent with the degradation of pure
436 starch and cellulose being less affected compared with Lucerne hay and barley straw.
437 Freezing in liquid nitrogen is preferred over storage at -20ºC (Prates et al., 2010) and
438 thawing of small volumes (approximately 20 ml) at 39ºC for 2 min. The time taken for
439 freezing and thawing appears to be as equally important as storage temperature. Overall,
440 studies suggest that preservation of rumen fluid at 0ºC for up to 6 h offered a practical
441 alternative, where necessary, to freshly collected inocula. Protozoa are lost after
442 freezing which could have an impact on the fermentation. There are, however, no
443 reports documenting the effect of different preservation methods on methanogenic
444 activity.
445
446 *RECOMMENDATION: Fresh rumen fluid maintained under anaerobic conditions at*
447 *39ºC should be inoculated into in vitro vessels as soon as possibly, ideally within 1 h*
448 *post collection. When this is not possible, rumen fluid can be preserved at 0-4 ºC for up*
449 *to 6 h or frozen in liquid nitrogen following addition of a cryoprotectant (15% glycerol*
450 *or 8% DMSO) for longer periods for use as inoculum. In either case, implementation of*

451 *standardized procedures to avoid undesirable variation in microbial activity is highly*
452 *recommended.*
453

454 Preparation of inoculum prior to incubation: Method of preparation also influences the
455 microbial activity of rumen inoculum. The rumen microbiome consists of three sub-
456 populations of microbes: those in the fluid phase, adherent to the particulate phase
457 (further divided into loosely- and firmly-associated with the feed particles) (Cheng et
458 al., 1993) or attached to the epithelium (Sadet et al., 2007). The latter tends to be
459 primarily involved in the release of ammonia from urea absorbed across the rumen
460 epithelium and, as such, has only a minor role in feed degradation, and for this reason
461 does not need to be sampled (Mueller et al., 1984). Fluid and particulate associated
462 bacterial populations (Kim et al., 2003) and methanogenic archaea (Shin et al., 2004)
463 differ in growth characteristics and in the activities of most enzymes (Moharrery and
464 Das, 2001). Microorganisms in rumen liquid (20 to 30% of total microbes) including
465 free-living bacteria and bacteria detached from solid substrate, have little direct
466 involvement in structural carbohydrate digestion (Miron et al., 2001). Microbes attached
467 to feed particles predominate (70 to 80% of microbial matter and microbial ATP
468 production) and play a key role in feed particle digestion in the rumen (Craig et al.,
469 1987; Miron et al., 2001; Trabalz Marinucci et al., 2006). Consequently, once samples
470 of whole rumen contents have been collected, the problem arises of how to effectively
471 detach the microorganisms associated with feed particles. Failure to do so will result in
472 a high proportion remaining to be attached after filtration, while the use of multiple
473 layers of cheesecloth, muslin or surgical gauze leads to different microbial fractions
474 being retained and inoculated *in vitro*. For example, use of a cloth with 50 µm pores
475 would substantially lower the number of large protozoa (which range from to 15–250

476 µm in size, Dehority, 2003) in inocula. Williams and Coleman (1991) reported that
477 most protozoa are retained using a 100 µm pore size filter. Therefore, if total protozoa
478 are to be included in the microbial culture, a 250 µm pore size cloth should be used.
479 Furthermore, some of the physical methods used to detach particle-associated microbes
480 (e.g., a stomacher or maceration of rumen contents in a food processor) may also cause
481 cell damage. Rymer et al. (1999) examined four methods of inoculum preparation:
482 strained, blended fluid, "stomacher" or strained plus blended residues. No consistent
483 treatment effect was observed, apart from blending which tended to decrease substrate
484 degradation. Authors concluded that there was little advantage from blending inoculum,
485 particularly in light of the risk of exposing microorganisms to oxygen. Even though
486 homogenizing rumen fluid may increase numbers of particle-associated bacteria in the
487 inoculum, Pell and Schofield (1993) excluded this step on the grounds that it i)
488 introduced an extra procedure into the laboratory protocol, ii) increased the risk of
489 exposing rumen microorganisms to oxygen, iii) increased the quantity of gas released
490 from the blanks, and iv) had no obvious effect on the results of IVGP experiments.

491 A number of techniques that have been proposed for detaching microorganisms
492 from rumen feed particles that involve various combinations of chemical and physical
493 treatments and h yield removal rates of between 20 and 80% (Kudo et al., 1987; Legay-
494 Carmier and Bauchart, 1989; Merry and McAllan, 1983; Whitehouse et al., 1994;
495 Hristov et al., 1999; Ranilla et al., 2001). While the use of these techniques is essential
496 when collecting microbial biomass to determine their composition to accurately assess
497 passage of nutrients of microbial origin to the intestine, it is not clear whether a standard
498 protocol for microbial detachment should be applied for *in vitro* methods. In recent
499 work, Soto et al. (2013) studied the development of the microbiota in different in vitro
500 rumen simulation systems inoculated with intact or filtered rumen fluid from goats.

501 Incubation of filtered rumen fluid fraction in batch culture resulted in lower microbial
502 diversity compared with non-filtered rumen fluid inoculum. Substantial growth of
503 fibrolytic bacteria and methanogenic archaea over the first 24 hpartially compensated
504 for low numbers in the inoculum due to filtering.
505
506 *RECOMMENDATION: Filtration of rumen fluid using the same pore size across*
507 *incubation runs is a straightforward method for preparing inoculum suitable for in*
508 *vitro experiments. A larger the pore size results in greater numbers of small particulate*
509 *associated bacteria and protozoa. A pore size of 250 μm is recommended. Use of*
510 *multiple layers of cheesecloth is not recommended due to inconsistencies in pore size.*
511
512 **3.4. Substrate and incubation buffer**
513 Substrate:
514 Provided donor animals are fed the same or a similar diet as that tested *in vitro*, there
515 remains an uncertainty of the choice of substrate to be used in the evaluation of
516 additives on CH_4 production *in vitro*. The composition of diets fermented *in vitro*
517 determines the production of dissolved H_2 that serves as a substrate for methanogens.
518 Most *in vitro* studies have tested additives in incubations with a single substrate, but
519 there are reports on the effects of additives using different fermentation substrates in a
520 single experiment. Machmüller et al. (2001) investigated effects of medium chain fatty
521 acids in incubations containing high or low amounts of fiber. The efficacy of monensin
522 has been examined using corn meal or timothy hay (Russell and Strobel, 1988) and corn
523 meal or soyabean hulls (Pellikaan et al., 2011) as substrates. In certain instances a
524 substrate by additive interaction has been observed. Pellikaan et al. (2011) reported a
525 complete inhibition of CH_4 production during the first 30 h of incubation, irrespective

526 of substrate composition. However, CH_4 production from soybean hulls was 65% lower
527 compared with other substrates after 72 h, suggesting an influence on adaptation,
528 whereas no CH_4 was produced when maize was incubated. Subsequent studies covering
529 a range of plant extracts and fatty acids (Castro-Montoya et al., 2012; Klevenhusen et
530 al., 2012; O'Brien et al., 2013) have provided further evidence that responses of CH_4 to
531 a given additive differs depending on the feed substrate incubated. For example,
532 addition of fatty acids were found more effective in lowering CH_4 during incubations
533 containing higher proportions of concentrate ingredients, an effect attributed to greater
534 protonation of fatty acids at a lower pH (e.g. Zhou et al. 2015). While specific
535 experiments have provided examples of substrate by additive interactions, drawing firm
536 conclusions on the magnitude of these effects remains challenging. Nevertheless, there
537 is a need for end users to recognize that specific characteristics of incubated substrates
538 impact on the outcomes of IVGP studies and be aware of a possible mismatch between
539 the diet of the donor animal and incubated substrate.

540 Implementing a standardized protocol for preparing substrates to be incubated is
541 also critical in allowing for between IVGP study comparisons (Rymer et al., 2005). The
542 most critical issue appears the methods used to dry fresh material, such as grass.
543 Comparisons of freeze-drying with oven drying at 60 or 105ºC are often contradictory.
544 There are reports on the effect of feed processing on CH_4 production. Nevertheless, *a*
545 *priori* freeze-drying is the method of choice for minimizing cell damage that potentially
546 alters the dynamics of microbial attachment and substrate degradation (Rymer et al.,
547 2005).

548

549 *RECOMMENDATION: It is recommended that a range of substrates that reflect the*
550 *types of feeds used in commercial production systems are used in the initial screening*

551 *of new additives, unless the objective of an in vitro experiment requires a predefined*
552 *substrate. Freeze-drying is preferred to oven drying for the drying of high moisture*
553 *substrates.*
554

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

556 There is considerable variation in the composition of the medium used for *in vitro*
557 studies reported in the literature. It is important to make a distinction between the term
558 'medium' (i.e., a solution containing a number of components including buffering
559 agents, trace elements, true protein and reducing agents) and 'buffer' (Williams, 1998).
560 The types of buffers used in IVGP and the implications on fermentation has been
561 comprehensively reviewed (Rymer et al., 2005). A medium with a high buffering
562 capacity, when used in IVGP studies, may be disadvantageous because it creates
563 conditions that are not representative of the rumen *in vivo*. This is particularly important
564 when assessing the effectiveness of CH_4 mitigations strategies that may rely on a
565 decrease in rumen pH that include an inhibition of fibrolytic bacteria and/or
566 methanogens (Argyle and Baldwin, 1988; Van Kessel and Russell, 1996; Navarro-Villa
567 et al., 2011). Furthermore, through the prevention of a sharp decline in pH, a highly
568 buffered medium may increase acetic:propionic acid ratios more than would otherwise
569 occur *in vivo*, which impacts on the availability of H_2 for CH_4 production (Lana et al.,
570 1998).

571 The effect of the incubation medium and rumen fluid to medium ratio (RF:M)
572 on IVGP has been investigated (Rymer et al., 1999; Pell and Schofield, 1993; Cone et
573 al., 2000). Such studies have demonstrated that increases in the proportion of rumen
574 fluid is associated with a decrease in lag phase and a higher rate of gas production,
575 while the effect on total gas production varies. Navarro-Villa et al. (2011) investigated

576 the effect of variable RF:M ratios (1:2, 1:4, and 1:6) in IVGP systems involving the
577 incubation of different amounts (0.3, 0.5, and 0.7 g) of three contrasting feeds (barley
578 grain, grass silage and barley straw) . The results indicated that CH_4 per unit of DM
579 degraded was a more appropriate unit for expressing *in vitro* CH_4 output than CH_4 per
580 unit of DM incubated. Incubation of 0.3 g dried milled feed in 50 ml of *in vitro* culture
581 containing 1:2 RF:M was an acceptable combination for 24 h incubations allowing for
582 a decline in pH declined and maximizing the difference in CH_4 output between
583 substrates.

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

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

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

617

618 **3.5. Incubation procedure and CH_4 measurements**

619 *In vitro* gas production systems are typically conducted over intervals of between 16 to
620 72 h. As such, IVGP systems do not mimic important physiological processes *in vivo*
621 such as ruminal digesta turnover. Removal of soluble particles in the liquid medium
622 may have adverse effects on microbial fermentation, by decreasing the amount of
623 soluble substrate available for microbial growth or may conversely stimulate activity
624 (Roger et al., 1990). Soto et al. (2013) reported that the numbers of all quantified
625 microorganisms (total bacteria, protozoa, methanogens, fungi, *Fibrobacter*

626 *succinogenes* and *Ruminococcus flavefaciens*) declined sharply during 24 h to 72 h of
627 incubation. This is likely due to the exhaustion of fermentable substrate and the
628 accumulation of fermentation end products. Different substrates (soluble carbohydrates,
629 starch, pectins, cellulose, hemicellulose and protein) that are fermented can be degraded
630 at different rates also have a variable contribution to CH_4 production (Colombato et al.,
631 2003; Bannink et al., 2006; 2011). In cows, mean retention time of NDF components in
632 the reticulorumen is around 28 h, being slightly longer than the standard *in vitro*
633 incubation time of 24 h (Schwarm et al., 2015). Retention time in the reticulorumen is
634 related to particle size (Schwarm et al. 2008). It can be argued that NDF fermentation
635 during in vitro incubations probably approaches a plateau after 24 h given that relatively
636 small feed particles (ca. 1 mm) are usually incubated.

637 Some IVGP trials have tested the effects of different substrates on CH_4
638 production based on the collection of a single gas sample after 24 h (García-González
639 et al., 2008). Such an approach may result in gas pressure in the headspace exceeding a
640 given threshold (48 kPa) and consequently an mpairment of microbial activity (Rymer
641 et al., 2005; Taglapietra et al., 2010). Several protocols, such as those described by
642 Theodorou et al., 1994, Cone et al., 1996 and Davies et al., 2000, stipulate that
643 headspace gases being released at pre-determined intervals or when a pre-set threshold
644 of pressure is reached (Hansen et al., 2010; Muetzel et al., 2014). Venting the gas
645 produced requires that CH_4 concentration is measured simultaneously, given that
646 different CH_4 concentrations can be expected depending on substrate. Nevertheless, it
647 is critical to measure total CH_4 produced *in vitro* in relation to incubated substrate, as
648 well as the amount degraded over time. For example, Navarro-Villa et al. (2011)
649 highlighted the importance of measuring substrate degradability over the incubation
650 period based on the observation that the ranking of CH_4 production potential of

651 incubated feeds (barley grain, grass silage, barley straw) differed depending on whether
652 CH_4 was expressed per unit DM incubated or DM degraded.

653 For successful IVGP studies, it is essential that experimental treatments are
654 randomly allocated to bottle positions. The order in which bottles are inoculated also
655 needs to be randomized across bottle positions and treatments. Randomization serves to
656 minimize possible confounding effects of bottle position (or water baths used),
657 treatments and timing of inoculation. Such an approach is analogous to the random
658 allocation of treatments to animals for *in vivo* experiments (Hammond et al.,
659 unpublished). It is also important to consider establishing incubations with a single,
660 identical source of medium, and a single, identical source of substrate.

661

662 *RECOMMENDATION: The duration of the incubation should be adjusted based on the*
663 *composition and physical properties of substrate incubated that determines the*
664 *frequency of gas sampling required for measuring CH_4 production. Gas composition*
665 *should be determined at the same time gas pressure is vented. Regardless of the*
666 *duration of the incubation in vitro, the amount of CH_4 produced should ideally be*
667 *expressed relative to the amount of substrate degraded, rather than the amount of*
668 *substrate incubated. It is highly desirable that the timing of inoculation and the*
669 *allocation of treatments with respect to bottle position are randomized as much as*
670 *possible. It is recommended that 3 bottles are used per unique treatment in each run*
671 *allowing possible outliers to be identified.*

672

673 **3.6. Headspace gas composition**

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

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

701 than N_2, total or net gas production was lower for the former compared with the latter.
702 Further investigations are *required to understand the impact of a mixture of* CO_2 *and* N_2
703 *that best mimics rumen gas composition in vivo.*
704
705 *RECOMMENDATION: Owing to the effect of headspace gas composition on gas*
706 *production, including* CH_4 *production, it is recommended that all future studies should*
707 *both consider this as an influencing factor and report headspace gas composition.*
708 *Rumen fluid should be flushed continuously, and once added into the medium,*
709 *continued to be flushed for at least 10 min before incubation is initiated. Following the*
710 *addition of inoculum into the bottle, the headspace should be flushed continuously until*
711 *the bottle is sealed.*
712
713 **Please insert Table 2 here**
714
715 **3.7. *In vitro* versus *in vivo***
716 Numerous studies have examined the influence of antimethanogenic compounds on
717 CH_4 production *in vitro*, but few have undertaken a simultaneous evaluation *in vivo* and
718 *in vitro*. Direct comparison of effects *in vitro* and *in vivo* would allow a better
719 interpretation of IVGP data and inform on the treatments suitable for further evaluation
720 *in vivo*. When addressing inconsistences between results from *in vitro* and *in vivo*
721 studies it is worthwhile considering:
722 i) The accuracy of *in vitro* systems to predict the CH_4 production of a given
723 diet per unit feed intake or digested matter

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

727 i) Bhatta et al. (2007) compared IVGP measurements of CH_4 production with the
728 SF_6-technique across a range of diets. Methane production estimated from 48 h *in vitro*
729 gas production was higher than measurements *in vivo* for all diets. Of particular note is
730 that the average of CH_4 production at 24 h and 48 h was closely correlated with values
731 based on SF_6 (R^2=0.78, 5 diets and 4 animals used) (Table 2).

732 Blümmel et al (2005) conducted a study to compare feed intake, digestibility and CH_4
733 production by open-circuit respiration measurements in sheep fed 15 untreated, sodium
734 hydroxide (NaOH) treated and anhydrous ammonia (NH_3) treated wheat, barley and oat
735 straws also evaluated using IVGP. Total daily CH_4 production, calculated from *in vitro*
736 fermentation characteristics (i.e., true degradability, SCFA ratio and efficiency of
737 microbial production) and OM intake were found to be closely correlated with CH_4
738 emissions measured in respiration chambers ($y = 2.5 + 0.86x$, $R^2 = 0.89$, $P < 0.001$, 15
739 diets and 4 animals used). Intake of OM measured *in vivo* OM intake was also used to
740 calculate CH_4 production *in vitro*. As such OM intake was common to both the
741 independent and the dependent variable, which could explain the close association
742 between in vitro and in vivo measurements.

743 More recently, Hatew et al. (2015) reported the first study to simultaneously compare
744 measurements of CH_4 production *in vitro* and in vivo. Animals adapted to the
745 experimental diet were used as a source of rumen inocula for 24 h *in vitro* incubations.
746 Measurements of CH_4 production *in vitro* (expressed per unit of OM incubated) were
747 found to be moderately correlated ($R^2 = 0.54$; $P = 0.04$, 10 diets and 16 animals used)
748 with *in vivo* CH_4 production (when expressed per unit of estimated rumen-fermentable

749 OM) across a range of diets differing in source and amount of starch in dietary
750 concentrates. However, no association was found when *in vivo* CH_4 production was
751 expressed per unit of ingested OM ($R^2 = 0.05$; $P = 0.87$).
752 ii) Few direct comparisons of antimethanogenic compounds *in vitro* and *in vivo* are
753 available (Table 2). Martínez-Fernández et al. (2013) compared the effectiveness of
754 bromchloromethane and propyl propane thiosulfinate to inhibit CH_4 production *in vitro*
755 and *in vivo*. Even though both compounds were found to decrease CH_4 production *in*
756 *vitro* by as much as 90% per unit of DM intake responses in goats were much lower (-
757 33%), although measurements *in vivo* and *in vitro* were not made simultaneously. Two
758 newly developed molecules (ethyl-3-nitrooxy propionate, ENP, and 3-nitrooxypropanol,
759 3NOP) have also been evaluated in vitro and in vivo (Martinez-Fernandez et al., 2014).
760 Both compounds were given to sheep at two different doses of 50 and 500 mg/animal
761 per day, corresponding to around 10 and 100 mg/L of rumen content or concentrations
762 of 68 and 681 μM, respectively. Administration of 500 mg/d of h 3NOP at decreased
763 CH_4 production by -29% on d 14, which was much lower than a value of -95%,
764 determined *in vitro* (Martínez-Fernández et al., 2014). Similar differences in responses
765 to Japanese horseradish oil have been reported between *in vitro* and *in vivo* studies
766 (Mohammed et al., 2004). Much larger decreases in CH_4 production were observed *in*
767 *vitro* (-89%) than *in vivo* (-18.7%, Table 2), findings that are in agreement a recent
768 meta-analysis (Hristov et al. 2012). Such discrepancies in the effectiveness of test
769 compounds when given in similar doses may be explained by a combination of several
770 factors: (1) test compounds used are typically administered in 1-2 pulses via the ruminal
771 cannula that often coincide with feeding times, and as a consequence not be rapidly and
772 well mixed with rumen contents; (2) differences in the degradation rate of the active
773 compounds *in vitro* and *in vivo*; (3) decrease in microbial density and changes in

774 bacterial community structure of rumen contents during processing as inoculum for *in*
775 *vitro* studies associated with the exposure of microorganisms to oxygen and the removal
776 of solids during filtration (Soto et al., 2012); (4) potential washout of these compounds
777 from the rumen or absorption through the rumen wall and (5) adaptation of the rumen
778 microbial ecosystem to the tested compound *in vivo* that is not emulated by inoculated
779 microbiota *in vitro*.
780 A different scenario as described above has been recently reported by Castro-Montoya
781 et al. (2015): a blend of essential oil was effective reducing daily emissions of methane
782 in dairy cattle and emissions relative to body weight in beef cattle, interestingly, these
783 effects were not observed *in vitro* regardless of the technique used to replicate *in vivo*
784 results (IVGP or consecutive batch culture). This might be due to differences in the
785 mode of action of the essential oils in vitro and in vivo, which merits attention for future
786 research.
787
788 *RECOMMENDATIONS:*
789 *In vitro CH_4 production is more closely correlated with in vivo CH_4 production across a*
790 *range of feeding regimes when in vivo CH_4 emissions are expressed per unit of*
791 *degraded material rather than per unit of material ingested. The ability of IVGP to*
792 *reliably predict effects in vivo is affected by adaptation of the rumen inoculum to the*
793 *substrate tested in vitro. IVGP offers a valuable tool for the study and screening of anti-*
794 *methanogenic additives before testing in vivo. It is recommended that in vitro data are*
795 *confirmed in vivo before conclusions on the effectiveness of feed ingredients or*
796 *additives for lowering CH_4 production are drawn given that inhibition potential is often*
797 *overestimated in vitro.*
798

799 **Please insert Table 3 here**

800

801 **4. Conclusion**

802 There is no standard protocol for assessing enteric methane mitigation in ruminants
803 using *in vitro* gas production technique, as conditions need to be adjusted according to
804 the research question. However, numerous technical issues relating to donor animals,
805 microbial inoculum and general procedures need to be considered (Table 3) to ensure
806 the objectives of experiments can be properly fulfilled. This would allow harmonization
807 of laboratory methods, better interpretation of results and facilitate inter-studies
808 comparisons. Results from *in vitro* gas production technique studies need to be carefully
809 interpreted before assessing mitigation strategies *in vivo.*

810

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819

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