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Use of blanks to determine *in vitro* net gas and methane production when using rumen fermentation modifiers

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ABSTRACT

Blanks (flasks without substrate containing only inoculum and medium) are used *in vitro* to correct for gas, CH_4 and residual organic matter (OM) fermented in inoculum. However inclusion of rumen fermentation modifiers may affect fermentation of OM in the substrate and inoculum. Thus, data correction using blanks that lack additives may result in inaccurate adjustment for background fermentation. Our objective was to evaluate impacts of using blanks containing additive (*i.e.*, specific blanks) or blanks without additive on estimation of *in vitro* net gas and CH_4 production. We used the semi-automatic *in vitro* gas production technique including monensin sodium at 2.08 mg/l of buffered rumen fluid (Experiment 1) or carvacrol, eugenol and 1,8-cineol at 667 mg/l (Experiment 2) in flasks with substrate and in blank flasks. At 16 h of incubation, monensin reduced ($P \leq 0.02$) total gas production in flasks containing substrate (162.0 ml *versus* 146.3 ml) and in blanks (84.4 ml *versus* 79.2 ml). Total methane production was also decreased ($P \leq 0.05$) by adding monensin to flasks containing substrate (15.7 ml *versus* 11.9 ml) as well as in blanks (6.4 ml *versus* 5.0 ml). Inclusion of carvacrol or eugenol reduced ($P \leq 0.05$) total gas and CH_4 production in flasks with substrate and in blanks, but in a more pronounced manner than monensin. For these three additives, correction for blank without additive resulted in lower net gas and CH_4 production than correction for a treatment specific blank. For instance, correcting carvacrol data using a blank without the additive resulted in negative net gas and CH_4 production (−6.5 and −1.5 ml, respectively). These biologically impossible results occurred because total gas and CH_4 production in blanks without carvacrol (46.1 and 2.1 ml, respectively) were higher than in flasks containing substrate plus carvacrol (39.7 and 0.6 ml, respectively). Results demonstrated that inclusion of rumen additives affected fermentation of OM in the substrate and the inoculum. Thus, correction of gas and CH_4 production using blanks without additives resulted in overestimation of these variables. Blanks containing the additive of interest should be included when rumen fermentation modifiers are evaluated *in vitro*.

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Abbreviations: ADFom, acid detergent fiber expressed exclusive of residual ash; aNDFom, neutral detergent fiber with heat stable α-amylase expressed exclusive of residual ash; CP, crude protein; DM, dry matter; EE, ether extract; OM, organic matter; TDOM, truly degraded OM.

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1. Introduction

In vitro gas production techniques are widely used to evaluate the antimethanogenic potential of rumen fermentation modifiers, such as plant secondary metabolites (Bodas et al., 2008; García-González et al., 2008; Soliva et al., 2008). *In vitro* CH_4 production is usually expressed as ml (or mmol) of CH_4/g of substrate incubated (Patra et al., 2006; Bodas et al., 2008), ml (or mmol) of CH_4/g of substrate degraded (García-González et al., 2008; Agarwal et al., 2009), or ml of CH_4/ml of gas produced (Goel et al., 2008; Soliva et al., 2008). As rumen fermentation modifiers (*e.g.*, ionophores, essential oils) affect *in vitro* degradation in a dose dependent manner (Russell and Strobel, 1988; Benchaar et al., 2008), CH_4 production is better expressed per unit of substrate degraded, rather than per unit of substrate incubated.

Excluding indirect CO_2 released from buffer, gas and CH_4 are produced *in vitro* by fermentation of organic matter (OM) in the substrate and inoculum (Getachew et al., 1998). To calculate the volume of CH_4 produced per unit of OM degraded, net values of gas and CH_4 production, as well as net amount of OM degraded, are needed. Consequently, blanks (*i.e.*, flasks without substrate which contain only inoculum and medium) are useful to correct for gas, CH_4 and fermentation of residual OM in the inoculum (Rymer et al., 2005).

Previous studies have used blanks without (Patra et al., 2006; Bodas et al., 2008) or with (Alexander et al., 2008; Goel et al., 2008) additive, whereas others did not report the use blanks (García-González et al., 2008; Soliva et al., 2008; Agarwal et al., 2009). Both substrate and inoculum OM fermentation are affected by inclusion of rumen fermentation modifiers. Thus, correcting gas and CH_4 production, as well as OM degradation, using blanks without additive may result in errors.

Using a semi-automatic *in vitro* gas production technique, we evaluated effects of monensin sodium and three plant secondary metabolites (*i.e.*, carvacrol, eugenol, 1,8-cineol) on rumen fermentation of flasks with substrate and in blanks.

2. Materials and methods

All animal use procedures followed guidelines recommended by the Internal Commission for Environmental Ethics and Experimentation with Animals of the Centre for Nuclear Energy in Agriculture (University of São Paulo, São Paulo, Brazil). Two experiments were independently completed. The first examined effects of monensin (2.08 mg/l) on fermentation in buffered rumen fluid. Monensin was selected due to its widespread use as an *in vitro* positive control for manipulating/modifying rumen fermentation. This dosage was chosen because it had previously been found to decrease gas and CH_4 production, increase propionate, and decrease acetate concentration with minimal effects on OM degradation (Araujo et al., 2009). The second experiment dealt with use of carvacrol, eugenol or 1,8-cineol (667 mg/l) in buffered rumen fluid. Essential oils were selected in view of the scientific attention which plant derived substances have received as rumen fermentation modifiers. Dosages were selected at levels that were certain to alter OM fermentation.

2.1. Experimental design

For monensin (Experiment 1), a randomized complete block design was used with incubation considered as a block repeated 10 times. In each incubation, three flasks were used per treatment and the mean of the flasks was considered the replicate. Treatments were defined as: Blank Control (B_{CTL}) – flask without substrate, containing inoculum + medium; Blank Monensin (B_{MON}) – B_{CTL} plus 0.156 mg of monensin; Control (CTL) – flask containing substrate + inoculum + medium; Monensin (MON) – CTL plus 0.156 mg of monensin.

A stock solution of monensin (M5273; Sigma–Aldrich Co., St. Louis, MO, USA; MW, 692.85) was prepared by diluting 15.6 mg in 1.0 ml of pure ethanol which was stored at −10 °C until used. Stock solution (10 μl) was added to each flask (50 ml of medium plus 25 ml of inoculum) to achieve a final monensin concentration of 2.08 mg/ml of buffered rumen fluid. According to Selje-Assmann et al. (2008),11.25 μl of ethanol in 75 ml of buffered rumen fluid had no effects on fermentation and so ethanol was not included in other treatments.

For the plant secondary metabolites (Experiment 2), a randomized complete block design was used with incubation considered as a block repeated thrice. In each incubation, three flasks were used per treatment, with the mean of three flasks considered as the replicate. Treatments were defined as: Blank Control (B_{CTL}) – flask without substrate, containing inoculum + medium; Blank Carvacrol (B_{CAR}) – B_{CTL} plus 50 mg of carvacrol; Blank Eugenol (B_{EUG}) – B_{CTL} plus 50 mg of eugenol; Blank Cineol (B_{CIN}) – B_{CTL} plus 50 mg of 1,8-cineol; Control (CTL) – flask containing substrate + inoculum + medium; Carvacrol (CAR) – CTL plus 50 mg of carvacrol; Eugenol (EUG) – CTL plus 50 mg of eugenol; Cineol (CIN) – CTL plus 50 mg of 1,8-cineol.

Final concentrations of all plant secondary metabolites were 667 mg/l of buffered rumen fluid. Carvacrol (2-methyl-5-isopropyl-1-phenol), eugenol (2-methoxy-4-(2-propenyl)-phenol) and 1,8-cineol (1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane) were provided by GRASP Ind. Com. Ltda. (Curitiba, Paraná, Brazil) as pure components.

2.2. Incubation conditions

An *in vitro* gas production technique (Theodorou et al., 1994) was adapted to a semi-automatic system (Maurício et al., 1999) using a pressure transducer and data logger (Pressure Press Data 800, LANA, CENA/USP, Piracicaba, SP, Brazil). Serum glass flasks of total volume 160 ml and head space 85 ml were filled sequentially with 500 mg of air dried substrate, 50 ml of incubation medium (Theodorou's medium described in Preston, 1995) and 25 ml of inoculum. Flasks were sealed imme-

diately with 20 mm butyl septum stoppers (Bellco Glass Inc., Vineland, NJ, USA), manually mixed, and incubated in a forced air oven (Marconi MA35, Piracicaba, SP, Brazil) at 39 °C for 16 h. Head space gas pressure was measured at 3, 6, 11 and 16 h of incubation. For CH_4 determination, 2.5 ml gas were sampled at each time using a 5 ml syringe (Becton-Dickson Indústria Cirúrgica Ltda, Curitiba, PR, Brazil) and stored in a 10 ml vacuum tube. After each gas sampling, flasks were vented, mixed and returned to the oven. After 16 h, flasks were placed in cold water (4 °C) followed by immediate addition of neutral detergent for determination of truly degraded OM (TDOM).

2.3. Description of substrate

The substrate was an 800:200 (w/w) concentrate:forage diet consisting of 200 g of coastcross (*Cynodon* sp.) hay, 627 g of ground corn, 150 g of soybean meal, 10 g of limestone, and 13 g of mineral premix/kg of dietary dry matter (DM). Dietary DM was 914 g/kg of the as fed diet and chemical composition was 157 g crude protein (CP), 33 g ether extract (EE), 54 g ash, 203 g neutral detergent fiber exclusive of residual ash and using heat stable α-amylase and sodium sulfite (aNDFom), and 88 g acid detergent fiber (ADFom) per kg of dietary DM. The diet was formulated to meet or exceed NRC (2007) recommendations using the Small Ruminant Nutrition System v. 1.8.0 (Cannas et al., 2004). The diet was ground in a Wiley mill (Marconi, Piracicaba SP, Brazil) to pass a 1 mm screen.

2.4. Inoculae preparation

For monensin, 3 rumen cannulated Santa Inês wethers of 50 kg body weight were penned and used as donors of rumen content. Wethers were individually fed 1.2 kg/d of the same incubated diet. Feed was provided twice daily at 07:00 and 17:00 h in equal portions with adaptation lasting at least 10 d. Wethers had free access to a mineral premix and fresh water. Ruminal liquid and solid fractions were collected separately from each wether before morning feeding and kept in pre-warmed thermo containers under anaerobic conditions. A liquid fraction was obtained by inserting a tube attached to a 60 ml syringe. Similar volumes (500:500, v/v) of both fractions were blended for 10 s, squeezed through 3 layers of cheesecloth, and maintained in a water bath at 39 °C under CO_2. Final inoculae consisted of a mixture of the 3 samples collected from each sheep.

For the experiment with plant secondary metabolites, the same 3 wethers were kept on pastures of signal grass (*Brachiaria decumbens*) and elephant grass (*Pennisetum purpureum*) with free access to a mineral premix and fresh water. Each was supplemented with 150 g/d of ground corn, 65 g/d of soybean meal, and 4.5 g/d of sugarcane molasses. Inoculae preparation followed the same procedures as described above.

2.5. Laboratory analyses and calculations

Gas production was calculated by the equation defined for our laboratory conditions as

$$V = 7.365 \times p \quad (n = 500;\ r^2 = 0.99)$$

where V = gas volume (ml); p = measured pressure (psi). Total gas production at 16 h of incubation was considered as the sum of partial gas production at each time interval. Net gas, expressed as ml/g OM degraded, was calculated by correcting values of gas production and OM degradation for the corresponding blank.

After incubation, 70 ml of neutral detergent (Van Soest et al., 1991) without heat stable α-amylase was included in each flask and incubated at 105 °C for 3 h. The residue was filtered in pre-weighed crucibles, washed with hot water and acetone and oven dried at 105 °C for 16 h. TDOM was determined after ashing at 550 °C for 4 h with correction for the corresponding blank.

Methane concentration was determined using a gas chromatograph (Shimadzu 2014, Tokyo, Japan) equipped with a Shincarbon ST 100/120 micro packed column (1.5875 mm OD, 1.0 mm ID, 1 m length; Ref. no. 19809; Restek, Bellefonte, PA, USA). Temperatures of column, injector, and flame ionization detector were 60, 200, and 240 °C, respectively. Helium at 10 ml/min was the carrier gas. Methane concentration was determined by external calibration using an analytical curve (0, 30, 60, 90, 120 ml/l) prepared with pure CH_4 (White Martins PRAXAIR Gases Industriais Inc., Osasco, SP, Brazil; 995 ml/l purity). Methane production was calculated according to Longo et al. (2006), as follows:

$$CH_4,\ \text{ml} = (\text{Total gas, ml} + \text{Head space},\ 85\ \text{ml}) \times CH_4\ \text{concentration (ml/ml)}$$

Net CH_4 production, expressed as ml/g OM degraded, was calculated by correcting values of CH_4 production and OM degradation for the corresponding blank.

For chemical analyses of substrate, dry matter (ID 934.01), ash (ID 942.05), N (ID 990.03; Leco FP528 combustion N analyzer; Leco Corporation, St. Joseph, MI, USA), and EE (ID 920.39) were determined according to AOAC (2006). Concentrations of aNDFom and ADFom were corrected for residual ash and determined by the non-sequential method using beakers according to Van Soest et al. (1991). The NDF determination was performed with addition of heat stable α-amylase (Ankom Technology, Tecnoglobo Equipamentos, Curitiba, PR, Brazil) and sodium sulfite.

Table 1
Effect of monensin on *in vitro* rumen fermentation in flasks with substrate and in blanks (Experiment 1).

	Treatment[a]				SEM	Contrast P		
	CTL	MON	B_{CTL}	B_{MON}		SED	CTL *versus* MON	B_{CTL} *versus* B_{MON}
Total Gas (ml)	162.0	146.3	84.4	79.2	4.66	2.16	<0.01	0.02
Total CH_4 (ml)	15.7	11.9	6.4	5.0	0.82	0.65	<0.01	0.05
Total residual OM (mg)	119.3	145.1	31.2	34.5	3.32	3.73	<0.01	0.39

[a] CTL and MON: Control and Monensin, flasks containing 500 mg of air dried substrate + 50 ml of medium + 25 ml of inoculum + no monensin or 0.156 mg of monensin; B_{CTL} and B_{MON}: Blank Control and Blank Monensin, flasks without substrate containing only 50 ml of medium + 25 ml of inoculum + no monensin or 0.156 mg of monensin.

2.6. Statistical analyses

For the monensin experiment, data were analyzed using the MIXED procedure of SAS (2004). Factors in the model were treatment as a fixed effect and block as a random effect. Means of gas production, CH_4 production, and residual OM were obtained by LSMEANS. Comparisons of CTL *versus* MON and B_{CTL} *versus* B_{MON} were by contrasts. Estimates of net gas production, net CH_4 production, and net residual OM were obtained by the ESTIMATE command subtracting B_{CTL} from CTL, and B_{MON} from MON, respectively. The contrast 'CTL − B_{CTL} *versus* MON − B_{CTL}' considered: +CTL − B_{CTL} − MON + B_{CTL}, which results in +CTL − MON. The contrast 'CTL − B_{CTL} *versus* MON − B_{MON}' was considered to be: +CTL − B_{CTL} − MON + B_{MON}. Differences were considered significant if P<0.05.

For the plant secondary metabolites experiment, data were analyzed using the MIXED procedure of SAS (2004). Factors in the model were treatment as a fixed effect and block as a random effect. Treatment means, contrasts, and estimates were obtained as described for the monensin experiment. Differences were considered significant if P<0.05.

3. Results

3.1. Monensin sodium Experiment 1

Compared with the respective control, monensin addition reduced (P<0.05) total gas and CH_4 production in flasks containing substrate and in blanks (Table 1). Therefore, MON treatment resulted in higher (P<0.01) total residual OM than CTL, whereas B_{MON} did not differ from B_{CTL}.

Effects of subtracting B_{CTL} or B_{MON} from flasks containing substrate (*i.e.*, CTL or MON) on net values of gas production, CH_4 production, residual OM and TDOM are in Table 2. The CTL corrected for B_{CTL} (herein referred as 'corrected CTL') resulted in higher ($P \leq 0.01$) net gas and CH_4 production than MON corrected for B_{CTL} or B_{MON}.

Net gas production, expressed as ml/g OM degraded, was higher (P<0.01) for corrected CTL than MON corrected for B_{CTL}. However, MON corrected for B_{MON} did not differ from corrected CTL. Net CH_4 production, expressed as ml/g OM degraded, was higher (P<0.01) for corrected CTL than MON corrected for B_{CTL} or B_{MON}.

Net residual OM (mg) was lower (P<0.01) for corrected CTL than MON corrected with B_{CTL} or B_{MON}. Finally, corrected CTL had the highest (P<0.01) TDOM, regardless of whether MON was corrected with B_{CTL} or B_{MON} (Table 2).

Table 2
Effect of subtracting Blank Control or Blank Monensin from flasks containing substrate in order to estimate net values of gas production, CH_4 production, and true OM degradation in *in vitro* rumen fermentations (Experiment 1).

	Estimate of contrast[a]			Contrast			
	With Blank Control		With Blank Monensin	CTL − B_{CTL} *versus* MON − B_{CTL}		CTL − B_{CTL} *versus* MON − B_{MON}	
	CTL − B_{CTL}	MON − B_{CTL}	MON − B_{MON}	SED	P	SED	P
Net Gas (ml)	77.6	61.9	67.1	2.16	<0.01	3.06	0.01
Net gas (ml/g OM degraded)	225.8	194.8	209.4	6.15	<0.01	9.14	0.11
Net CH_4 (ml)	9.4	5.6	6.9	0.65	<0.01	0.93	0.01
Net CH_4 (ml/g OM degraded)	27.3	17.5	21.5	1.72	<0.01	1.33	<0.01
Net residual OM (mg)	88.1	113.9	110.6	3.73	<0.01	5.27	<0.01
TDOM (g/g incubated)[b]	0.796	0.736	0.744	0.0052	<0.01	0.0066	<0.01

[a] CTL − B_{CTL}: Control corrected for Blank Control; MON − B_{CTL}: Monensin corrected for Blank Control; MON − B_{MON}: Monensin corrected for Blank Monensin.
[b] Total OM content per flask was 432.3 mg (in DM basis) in 500 mg of air-dried substrate.

Table 3
Effect of carvacrol, eugenol, or 1,8-cineol on *in vitro* rumen fermentation in flasks with substrate and in blanks (Experiment 2).

	Treatment[a]								SEM	Contrast						
	With substrate				Blank					SED	P for CTL *versus*			P for B_{CTL} *versus*		
	CTL	CAR	EUG	CIN	B_{CTL}	B_{CAR}	B_{EUG}	B_{CIN}			CAR	EUG	CIN	B_{CAR}	B_{EUG}	B_{CIN}
Total Gas (ml)	115.0	39.7	97.9	112.3	46.1	32.2	39.2	42.7	2.05	2.42	<0.01	<0.01	0.27	<0.01	0.01	0.18
Total CH_4 (ml)	11.2	0.6	3.7	9.7	2.1	0.4	0.9	2.1	0.40	0.54	<0.01	<0.01	0.02	<0.01	0.05	0.95
Total residual OM (mg)	131.0	235.0	164.8	156.3	25.7	31.0	30.4	29.1	4.45	6.28	<0.01	<0.01	<0.01	0.42	0.47	0.60

[a] CTL, CAR, EUG, CIN: Control, Carvacrol, Eugenol, and 1,8-Cineol, flasks containing 500 mg of air-dried substrate + 50 ml of medium + 25 ml of inoculum + no additive or 50 mg of carvacrol, eugenol, or 1,8-cineol, respectively; B_{CTL}, B_{CAR}, B_{EUG}, B_{CIN}: Blank Control, Blank Carvacrol, Blank Eugenol, and Blank 1,8-Cineol, flasks without substrate containing only 50 ml of medium + 25 ml of inoculum + no additive or 50 mg of carvacrol, eugenol, or 1,8-cineol, respectively.

3.2. Plant secondary metabolites trial (Experiment 2)

There was a decrease ($P \leq 0.05$) in total gas and CH_4 production with inclusion of carvacrol or eugenol in either substrate or blank flasks (Table 3). In contrast, 1,8-cineol had no affect on total gas production in flasks containing substrate or in blanks. However, 1,8-cineol reduced total CH_4 production in flasks containing substrate ($P<0.05$), but not in blanks.

Addition of carvacrol, eugenol, or 1,8-cineol with substrate resulted in higher ($P<0.01$) total residual OM (mg) in comparison with CTL, but this was not affected by inclusion of essential oils in blanks (Table 3).

Net gas and CH_4 production (ml) were higher ($P<0.01$) for corrected CTL than CAR or EUG corrected for either B_{CTL} or additive specific blanks (Table 4). Correction of CAR for B_{CTL} resulted in biologically unrealistic negative estimates for these variables as B_{CTL} had higher gas and CH_4 production than CAR (46.1 and 2.1 ml for B_{CTL} *versus* 39.7 and 0.6 ml for CAR, respectively). In contrast, no difference occurred in net gas production (ml) when corrected CTL was compared with CIN adjusted for either B_{CTL} or B_{CIN}. However, CIN corrected for B_{CTL} had lower ($P=0.02$) net CH_4 production (ml) compared with corrected CTL, but no difference occurred when CIN was corrected for B_{CIN}.

Corrected CTL had higher ($P \leq 0.02$) net gas and CH_4 production, expressed as ml/g OM degraded, compared with CAR or EUG corrected for either B_{CTL} or additive specific blanks (Table 4). Again, correcting CAR for B_{CTL} resulted in a negative estimate of net gas and CH_4 production (ml/g OM degraded), which was a consequence of higher total gas and CH_4 production in B_{CTL} compared with CAR. Conversely, correction of CIN for B_{CIN} resulted in higher ($P<0.01$) net gas production (as ml/g OM degraded) than corrected CTL, whereas no difference occurred after correcting CIN for B_{CTL}. Net CH_4 production, expressed as ml/g OM degraded, was not influenced by 1,8-cineol addition, regardless of whether this estimate was corrected using B_{CTL} or B_{CIN} (Table 4).

Inclusion of carvacrol, eugenol, or 1,8-cineol resulted in higher ($P \leq 0.02$) net residual OM compared to corrected CTL. As a result, TDOM was highest ($P \leq 0.02$) for corrected CTL in spite of correcting CAR, EUG, or CIN for B_{CTL} or additive specific blanks (Table 4).

4. Discussion

For monensin, there are two explanations for the decrease in total gas and CH_4 production and the increase in total residual OM (Table 1). First, monensin increases propionate concentration (Russell and Strobel, 1988) and, according to the stoichiometry of gas production, propionate formation is associated with decreases in gas and CH_4 production (Wolin, 1960). Methane production was 27.3 ml/g OM degraded for corrected CTL and 21.5 ml/g OM degraded for MON (2.08 mg/l) corrected for B_{MON}; a 21% reduction. Reductions in methane production of 48, 52 and 58% were reported using hay as substrate when 2.5, 5.0, and 12.5 mg/l of monensin where added *in vitro* (Russell and Strobel, 1988). Secondly, monensin decreases *in vitro* OM degradation, which is supported by higher residual OM for MON (145.1 mg) compared with CTL (119.3 mg). This reduction in OM degradation is a typical limitation of short term *in vitro* experiments because monensin inhibits gram positive bacteria involved in fermentation, including some cellulolytic ruminococci (Chen and Wolin, 1979). Unfortunately, we did not measure NDF concentrations in residues, which might support the explanation that monensin impaired fiber degradation. In contrast, monensin's detrimental effects on fiber degradation have not been observed *in vivo* because some monensin tolerant cellulolytic species are able to replace the sensitive species of cellulolytic bacteria *in vivo* (Russell and Strobel, 1988).

For carvacrol and eugenol, the reduction in total gas and CH_4 production was associated with an increase in total residual OM due to the antimicrobial and antimethanogenic activities of these substances. Calsamiglia et al. (2007) and Benchaar et al. (2008) reviewed the effects of carvacrol and eugenol on ruminal fermentation. Effects of 1,8-cineol on fermentation were less evident in our study, demonstrating that 1,8-cineol does not possess potent antimicrobial activity. However Tatsuoka et al. (2008) reported minor effects of cineol cyclodextrins on *in vitro* rumen fermentation, with an increase in CH_4 production.

The reduction in gas and CH_4 production of blanks after inclusion of monensin, carvacrol or eugenol indicates that these additives were able to affect OM fermentation in the inoculum. It is evident that if rumen additives are developed to modify rumen fermentation, they may impact fermentation of OM in substrate and inoculum. However, inoculum feed particles were

Table 4
Effect of subtracting blank controls or additive specific blanks from flasks containing substrate in order to estimate net values of gas production, CH_4 production, and truly OM degradation in *in vitro* rumen fermentations (Experiment 2).

	Estimate of contrast[a]							Contrast P							
	Blank Control				Additive Specific Blanks			$CTL - B_{CTL}$ *versus*				$CTL - B_{CTL}$ *versus*			
	$CTL - B_{CTL}$	$CAR - B_{CTL}$	$EUG - B_{CTL}$	$CIN - B_{CTL}$	$CAR - B_{CAR}$	$EUG - B_{EUG}$	$CIN - B_{CIN}$	SED	$CAR - B_{CTL}$	$EUG - B_{CTL}$	$CIN - B_{CTL}$	SED	$CAR - B_{CAR}$	$EUG - B_{EUG}$	$CIN - B_{CIN}$
Net Gas (ml)	68.9	−6.5	51.7	66.1	7.5	58.7	69.5	2.42	<0.01	<0.01	0.27	3.42	<0.01	<0.01	0.85
Net gas (ml/g OM degraded)	213.0	−30.4	178.8	222.0	27.3	199.6	231.0	5.82	<0.01	<0.01	0.17	4.43	<0.01	0.02	<0.01
Net CH_4 (ml)	9.1	−1.5	1.6	7.6	0.2	2.7	7.6	0.54	<0.01	<0.01	0.02	0.76	<0.01	<0.01	0.07
Net CH_4 (ml/g OM degraded)	28.2	−6.7	5.6	25.7	0.7	9.3	25.2	2.06	<0.01	<0.01	0.26	2.32	<0.01	<0.01	0.23
Net residual OM (mg)	105.2	209.2	139.0	130.6	204.0	134.4	127.2	6.28	<0.01	<0.01	<0.01	8.89	<0.01	<0.01	0.02
TDOM (g/g incubated)[b]	0.754	0.512	0.676	0.695	0.524	0.686	0.703	0.0160	<0.01	<0.01	0.01	0.0158	<0.01	<0.01	0.02

[a] $CTL - B_{CTL}$: Control corrected for Blank Control; $CAR - B_{CTL}$, $EUG - B_{CTL}$, $CIN - B_{CTL}$: Carvacrol, Eugenol or 1,8-Cineol corrected for Blank Control; $CAR - B_{CAR}$, $EUG - B_{EUG}$, $CIN - B_{CIN}$: Carvacrol, Eugenol or 1,8-Cineol corrected for additive specific blank.

[b] Total OM content per flask was 432.3 mg (in DM basis) in 500 mg of air-dried substrate.

undergoing fermentation prior to incubation *in vitro* as they would already have been colonized by ruminal microorganisms while in the host animal. Moreover, much of the gas produced from fermentation of OM in the inoculum is released during the first few hours of incubation. Thus, considering that a period of time is required for appearance of additive effects, it was expected that additives would be less effective in blanks than in flasks with substrate. Such a pattern was only confirmed for carvacrol, the additive which had the most pronounced effect on fermentation. For carvacrol, gas and CH_4 production were reduced by 65.5 and 94.6% in flasks with substrate and by 30.2 and 81.0% in blanks. However in the other treatments, effects of additives were similar in flasks with substrate and in blanks. For instance, monensin inclusion reduced gas and CH_4 production by 9.7 and 24.2% in flasks with substrate, and by 6.2 and 21.9% in blanks. For eugenol, gas production was reduced by 15.0% in flasks with substrate as well as in blanks. These inconsistencies are probably a reflection of differences in antimicrobial activity among the additives.

Effects of monensin, carvacrol, or eugenol on fermentation of blanks confirm that correction of gas and CH_4 production using B_{CTL} (*i.e.*, blanks without additive) is an inappropriate approach. It is clear that correction using B_{CTL} leads to overestimation of additive effects when compared with those corrected for additive specific blanks. For example, net gas production was 225.8 and 194.8 ml/g OM degraded for corrected CTL and MON corrected for B_{CTL}, respectively. However, the estimate of net gas production increased to 209.4 ml/g OM degraded when MON was corrected for B_{MON}, which was not different than corrected CTL. Similarly, in the second experiment, net gas production was 213.0 and 222.0 ml/g OM degraded for correct CTL and CIN adjusted to B_{CTL}. In this case, net gas production increased to 231.0 ml/g OM degraded when CIN was corrected for B_{CIN}, an estimate that was higher than corrected CTL.

Additives included at a higher dosage, or exhibiting higher biological activity, increase the error associated with correction for B_{CTL}. In our case, this was confirmed for CAR, the additive which had the highest antimicrobial activity. Correcting gas and CH_4 production of CAR using B_{CTL} resulted in unrealistic negative estimates of these variables.

Using additive specific blanks presents additional advantages, particularly if the additive can be used as a substrate by microbial populations. For example, crude plant extracts may contain fermentable sugars. Thus, an additive specific blank would also correct for gas and CH_4 that arises from the presence of this substrate. Such an approach has been employed previously using ground plant material that could potentially modify fermentation (Goel et al., 2008).

In some instances additive specific blanks may not be necessary or practical. For example, 1,8-cineol had minimal affects on ruminal fermentation. Inclusion of additive specific blanks also increases the number of incubation flasks, a relevant consideration in large scale programs screening for antimethanogenic plant additives such as those conducted by Bodas et al. (2008), García-González et al. (2008) and Soliva et al. (2008). In addition, efforts to minimize inoculum OM content would reduce the necessity of an additive specific blank.

Finally, net gas production for CAR corrected for B_{CAR} (7.5 ml) was biologically too low when related to TDOM (0.524 g/g incubated). Consequently, net gas production was only 27.3 ml/g OM degraded, which is much lower than 213 ml/g OM degraded for CTL. Similar results occurred for net CH_4 production expressed as ml/g OM degraded. Degradation was much higher than gas production because undegraded substrate (800:200 concentrate:forage diet with 627 g of corn/kg of DM) was partially solubilized by the incubation medium and NDF solution used to estimate true microbial degradation. Addition of 50 mg of carvacrol/flask impacted microbial activity the most among our additives. As a consequence, more undegraded substrate remained after 16 h of fermentation. It is known that ND was developed to solubilize plant cell content, but it also solubilizes starch to some extent (Van Soest et al., 1991). As a result, TDOM was overestimated for CAR.

5. Conclusions

We demonstrated that inclusion of rumen fermentation modifiers affects substrate as well as inoculum OM fermentation. Therefore, correction of gas and CH_4 production using blanks without additive results in overestimation of these variables. In addition, additives included at a high dosage, or exhibiting high biological activity, increase the error associated with correction for a blank without additive. Thus to obtain net values of gas production, CH_4 production, and truly degraded OM, we suggest utilization of additive specific blanks, which is a flask without substrate containing only inoculum, incubation medium, and the additive being evaluated.

Conflicts of interest

None.

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