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Title

Comparison of five methods for the estimation of methane production from vented *in vitro* systems.

Running title

The estimation of methane production from vented *in vitro* systems.

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ABSTRACT

BACKGROUND There are several methods for estimating methane production (MP) from feedstuffs in vented *in vitro* systems. One method (A; "gold standard") measures methane proportions in the incubation bottle's head space (HS) and in the vented gas collected in gas bags. Four other methods (B, C, D and E) measure methane proportion in a single gas sample from HS. Method B assumes the same methane proportion in the vented gas as in HS, method C assumes constant methane to carbon dioxide ratio, method D has been developed based on empirical data and method E assumes constant individual venting volumes. This study aimed to compare the MP predictions from these methods to that of the gold standard method under different incubation scenarios, to validate these methods based on their concordance with a gold standard method.

RESULTS Methods C, D and E had greater concordance (0.85, 0.88 and 0.81), lower root mean square error (RMSE) (0.80, 0.72 and 0.85) and lower mean bias (0.20, 0.35, -0.35) with the gold standard than did method B (concordance 0.67, RMSE 1.49 and mean bias 1.26). Methods D and E were simpler to perform than method C and method D was slightly more accurate than method E.

CONCLUSION Based on precision, accuracy and simplicity of implementation, it is recommended that, when method A cannot be used, methods D and E are preferred to estimate MP from vented *in vitro* systems.

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Keywords: *In vitro* fermentation; methane estimation; vented *in vitro* systems; Ankom GP; ruminants.

INTRODUCTION

Methane is a potent greenhouse gas with a global warming potential that is 34 times greater than that of carbon dioxide.[1,2] The livestock sector is responsible for producing approximately 15% of global greenhouse gas (GHG) emissions.[3,4,5] Currently, 80% of the GHG emissions generated by livestock consist of methane from enteric sources.[5,6] Enteric methane is produced when ingested plant compounds are fermented in the rumen to produce intermediary products such as carbon dioxide and hydrogen, which are then captured by the rumen methanogens and converted to methane, and this is then excreted mostly by eructation.[7,8,9]

Several strategies have been proposed for reducing enteric methane production (MP), and in order to assess these strategies, it is important to accurately quantify MP.[10,11] *In vitro* fermentation is a fast and low-cost method to screen feeds and feed additives for their methane mitigation potential.[10,12,13] The *in vitro* method involves incubating ruminal fluid with the desired substrate, a buffering solution along with the desired methane mitigant in a culture bottle kept at 39ºC; total gas production (GP) is then measured and gas samples are taken to be measured for methane proportion to enable the estimation of MP.[11,14]

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Vented *in vitro* systems have become popular for studies measuring MP.[15,16] Vented systems periodically release fermented gas from the system to prevent build-up of excessive gas pressure and thus prevent gas from diffusing into the ruminal fluid and inhibiting the fermentation and GP process.[17,18,19] In most *in vitro* systems, in order to ensure an anaerobic fermentation, carbon dioxide is used to flush the head space and incubation medium prior to the start of the incubation.[10] Thus, during early incubation, the head space gas and vented gas mostly comprise the carbon dioxide that was used to flush the system. As the incubation progresses and fermentation gas is produced, the proportion of methane in the head space gas ($HSCH_4$) and in the vented gas (VCH_4) increases.[21] Thus, the changing proportions of $HSCH_4$ and VCH_4 complicate the measurement of MP from vented systems.

The gold standard method for estimating MP involves measurement of total gas production, collection of all the vented gas in a gas-tight collection bag and measurement of $HSCH_4$ and VCH_4.[19] Four methods that use a single gas sample taken from the head space at the end of the incubation have been proposed.[22,23,24] One of these methods involves estimating MP as the sum of the headspace gas volume in the incubation vessel, plus the volume of gas produced, multiplied by the proportion of methane in the headspace at the termination of the incubation.[22] Two of these methods are calculated through algorithms and rely on the assumption of a constant methane to carbon dioxide ratio.[24] A fourth method involves an empirical equation, for predicting methane production.[23] To our knowledge there are no studies that have compared these different methods and tested or ranked their agreement against the gold standard method under a wide range of incubation scenarios.

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The work reported here aimed to compare the MP predictions of a series of methods that use a single head space sample to the MP measured by the gold standard method under a wide variety of different incubation scenarios, in order to validate these methods by evaluating their concordance with a gold standard method. It was hypothesized that MP estimation from the four methods relying on a single head space gas sample would be concordant with the gold standard (method A), but that some methods would be more closely concordant than others. Since such methods require assumptions about the pattern of methane mixing ratio throughout fermentation, it was further hypothesized that discordance would depend on substrate material as well as measurement error associated with individual units.

MATERIALS AND METHOD

All experiments involving sampling of ruminal fluid from rumen cannulated cows were approved by the Animal Ethics Committee of the Department of Economic Development Jobs Transport and Resources – Victoria. Cows were cared for according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (https://www.nhmrc.gov.au).

Methods for estimating methane production

Method A was considered the gold standard since it involved collecting all the vented gas in a gas-tight gas collection bag, measuring the volume of gas vented and measuring $HSCH_4$ and VCH_4.[19] Methane production, MP (mL) was calculated as:

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where HSV = head space volume (mL), $HSCH_4$ = methane proportion (also called mixing ratio, L/L) in head space, VGV = total vented gas volume (mL) which was also called total gas production (GP), VCH_4 = methane proportion in vented gas.

For method B, the proportion of $HSCH_4$ was multiplied by the sum of the incubation bottle's head space volume and the total volume of GP.[22]

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interval times, so that the difference, Pa_i - Qa_i, measures absorbance during the same intervals.

Method D was an empirical method developed using the Ankom GP system.[23] It also used GP and $HSCH_4$ to estimate MP.

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g DM of lucerne hay, 2) 1 g DM of lucerne hay, 3) 1.5 g DM of lucerne hay, 4) 0.5 g DM of wheat, 5) 1 g DM of wheat, 6) 1.5 g DM of wheat. In experiment III, eight treatments which involved four different feed types with different types of processing were incubated for 48 hours: 1) 1 g DM ground lucerne hay, 2) 1 g DM of ground wheat, 3) 1 g DM of crushed wheat, 4) 1 g DM of ground barley, 5) 1 g DM of crushed barley, 6) 1 g DM of rolled barley, 7) 1 g DM of ground corn, 8) 1 g DM of crushed wheat. Each incubation was replicated eight times in a single run. In each experiment, incubations were made in two separate *in vitro* fermentation systems, where each system was composed of independent water baths, transceiver and computer for data recording.

///Insert Table 1 around here///

***In vitro* fermentations**

The *in vitro* experiment was run using the automated GP system developed by Ankom (Ankom Technology, New York, USA) as described in previous studies.[25,26] At the end of the incubation period, a gas sample was collected from the head space of each bottle and from the gas bag attached to each Ankom GP module with the use of a needle and an air tight glass syringe (SGE International Pty Ltd, Ringwood, Vic, Australia), and the gas samples were transferred into separate Exetainers® (12 mL soda glass vial Labco ltd. Buckinghamshire, UK). Methane and carbon dioxide proportions in the samples were determined by gas chromatography. Briefly, a gas chromatograph (7890A Agilent, Santa Clara, CA, USA) fitted with an autosampler (Gilson GX-271, Gilson Inc., Middleton, WI, USA), and equipped with a HayeSep® N 80/100 mesh pre-column (0.5 m × 3 mm stainless steel, Agilent, Santa Clara,

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CA, USA), a Porapak® QS 80/100 mesh column (2 m × 3 mm stainless steel, Agilent, Santa Clara, CA, USA) and a flame ionisation detector was used. The injector and gas chromatograph oven were maintained at 70°C. Interpretation of GC results was via seven standards (Air Liquide, Air Liquide Australia, Melbourne, Vic, Australia) consisting in 0, 25, 50, 100, 150, 200 and 250 mL methane L^{-1} nitrogen; standards had a purity of 99.95%. Ultra high purity helium (999.99 g/kg He) was used as the carrier gas.

Statistical Analysis

Incubation bottle with its respective Ankom GP module and where relevant, its associated gas collection bag was used as the experimental unit.[27] In each experiment, each feed was replicated eight times. Data were checked for outliers and normality. Across the three experiments, out of 152 modules, data from nine modules were removed from the study, two caused by faulty batteries, five caused by temporary loss of connection between the pressure transducer and the computer and two caused by faulty modules. Methane production estimation through methods A, B, D and E were calculated in Excel using Eqn (1), (2), (4) and (5), respectively. Methane production estimation through method C was calculated using Eqn (3) in R Studio 3.2.3 (version 0.99.491 Integrated Development for R. RStudio, Inc., Boston, MA) with an existing R script,[24] as this method requires counting the number of ventings that occur in each incubation bottle during an incubation. The statistical analyses (ANOVA, Lin’s concordance, ReML, regression analysis and Pearson correlation) for all experiments were performed with the Gentstat statistical package (64 bit Release 16.1; VSN International, Hemel Hempstead, UK). In each experiment, differences between feeds

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in methane production and in GP were tested by ANOVA with substrate as treatment factor and a blocking structure of system and bottle within system using the model,

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D_{ijk} = ´ + Ą+ (8)

where D_{ijk} are the difference data, $y_m - y_A$, between MP estimated and that measured by method A, ´ = the mean difference (i.e. mean bias for the method), Ą = an effect for substrate treatment i (1-16), and μ_{ijk} = a deviation for bottle j (1-4) of treatment i and system k (1-2). Both Ą and μ_{ijk} were fitted as random effects, and variance components, ^{2} and ^{2}, expressed on the square-root scale as standard deviations, used to summarize variation in bias due to treatments and bottles respectively. Under this model, MSE = $'^2 + {}^2 + {}^2$. The significance of treatment effects was tested by change in deviance chi-square on removing Ą from the model.

The differences, D_{ij} as defined above, between MP predicted by each method and actual MP were also subjected to the ANOVA under model equation 8, and 95% confidence intervals were computed for the treatment means, for graphical presentation.

Nominal standard errors of treatment means (SEM) of MP were summarized from the ANOVAs of MP using an error mean square pooled from the three experiments as follows: Nominal SEM = $_p \big/ \sqrt{n}$ where $^2_p \quad \sum_{i\,1}^{3} d_i \; ^2_i \Big/ \sum_{i\,1}^{3} d_i$, where ^2_i and d_i are the residual mean square and residual degrees of freedom, respectively, for each experiment, and n = 8 is the replication. The nominal SEM estimates the incorrect SEM that would be obtained using the method B, C, D or E in practice. In practice these would be calculated by ANOVA without reference to the actual MP, as measured by Method A, and the treatment bias would be unknown and incorrectly assumed zero. Accordingly, and for comparison, an estimate of

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actual SEM was also calculated by including the variance component for treatment bias, ^{2}, in the calculation of SEM as follows,

$$\text{Actual SEM} = \sqrt{\ ^{2}\quad {}_{p}^{2}/n} \qquad (9)$$

where ${}_{p}^{2}$ and ^{2} are described above.

Pearson correlation coefficients were calculated between the substrate treatment means for each pair of the five methods

RESULTS

Mean data on $HSCH_4$, VCH_4, GP and MP for experiments I, II and III are presented in Table 2. Across all experiments, the mean $HSCH_4$, was 3.6 ± SD 0.39 % whereas for VCH_4, it was 2.2 ± SD 0.86 %. Methane production ranged from 6.1 to 12.6 mL. In experiments I and II, wheat produced more methane than lucerne hay. However, in experiment III, wheat produced less methane than lucerne hay. In experiment III, ground wheat also produced less methane than either ground barley or ground corn, but crushed wheat, barley and corn all produced similar amounts of methane. In experiment III, ground wheat, barley and corn all produced less methane than their corresponding crushed grains. In experiment II, MP increased with substrate mass, but was not commensurate with the increase in mass. The mean ratio of $VCH_4/HSCH_4$ was 0.62 ± 0.209 and this ratio was related to GP ($R^2 = 0.685$; $P < 0.001$) (Fig. 1).

///Insert Table 2 around here///

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///Insert Figure 1 around here///

Results from the concordance analysis are presented in Table 3. Methods C, D and E presented a greater concordance in MP with the gold standard (method A), than did method B. The Pearson correlation was similar for all methods. The Cb was also similar for all methods, except for method B which was lower, indicating greater bias, visible in Fig. 2a, which accounted for the reduced concordance observed for this method.

///Insert table 3 around here///

Methane production estimates for each bottle from the three experiments using methods B, C, D and E are shown in Fig. 2, each graphed against MP using method A. The data for method B was consistently above the 1:1 line of agreement ($P < 0.001$) while the data for methods C, D and E were all distributed about the line of agreement, but with negative slope biases (each $P < 0.005$).

///Insert Figure 2 around here///

Table 4 shows the RMSE and its components estimated under statistics from the model given by Eqn (6). These summarise the difference between MP estimated by the methods B, C, D and E compared to actual MP, measured by method A. It was observed that method B presented a statistically significant ($P < 0.001$) positive mean bias that was 0.9 to 1.0 mL methane greater than mean biases for methods C, D and E, that varied in statistical significance ($P = 0.085$, $P = 0.003$ and $P = 0.014$, respectively). The variance components for treatment were significant ($P < 0.001$) under all methods B, C, D and E. The square roots of

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these are shown in Table 4 as standard deviations, and indicate that variation in bias of up to about ± 1 mL methane (i.e. 2 standard deviations) was associated with substrate treatments, in addition to the mean bias for each method. The residual standard deviations were of a similar order of magnitude to one another under the different methods and were generally similar, though slightly larger than standard deviations due to treatment.

///Insert Table 4 around here///

Figure 3 illustrates the bias associated with each incubation substrate. The mean bias differed between methods; method B presented a greater bias than the other methods across all substrates, and method E had a slightly negative mean bias. The bias also depended on substrate, and the pattern of dependence was similar though not identical for the four methods. Barley presented the greatest bias in method B, wheat and barley had the greatest bias in method C, barley had the greatest bias in method D, while wheat had the greatest (negative) bias in method E.

///Insert Figure 3 around here///

Table 5 shows correlations between the substrate treatment means for the five methods. Correlations between methods B, C, D, and E were all above 0.88. The correlations between each of these methods and method A were, between 0.88 and 0.93. Table 4 also shows the nominal SEM and actual SEM allowing for a random treatment bias. The actual SEM ranged from 31% to 89% larger than the nominal SEM.

///Insert Table 5 around here///

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DISCUSSION

In the three *in vitro* experiments used in this study, there were substantial differences in $HSCH_4$, VCH_4, MP and GP between type of substrate, amount of substrate and processing of substrates. These factors were not the focus of this research, the substrate types, amounts of substrate and processing methods having been chosen purely to achieve a wide range in MP and GP in order to test if Eqn (2 – 5) could accurately predict a wide range of MP. The MP and GP measured in these experiments encompass values reported in the scientific literature.[15,16,29]

In the present study, method A was considered the most accurate (gold standard) method for measuring total methane production. It involved collection of all the gas produced and, through the measurement of volumes and the analysis of two gas samples, constitutes the most reliable method available for establishing the total amount of methane produced. The main problem with this method is that for every incubation, it involves measurement of methane content in two gas samples. This makes this method more onerous and expensive than methods that involve measurement of methane content in just a single sample of headspace gas taken at the conclusion of the incubation. The four methods compared in this study (B, C, D and E) presented similar results to method A, with high correlation and concordance. Thus, we accept our first hypothesis that these methods are to a large degree concordant. There are other methods not analyzed here that possibly approach method A for accuracy, but these appear to be more onerous and expensive than any of the methods described here. For example, in previously published methods, each incubation involves the collection during the incubation period of up to 12 gas samples from the head space of the

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incubation bottle, and all these gas samples must then be separately analyzed for methane proportions.[30]

When comparing between methods, it was observed that compared to methods C, D and E, method B produced the lowest concordance and greatest bias relative to method A, and was therefore considered inferior to methods C, D and E. Method B was based on using a single gas sample taken from the incubation bottle's head space at the conclusion of the incubation period. This method relies on the implausible assumption that the methane concentration in the vented gas is the same as the methane concentration in the headspace of the incubation vessel at the termination of the incubation and this method has not been validated against the gold standard method.[22] This is most unlikely as the initial ventings would have comprised mostly the carbon dioxide that had originally been used to flush the head space of the incubation bottle.[21] Indeed, the data in Table 2 show that at the end of each incubation, the VCH_4 was always less than the corresponding $HSCH_4$ and this is especially the case for treatments producing less GP (Fig. 1). Consequently, MP was systematically overestimated in method B, as observed in Fig. 2, where MP estimations of method B were consistently above the line of agreement with method A. These results demonstrate that method B will not be an accurate method for studying the effect of *in vitro* incubation on MP. However, it should be considered that method B also had a strong correlation with method A (Table 3; 0.89). It is therefore likely that in previous published studies that have used this approach,[15,16,29] the rankings of treatments and therefore conclusions involving comparisons between substrate treatments would not have been very different even if the authors had used method A.

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It has been suggested that Method C could infer MP with adequate accuracy based on a single gas sample taken from the incubation bottle's head space after the incubation period, by taking into consideration the venting history.[24] This method has been been tested for its accuracy, but the test involved simulated data and the researchers concluded that: "No validation data were available. Validation is required to determine how accurate and appropriate are the proposed algorithms in practice for *in vitro* studies employing the AnkomRF system." [24] The major benefit of this method was that, like methods B, D and E, it allowed for the calculation of MP from vented systems without the collection of the vented gas, ultimately reducing costs and labour. One issue associated with this method was that it required counting the number of ventings that occur in each incubation bottle during an incubation, this can be onerous and error prone if done manually. Four other algorithms were postulated for calculating MP in the original study.[24] In this analysis we presented MP data obtained through their Eqn (3), the most detailed.[24] However, all four algorithms presented essentially identical conclusions (data not shown).

Methods D and E allowed for the calculation of MP from vented systems without the collection of the vented gas and without counting the number of ventings, ultimately reducing costs and labour. Method D had been validated in a previous study, however, the development of the equation was never described and the authors provided only a very limited description of a validation study involving just 3 concentrates and 4 forages in 42 bottles.[23] Method E is based on the assumption that the $VCH_4/HSCH_4$ is approximately equal to 0.5. However, in this research, the average value for this ratio was 0.62 ± 0.21, Furthermore, as shown in Fig. 1, this ratio was positively correlated with GP. These findings

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explain some of the discrepancies between method A and method E. We do not discount the possibility that another method more accurate than method E, and based on the relationship shown in Fig. 1, could be developed. Indeed, using Eqn (1) and the equation shown in Fig. 1, it can be shown that MP is quadratically related to GP :

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measured by method A (Table 5), is in part due to the fact that MP ranged from 6.1 to 12.6 mL methane, a 200% difference. This study therefore covered a much broader range than typical *in vitro* studies.[15,19,24] For these kinds of differences methods B, C, D and E may be adequate, depending on the purposes of the analyses and the accuracy required.

The residual standard deviation, accounting for variation between bottles, was in the order of 0.51 to 0.62 mL methane (Table 4). However, this variation is not as critical as the treatment bias, as Eqn (7) shows that it can in principle be controlled through increasing replication of bottles. A further limitation of using methods B, C, D and E, is that the nominal SEM, that would result from statistical analysis of the predicted MP data, accounts for variation between bottles only. It does not account for treatment bias. If it is to be useful however, the SEM should account for both these sources of variation. Thus, highlighted by our analysis, is that the nominal SEM, as customarily calculated, underestimates the true SEM (Table 5). Treatment bias does not diminish with increasing replication. The consequent underestimation by the nominal SEM may be as large as 0.5 mL methane and would manifest particularly in a highly replicated experiment in which the apparent (nominal) SEM would be quite small. In our experiments, with 8 replicates, the under-estimation of SEM was at most 0.3 mL methane (an apparent SEM of 0.35 instead of the actual SEM of 0.67, in the case of method E). Note however that our experiments did entail diverse substrate treatments. In an experiment with less diversity, a smaller bias variance could possibly be expected.

Although all methods presented similar results and approximated method A, an ideal method should be cost efficient, accurate and simple to perform. While Method A was considered the gold standard in this case, it is the most expensive of the methods considered

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here, having double the number of gas samples to analyze, compared with methods B, C, D and E. Method B had a consistently greater bias than the other methods, meaning that it was the least accurate and method C required counting the total number of ventings which makes it less simple to perform. Method E was slightly less accurate than method D (Table 3) and presented a slight negative bias for both corn and wheat (Fig. 3). Methods D and E were less expensive and less accurate than method A. However, methods D and E were simple to perform and can be considered to be the more accurate methods for estimating MP from vented systems when using a single gas sample taken from the headspace of the incubation vessel at the conclusion of each incubation.

In this study we have compared method B to the gold standard method and showed that it is systematically biased in that it over estimates MP. We have compared methods C and E, which required further validation using real data and a wide range of incubation scenarios, with the gold standard method and showed that these two methods are highly concordant with the gold standard method. Furthermore, we have compared the empirical method D to the gold standard to provide the first comprehensive validation of this method.

CONCLUSIONS

It is concluded that the methane proportion in a gas sample taken from the incubation bottle's head space after the incubation period is greater than the methane proportion in the vented gas. Therefore, methods relying on a simple multiplication of $HSCH_4$ at the end of incubation by GP, such as method B, results in overestimation of MP. Although methods C, D or E did not perfectly match method A, the gold standard, these methods were all more

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accurate than method B for estimating MP. When method A cannot be used, methods D and E are the preferred methods based on a combination of accuracy and simplicity of implementation.

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Table 1. Composition of feeds used in the in vitro experiments

Item	Lucerne hay	Wheat	Barley	Corn
Composition (g/kgDM, unless noted)				
CP	178	145	100	103
ADF	369	32	66	23
NDF	460	87	173	65
Lignin	93	14	15	9
NFC	244	725	673	779
Starch	15	603	532	691
Ash	90	21	25	15
Crude fat	27.6	21.8	29	37.5

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Table 2. Methane in headspace gas ($HSCH_4$, %), methane in vented gas (VCH_4, %), total gas production (GP, mL), and methane production (MP, mL) as calculated by method A[1] (gold standard) for experiments I, II and III

Substrate	Amount (g)	$HSCH_4$ (%)	VCH_4 (%)	GP (mL)	MP (mL)
		Experiment I			
Ground Lucerne hay	1	3.5	1.2[a]	82[a]	8.4[a]
Ground Wheat	1	3.7	2.5[b]	119[b]	10.7[b]
Mean		3.6	1.9	100	9.7
SED		0.16	0.2	3.2	0.2
P value		0.37	<0.001	<0.001	<0.001
		Experiment II			
Ground Lucerne hay	0.5	2.8[b]	0.6[a]	36[a]	6.1[a]
Ground Lucerne hay	1	3.4[a]	1.0[b]	72[b]	7.7[b]
Ground Lucerne hay	1.5	3.5[a]	1.6[c]	94[c]	9.0[c]
Ground Wheat	0.5	3.4[a]	2.1[d]	81[d]	8.9[c]
Ground Wheat	1	3.3[a]	2.6[e]	117[e]	10.1[d]
Ground Wheat	1.5	3.3[a]	2.6[e]	132[f]	10.4[d]
Mean		3.6	1.9	100	9.7
SED		0.16	0.2	3.2	0.2
P value		0.005	<0.001	<0.001	<0.001
		Experiment III			
Ground Lucerne hay	1	4.3[e]	2.7[cd]	93[a]	11.5[bcd]
Ground Wheat	1	3.2[a]	1.8[a]	125[cd]	8.9[a]
Crushed Wheat	1	3.7[bc]	3.3[de]	142[e]	12.4[cd]
Ground Barley	1	4.0[cde]	2.0[ab]	125[c]	10.7[b]
Crushed barley	1	4.1[de]	3.5[e]	123[bc]	12.8[d]
Rolled Barley	1	4.0[cde]	3.3[de]	120[b]	12.2[cd]
Ground Corn	1	3.5[b]	2.6[bc]	131[d]	10.8[bc]
Crushed Corn	1	3.9[de]	3.0[cde]	148[e]	12.6[d]
Mean		3.8	2.8	126	11.5
SED		0.17	0.34	12.3	0.85

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P value	<0.001	<0.001	0.003	<0.001

Means in the same column followed by different supercripts differ (*P* d 0.05)

[1] Cattani et al. (2014)

Table 3. Lin's concordance correlation with confidence interval and its constituent Pearson correlation coefficient and bias correction factor (Cb) of methane production (mL) calculated with methods B[1], C[2], D[3] and E[2] compared to that of the gold standard method A[4]

Method	Concordance	Lower 95%	Upper 95%	Correlation	Cb
Method B	0.67	0.59	0.73	0.89	0.74
Method C	0.85	0.78	0.89	0.85	0.99
Method D	0.88	0.82	0.91	0.89	0.98
Method E	0.81	0.74	0.86	0.85	0.95

[1] Lopez et al. (2007)

[2] Hannah et al. (2016)

[3] Cattani et al. (2016)

[4] Cattani et al. (2014)

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Table 4. Summary statistics from analysis of the difference between estimated methane production (mL) by each method B[1], C[2], D[3] and E[2], and actual methane production (mL) measured by method A4. The model applied to the difference data comprised a mean bias, root-mean-square-error (RMSE) and random effects for incubation treatment and for bottle summarised here by their standard deviation (SD) and residual standard deviation (RSD) respectively

Method	Mean bias (mL methane)	RMSE	SD of incubation (mL methane)	RSD of bottle (mL methane)
B	1.26	1.45	0.37	0.62
C	0.20	0.82	0.55	0.57
D	0.35	0.74	0.41	0.51
E	-0.35	0.84	0.57	0.51

[1] Lopez et al. (2007)

[2] Hannah et al. (2016)

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[3] Cattani et al. (2016)

[4] Cattani et al. (2014)

Table 5. Pearson correlation between substrate treatment means for methane production (mL) measured by method A[4] and estimated by methods, B[1], C[2], D[3] and E[2]. The nominal SEM is the standard error of mean derived from ANOVA of the methane data under each method. The actual SEM was derived after allowing for the estimated random bias associated with substrate treatment

	A	B	C	D	Nominal SEM	Actual SEM
A					0.31	0.31
B	0.93				0.43	0.57
C	0.88	0.94			0.38	0.67
D	0.93	1.00	0.94		0.37	0.55

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E	0.88	0.98	0.98	0.98	0.35	0.67

Figure 1. Vented gas methane to head space methane ratio ($VCH_4/HSCH_4$) against total gas production (GP; mL) from experiments I, II and III.

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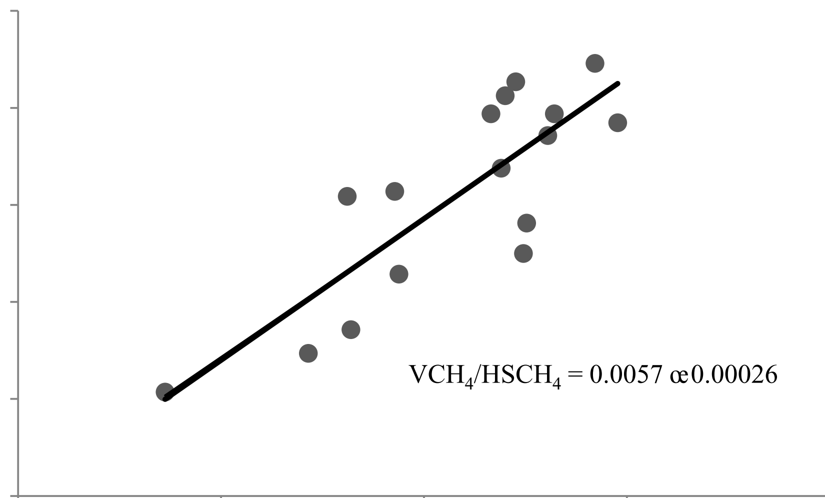

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Figure 2. Methane production (mL) estimates from the three *in vitro* experiments using method B[1] (a), method C[2] (b), method D[3] (c) and method E[2] (d) against the gold standard method A[4]. [1] Lopez et al. (2007); [2] Hannah et al. (2016); [3] Cattani et al. (2016); [4] Cattani et al. (2014) MB: Mean bias, SB: Slope bias

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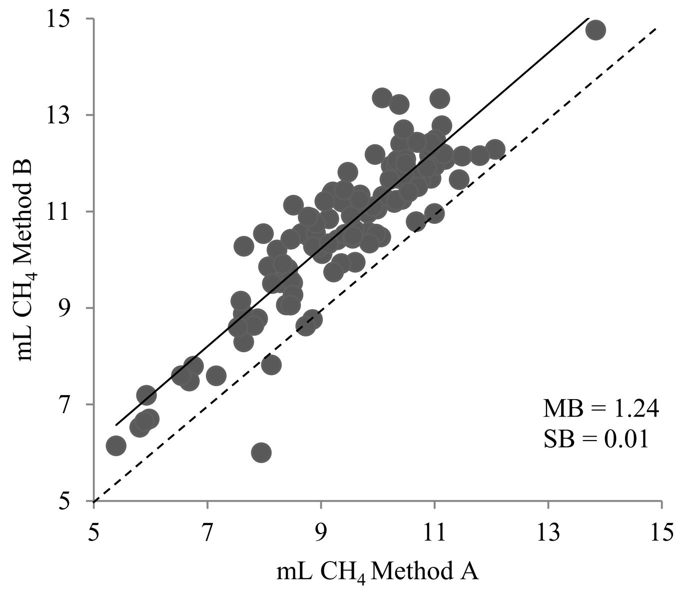
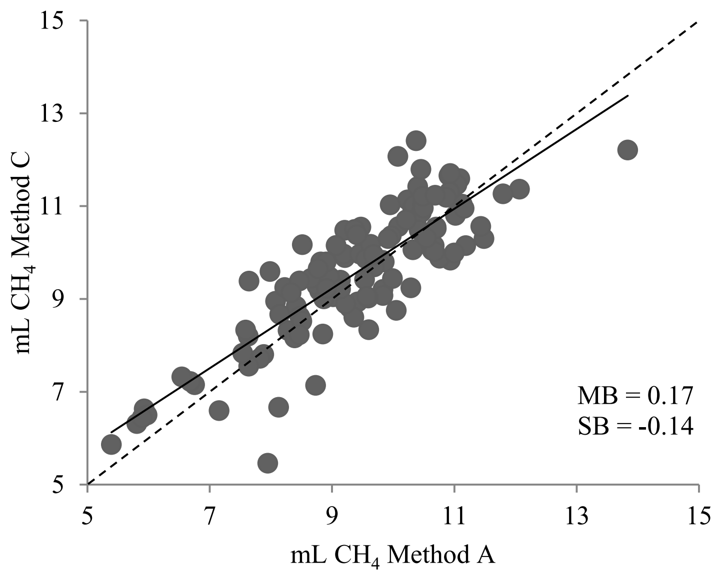
a)



b)



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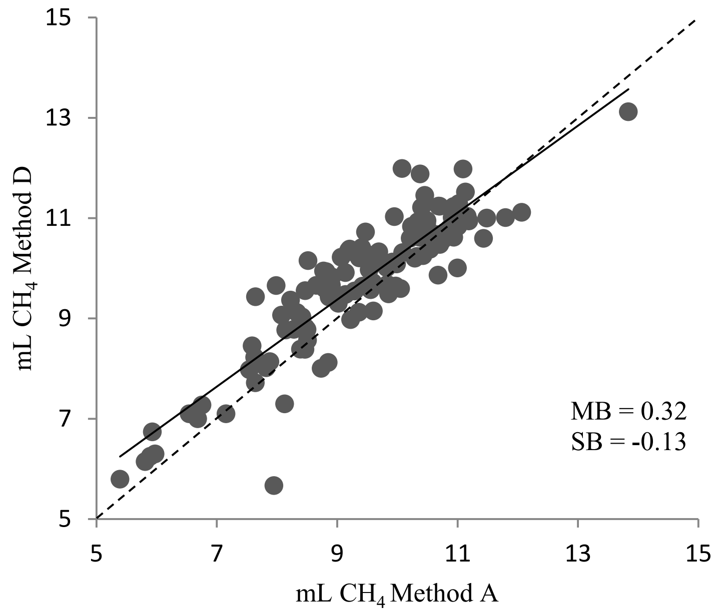
c)



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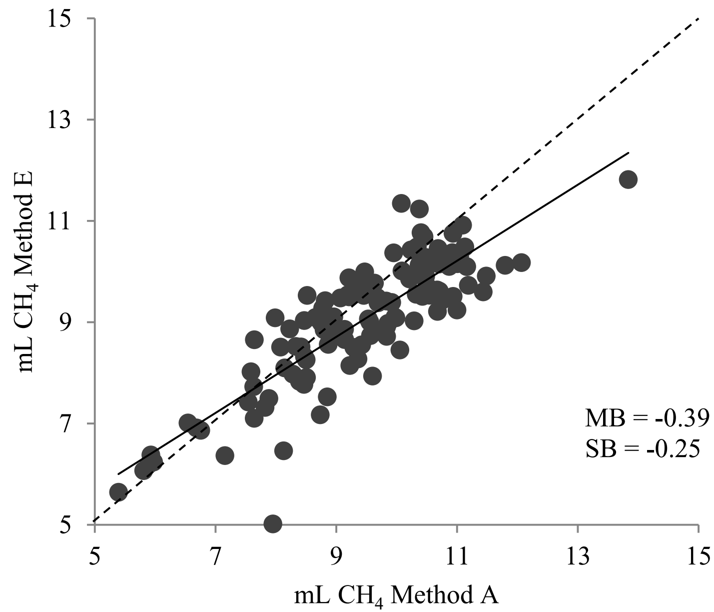

Figure 3. Mean differences between estimated methane production for each incubation by methods B[1], C[2], D[3] and E[2] and actual methane production, measured by method A[4], with 95% confidence intervals.[1] Lopez et al. (2007); [2] Hannah et al. (2016); [3] Cattani et al. (2016); [4] Cattani et al. (2014)

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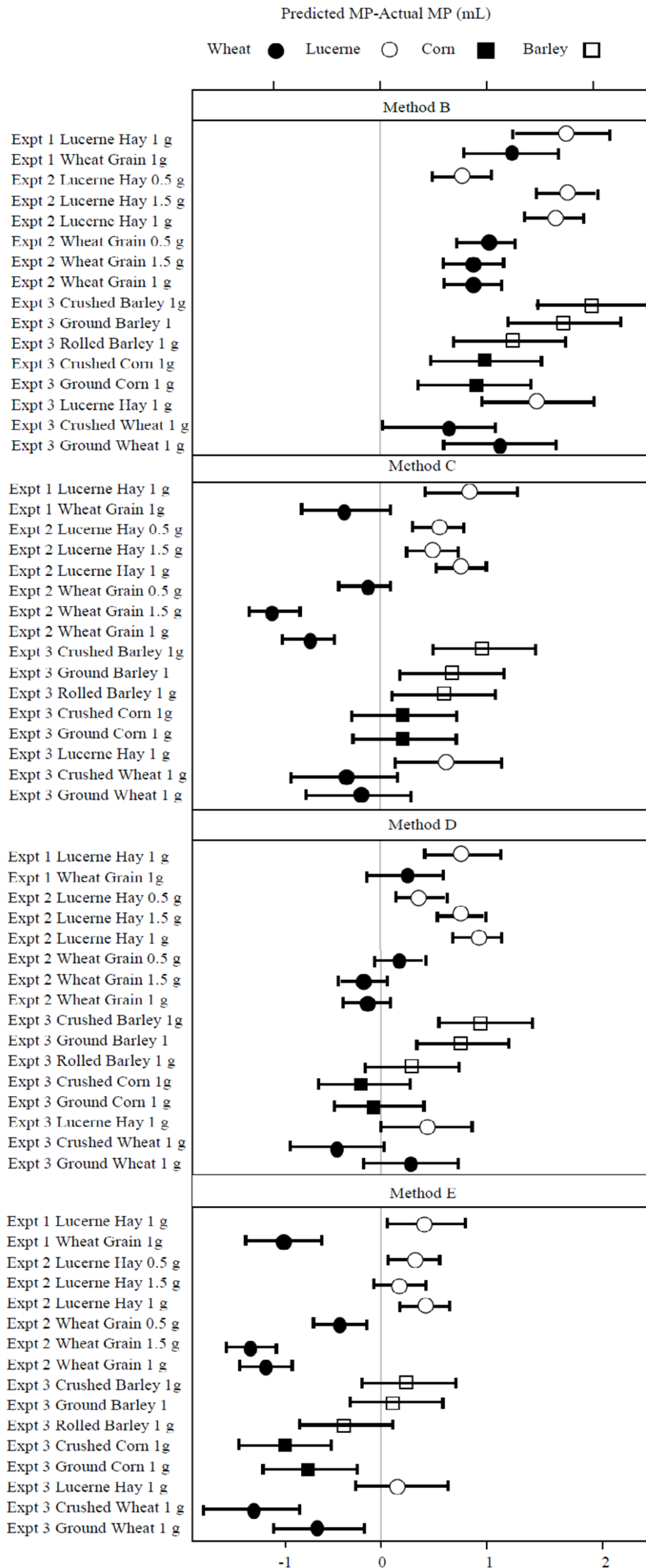

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