Final report

Screening feed additives for methane mitigation using in vitro studies

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Abstract

Reducing enteric methane (CH_4) emissions from ruminants is a key priority to improve environmental sustainability and consumer perception of Australian red meat production. This project used high throughput laboratory screening methodology to identify feed additives with potential to supress enteric CH_4 production, whilst maintaining favourable ruminal digestion and fermentation. Nine additives were selected for evaluation based on previous literature. Additives, at doses recommended by manufacturers, were combined with Rhodes grass hay and subjected to a 48h batch culture incubation. The four additives which exhibited the greatest CH_4 mitigation potential included two biochar samples, Citral, and Sandalwood oil, both of which also affected rumen fermentation parameters, to varying degrees. Citral and Sandalwood oil had detrimental effects on digestibility, which when combined with the price of these supplements made them unviable for livestock feed supplements in their current state. Detailed knowledge of biochar composition and the additional compounds it contained for this study (KNO_3 and asparagopsis), would facilitate an understanding of how these products are able to elicit changes in CH_4, but were considered to have confounding effects in the current trial, with the source of the effect unknown. As such, no additives studied in this project met the criteria to be further evaluated in the RUSITEC system.

Executive summary

Background

The Australian Red Meat Industry has set the aspirational target to be carbon neutral by 2030 (CN30). Currently, 78% of greenhouse gas (GHG) emissions are from pasture raised beef (Mayberry et al, 2018). Most of these emissions are from enteric CH_4, which is gas exhaled by ruminants as a natural part of the digestion process. This project sought to identify potential feed additives that mitigate enteric CH_4 production, whilst maintaining favourable ruminal digestion and fermentation.

Aims/objectives

- Determine the effect of graded levels (dose response) of provided feed additives on batch-culture fermentation utilizing roughage diets.
- Based on the results of the batch culture, select the two most promising additives for screening by RUSITEC (in-vitro rumen simulation technique)
- Determine the effect of the graded levels of the two most promising additives on RUSITEC fermentation.
- Determine changes in the rumen microbial population (abundance and diversity) associated with treatment responses from RUSITEC fermentation.

Methodology

Feed additives were selected by Meat & Livestock Australia by an open call process. Products were prioritized according to potential biological mode of action, safety, ease of manufacture and dietary inclusion rate. A literature review of published research to-date regarding the 26 products was undertaken to allow selection of the most promising additives for investigation using in-vitro batch cultures. Batch cultures were conducted with Rhodes grass hay as the basal diet.

Results/key findings

The four additives which exhibited the greatest CH_4 mitigation potential included two biochar samples, Citral, and Sandalwood oil, also affected rumen fermentation parameters, to varying degrees. When combined with additional information on product availability for livestock and price, they were not considered viable options to progress to the RUSITEC evaluation.

Recommendations

The planned RUSITEC trials did not proceed. A thorough economic assessment of future promising additives in addition to in-vitro screening would facilitate an evaluation of the feasibility of industry adoption.

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1. Background

Global meat production is required to increase exponentially to meet the increasing demand from a world population forecast to exceed 8.6 billion by 2030 (Davison et al., 2020). At the same time, there is a growing consumer awareness of the contribution of the red meat industry to climate change. Both aspects are transforming the industry, as producers are required to rapidly increase production, while reducing emissions of greenhouse gases (GHG) such as methane (CH_4; Johnson and Johnson, 1995). In Australia, these challenges have been considered with priority. In fact, the Australian Red Meat Industry has implemented an ambitious target to become carbon neutral by 2030 (CN30).

Currently, there are a wide range of commercial dietary additives that have been studied in *in vitro* and *in vivo* experiments. Most additives have shown inconsistent results against CH_4 production and/or concentration, and in some cases, these alternatives negatively affect rumen fermentation, nutrient digestibility, growth performance, and feed efficiency. Conversely, other dietary additives such as 3-nitrooxypropanol (3-NOP) have reduced CH_4 production by 30 and 42% in forage and concentrate based diets, respectively, without altering rumen fermentation, and promoting daily weight gain and/or feed efficiency (Martinez-Fernandez et al., 2018; Vyas et al., 2018; Meale et al., 2021). The macroalgae *Asparigopsis* (*taxiformis* and *armate*) has also been shown to reduce CH_4 under *in vitro* conditions between 95 to 99% (Machado et al., 2016a; Roque et al., 2019a), and under *in vivo* conditions by 67.7% for dairy cattle, and up to 80% for beef cattle and sheep (Li et al., 2018; Roque et al., 2019b; Roque et al., 2020). In general terms, both additives have the potential to inactivate methyl coenzyme-M reductase (MCR) and its reactions, which are essential in the terminal enzymatic step of methanogenesis (Liu et al., 2011; Duin et al., 2016). For the latter additive, the halomethane compounds (e.g. bromoform and bromochloromethane) can also inhibit cobamide-dependent methyl transferase, which has a fundamental role in the last step in the methanogenesis pathway (Machado et al., 2016b), subsequently, both additives can directly inhibit methanogenesis.

However, as neither of these additives are currently commercially available in Australia, the aim of this project was to screen additives that are commercially available or very close to commercially available for their effect on CH_4 and rumen fermentation characteristics. The aim was to identify additives which could have an immediate impact on the GHG emissions from the red meat industry.

2. Objectives

The objectives of the study were to determine the effect of graded levels (dose response) of 10 feed additives on batch-culture fermentation utilizing roughage diets. This objective was successfully achieved for 9 additives due to the unavailability of the remaining selected additive. A further objective was to select the two most promising additives from the batch culture for screening by RUSITEC (in-vitro rumen simulation technique). The two most promising additives for CH_4 reduction were selected, however they exhibited detrimental effects on fermentation parameters and were not considered viable options for large scale uptake in the red meat industry, so the RUSITEC was not carried out. The final two objectives: 1) Determine the effect of the graded levels of the two most promising additives on RUSITEC fermentation; and 2) Determine changes in the rumen microbial population (abundance and diversity) associated with treatment responses from RUSITEC fermentation, were not completed.

3. Methodology

3.1 Feed Additive Selection

Feed additives were selected by Meat & Livestock Australia by an open call process. A list of 26 additives was supplied to UQ for independent assessment of the biological mode of action to supress enteric CH_4 via an extensive review of previous literature. Products were prioritized according to potential biological mode of action, cost, safety, and with a small consideration of dietary inclusion rate as not all additives were supplied with current inclusion rates. Potential animal, environmental and human safety implications were also identified.

Ten additives were recommended for in-vitro screening based on an extensive literature review. The selected additives had shown promising results to reduce CH_4 and elicited only small changes in rumen fermentation parameters and diet digestibility. Unfortunately, it was not possible to evaluate all of the selected additives in the batch culture incubation. Grape marc (Grap' Tan PC) is no longer commercially available and could not be sourced. In the case of *B. pelecinus* and *E. glabra*, a complex and time-consuming extraction of their bioactive compounds was required, and this was not able to be conducted prior to incubations commencing. Additionally, sesamin, the fraction of *E. glabra* that we planned to evaluate, required importation from United States. As such, in consultation with MLA it was decided that these additives were not available for further testing. As a result, we proceeded to evaluate two biochar additives with different compositions, in addition to two presentations of a Commercial additive (powder and liquid), which as indicated by the manufacturer, may alter the ability to reduce CH_4. Another Commercial additive was included in the final list of tested additives, as in previous studies it had shown inconsistent anti-methanogenic effects, with no effects on other rumen fermentation parameters.

3.2 In-vitro Batch Culture Incubations

The batch culture system facilitates a rapid and relatively inexpensive assessment of a large number of additives under simulated ruminal conditions. As rumen fluid is collected from live animals adapted to the basal diet, the additives are subjected to the normal suite of microbes present in the rumen, allowing an additives effect on rumen fermentation to be quantified and to identify additives with the potential to reduce CH_4 production. The batch culture is a static system, however, with no inflow or outflow of saliva and feed, limiting microbial turnover and necessitating a short incubation period. Nevertheless, is a useful screening step employed prior to embarking on more expensive and time-consuming methods, such as the Rumen Simulation Technique (RUSITEC) which replicates saliva and feed in flux on a daily basis to facilitate incubations of 14 d allowing microbial adaptation to new additives to be assessed.

Animal ethics approval was obtained for rumen fluid collection for the *in vitro* batch culture studies under the guidelines of the UQ Production Animal Ethics Committee (AEC# SA 2019/08/707, ratified by UQ, approval #2021/AE000823).

Briefly, Rhodes grass hay was selected as the basal roughage source to represent the pasture available in Northern QLD. Inclusion levels of each additive were determined based on the manufacturer's recommended dose and/or literature available. With the recommended dose used as dose level 2, and dose 1 and 3 being above and below the recommended dose, respectively, unless three doses were provided by the manufacturer (Table 1 and 2).

Table 1. Doses of additives used in the in-vitro batch culture experiments and concentration replacing the substrate dry matter (DM).

Additives	Concentration, %DM		
	Dose 1	Dose 2	Dose 3
Commercial additive A (powder)	0.025	0.05	0.1
Commercial additive B	1	3	5
Commercial additive C	0.05	0.25	0.5
Bovacillus®	0.25	0.5	0.75
Biochar A	2	5	8
Biochar B	1	3	5

Table 2. Doses of additives used in the in-vitro batch culture experiments and concentration of media (rumen fluid + buffer).

Additives	Concentration, % rumen fluid volume		
	Dose 1	Dose 2	Dose 3
Citral	0.1	0.5	1
Sandalwood oil	1	3	5
Commercial additive A (liquid)	0.25	0.5	1

The effects of the additives on the functionality of the rumen microbial system were assessed by measuring common fermentation parameters including microbial gas production and composition (methane; 24 and 48 h), pH, volatile fatty acids (VFA) and in vitro dry matter digestibility (IVDMD) at 48 h of incubation.

Calculations for IVDMD were adjusted for powder additives as some of the additives had a small particle size and were able to leave the bag during incubation, which could bias IVDMD estimation (Table 3). A similar approach as used at Teoh et al. (2019).

Soluble fraction (%DM) = percentage of additives that left the bags at time 0h after bags being washed continuously until water ran clear under the tap

Amount of soluble fraction (in g) = Soluble fraction (%DM) × amount of additives added

Corrected residues (g) = [(Bags + residues after 48h incubation) – bags weight] + Amount of soluble fraction (in g)

Digestible DM (g) = (Substrate + additives; DM basis) - corrected residues

IVDMD = Digestible DM (g) / (Substrate + additives; DM basis) × 100

Table 3. Soluble fraction of powder additives evaluated in in-vitro batch culture incubation

Additive	Soluble fraction, (%DM)
Biochar A	31.2
Biochar B	63.5
Commercial additive B	76.0
Bovacillus®	7.7
Commercial additive A (powder)	99.4
Commercial additive C	33.9

3.3 Statistical analysis

The univariate procedure of SAS was used to test for normal distribution of the data. The 3 replicate bags were averaged prior to statistical analysis and those averages, within run, were the statistical unit. Data were analysed with mixed model procedures of (SAS Inc., 2021). The data were analysed as a randomized complete design using PROC MIXED (SAS) with treatment in the model as fixed effects and run and the run by treatment interaction as random effects. The run × treatment interaction was used as the error term to test the treatment effect. Treatment means for each concentration were compared against the control using the least squares mean linear hypothesis test (LSMEANS/DIFF) with the Dunnett adjustment with significance declared if $P<0.05$.

As Biochar A and B, Citral and Sandalwood oil had lower ($P<0.05$) CH_4 outputs, these data were analysed again using orthogonal contrast testing linear and quadratic effects. Biochar B and Sandalwood were analysed together as the doses used were the same (0, 1, 3, and 5%), but Citral (0, 0.01, 0.05 and 0.10%) and biochar A (0, 2, 5 and 8%) were analysed separately.

3.4 Rumen Simulation Technique (RUSITEC)

The use of the RUSITEC to assess the two most promising additives from the batch culture incubations was intended, however due to a lack of promising candidates this was not conducted. The RUSITEC is a continuous culture system initiated using a mixed rumen population from donor animals, with substrate added daily, and buffer infused, and end-products removed continuously. This methodology allows microbial adaptation to new additives, identifying if the effect of an additive can persist over time, or if microbes will adapt and diminish its effects with constant supplementation.

Briefly, after a period of adaptation (7 days), two selected additives were to be tested over 7 days, with daily supplementation of fresh substrate and additive, buffer and removal of end-products. Additives were to be tested at 3 doses, including a control with no additive. Samples were to be collected daily for assessment of functionality of the rumen microbial system (gas, CH_4, pH, VFAs, IVDMD, and microbial diversity).

4. Project outcomes

4.1 Additive selection

Following an extensive review of the literature on the submitted additives it is evident that multiple mechanisms exist to reduce CH_4 under *in vitro* and *in vivo* conditions. Additives can either directly reduce methanogenic growth and methanogenesis; or indirectly reduce CH_4 by creating alternative H_2 sinks, or altering rumen bacterial and protozoal communities. Unfortunately, a common factor among many additives is the depletion of rumen fermentation, which is not desirable in a potential additive to mitigate CH_4. Despite this, our review highlighted several additives that showed the capability of mitigating CH_4 with only minor impacts on rumen fermentation parameters, and some positive effects on feed efficiency (Figure 1).

Additives selected for in-vitro screening based on the literature review of submitted additives included Grap' Tan PC, Commercial additive B, Commercial additive D, Biochar, S. *spicatum* oil (Sandalwood oil), *E. glabra* , *B. pelecinus,* Citral, Bovacillus®, and Commercial additive A. Due to timing and availability constraints the final list of nine additives evaluated in the in-vitro batch cultures included Citral, Sandalwood oil (*S. spicatum*), biochar A and biochar B, Commercial additive B, Commercial additive A in liquid and powder formats, Commercial additive C, and Bovacillus®.

4.2 In-vitro incubations

With a basal substrate of Rhodes grass hay, Commercial additive A (both liquid and powder forms), Commercial additive C, and Bovacillus® were unable to alter rumen fermentation, including CH_4 concentration during 48 h of incubation (Table 4-6). It is possible that with longer adaptation periods these supplements may be able to elicit effects on fermentation, however, that was not possible to determine using a batch culture incubation.

Commercial additive Acontains multiple secondary compounds, such as phenolic acids and flavonoids, which separately have the potential to reduce CH_4 under *in vitro* conditions without changing rumen fermentation (Oskoueian et al., 2013). These compounds can deplete ruminal fibrolytic bacteria, protozoal and fungi communities, but their effects are highly dependent on the inclusion rate, source, and diet/substrate. These compounds appear to have modes of actions which are capable of inhibiting CH_4 due to their direct effect on methanogens (e.g. creating a hydrogen sink), and indirectly by depleting rumen cellulolytic bacteria growth and activities, decreasing fibre digestibility and shifting VFA's profile. This was the first study to examine the effect of Commercial additive A in liquid and powder formats, on CH_4 (Table 4 and 5).

Commercial additive Cis a blend of essential oils that has previously been examined with variable results on CH_4 and performance. It has been evaluated at doses up to 30 ppm (m/v) in two consecutive batch culture incubations (96 h and 14 d) with no change observed in CH_4 concentrations, total VFAs, and individual molar proportions of VFAs. Conversely, using the gas production technique for 72 h, Commercial additive Cat 30 ppm successfully reduced CH_4 concentration by ~17% after 30 h, but this effect tended to disappear after 60 h.

Bovacillus® contains a combination of *B. licheniformis* and *B. subtilis* and to-date had limited evidence to confirm its anti-methanogenic properties, effects on rumen fermentation, growth performance or feed efficiency. The two *Bacillus* strains comprising **Bovacillus**® have individually shown the capability to reduce CH_4 by 8.3% (Wang et al., 2016; Deng et al., 2018) due to the synthesis of bacteriocin-like compounds which directly inhibit methanogenic growth and promote H_2 bacterial utilisation.

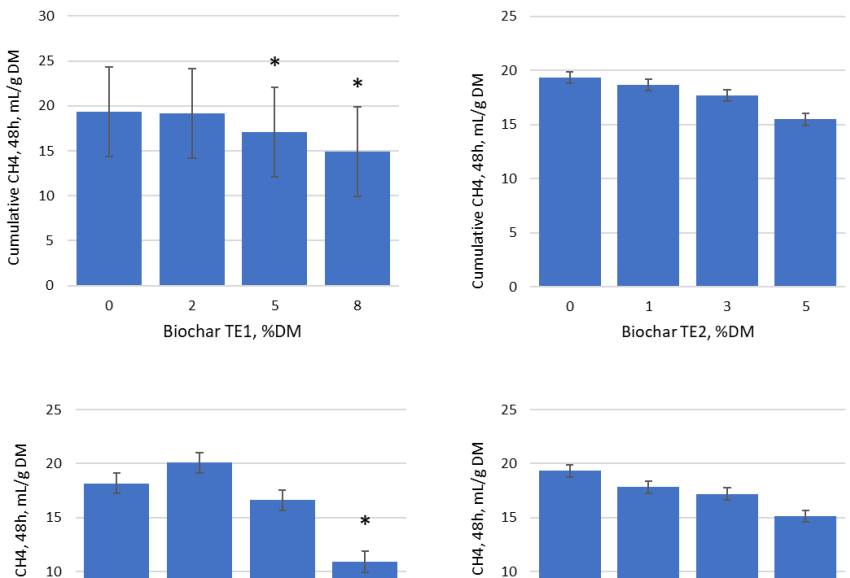
Commercial additive B**,** comprised of multiple organosulphur compounds which inhibit methanogenic growth by disrupting the HMG-CoA enzyme essential for a methanogens membrane integrity. Itwas evaluated with and without air exposure prior to incubation, to determine differences in additive effectivity and stability, with neither leading to altered rumen fermentation, IVDMD or CH_4. However, when exposed to air, reduced total gas production was achieved at 48 h by 24% ($P < 0.05$) when included at 5% DM (Table 6).

Citral is the main essential oil compound in lemongrass (80%), and green tea tree (*Melaleuca teretifolia;* Joch et al., 2016). Its inclusion at 0.1% media volume linearly reduced total gas and CH_4 production, as well as IVDMD and pH (Table 6-7), indicating that although CH_4 was inhibited rumen fermentation was also detrimentally affected. The interaction of citral aldehydes with carbon or double carbon bounds, increases electronegative compounds which can supress electro transfers, inhibiting biological activities and thus, rumen bacterial growth (Dorman and Deans, 2000). Here, Citral at 0.05% of media volume decreased cumulative CH_4 production, compared to the lowest dose (0.01% v/v), but did not differ from the control. Inclusion at 0.05% of media volume linearly decreased cumulative gas production (mL and mL/g DM) and IVDMD, and linearly increased rumen pH, compared to the control, yet its effects were less severe than inclusion at the highest dose.

Previous reports (Pawar et al., 2014) have shown a similar dose dependant response, where at the lowest inclusion of lemongrass (167 ul/ DMi) caused a 7% reduction in CH_4 without altering rumen fermentation parameters. While at higher doses (333, 500, 667 and 833 ul/ DMi) decreased CH_4 (mL/gDMi) up to 91%, but reduced gas production, and total VFAs (up to 56%, and 32%, respectively) suggesting the dose administered is crucial when considering the use of citral as an anti-methanogenic additive.

Biochar

The ability of biochar to inhibit CH_4 emissions in previous studies, results from its highly porous structure and large internal surface area (Leng et al., 2012). Both characteristics may facilitate the development of diverse microbial biofilms, and act as an electron mediator in redox reactions between microbial species, resulting in more efficient digestion and utilisation of energy. These properties may promote rumen bacterial growth and synthesis of substrates which have the potential to sink hydrogen, however results on methane reductions have been inconsistent.



Here, cumulative CH_4 at 48h (mL) decreased (P<0.01) with both biochar additives included at the highest dose of 8% DM (Table 4, and 8-9). A linear decrease in $CH_4$was observed with Biochar A. However, analysis suggested that Biochar A at 5% DM caused a reduction in CH_4, compared with the control, and Biochar A at 2% DM, but this effect was lower than the highest concentration used (8%). Interestingly, a dose effect was observed for both biochar additives, indicating a linear decrease in total gas and CH_4 parameters, and a linear increase in rumen pH, and acetate as a % of total VFA.

Sandalwood (S. *spicatum*) oil at 5% inclusion reduced cumulative CH_4 at 48h (mL/g DM) by 21.6%, compared to the control. However, at 3% and 5% of total media volume it also reduced IVDMD by 12.4% and 13.3%, respectively (Table 5 and 9) and increased rumen pH (P<0.01) by an average 0.14, compared with the control (Table 5 and 9). The reduction in CH_4 agrees with previous reports which achieved an average 40% reduction in CH_4, with corresponding reductions in total VFA and acetate, by 14.1% and 5.5%, respectively (Durmic et al., 2014; Jahani-Azizabadi et al., 2019). Though, this essential oil may increase propionate concentrations, this was not observed in the current in-vitro incubation. The specific mode of action for Sandalwood oil is unknown.

4.3 Commercial considerations

Biochar (A and B), Citral and Sandalwood oil are not currently available/licensed as commercial livestock feed additives, and/or on a scale suitable for use in the livestock industry. Commonly Sandalwood oil is produced for human use, while both Biochar's are currently still in the formula optimization stage. The cost in Australian dollars for the biochar additives is projected to be $1.25/kg, and Sandalwood oil is $175/kg + *GST*. Citral is produced by multiple companies with different purities. For the current *in vitro* incubations, Citral was purchased from Sigma-Aldrich with a cost around $434/L. Another important factor is the potential production capacity of products as this can limit distribution. The current manufacturers production capacity projection for both biochar and Sandalwood additives stand at 10,000 tons per year. In relation with Citral, the potential production is not available for public knowledge. Considering that Sandalwood oil is not distributed on a wide scale currently, and large-scale production for animal use is not an immediate target of the manufacturer, production capacity is up to 1 tonne per year, but this capacity is variable each year. Nevertheless, the amount of Sandalwood harvested per year around Australia is approximately 500 tonnes, indicating that the capacity of oil production could increase. Importantly to note, the recommended inclusion rate of Sandalwood oil is limited to 5% DM to avoid negatively supressing rumen fermentation. Further, knowledge of the exact components within both Biochar samples would be of benefit, to determine which components are eliciting the maximum effects on rumen fermentation and to further determine the optimal dose rate.

5. Conclusion

The four additives which showed the most promising CH_4 mitigation effect [Biochar (A and B), Citral, and Sandalwood oil], also affected rumen fermentation parameters, to varying degrees. Biochar A which contained KNO_3, decreased CH_4 when used at 8% DM. This CH_4 inhibition was accompanied with an increased pH and reduction of valerate % of total VFA. Biochar B, containing KNO_3+*asparagopsis*, at 5% DM reduced cumulative CH_4 production without altering IVDMD but increased pH. The CH_4 mitigation effect of Sandalwood oil at 5% DM was observed in the final period of the incubation (24 to 48h), suggesting a possible lag in effect. The strong negative effect on IVDMD limited the consideration of Sandalwood oil and Citral as potential additives for livestock, however, total and cumulative gas production, indicators of rumen fermentation, were not affected.

Important considerations for the use of these additives include the price of Sandalwood oil, and its capped inclusion rate (max 5% DM) to avoid supressing rumen fermentation as an oil product. Detailed knowledge of the biochar composition and the additional compounds (KNO_3 and *asparagopsis*), on a DM basis would further facilitate an understanding of how these products are able to elicit changes in methane. There is a confounding effect of these additional compounds and the biochar itself, which would benefit from further investigation. Knowledge of the metabolites into which these compounds are broken down are of relevance, as nitrates may cause nitrate toxicity and hypoxia. Furthermore, *Asparagopsis* contains bromoform, dibromochloromethane, bromochloroacetic acid and dibromoacetic acid, which animals have shown hesitance to consuming, and can be excreted in milk (Muizelaar et al. 2021), which may limit its adoption by producers. Despite low inclusion rates, care should be taken as negative media attention associated with the inclusion of bromoform in ruminant diets would limit its adoption by the industry. This product is also not available at commercial scale, and the form (wet or dry) and amount at which it was included into the biochar is unknown. Citral at 0.1% was not considered a viable additive to be used for CH_4 mitigation due to its strong negative effects on total gas, pH and IVDMD. However, results from orthogonal analysis suggests that it may be worth testing Citral between 0.05% and 0.1% of media volume in further in-vitro evaluations.

5. Benefits to industry

Most of the additives evaluated in this project are commercially available, significantly increasing the distribution potential around Australia and increasing accessibility to producers wishing to adopt methane mitigation strategies, although they may not be currently available at the scale and price required to be feasible as a livestock feed supplement. As such, the evaluation of non-commercial compounds expands our knowledge on potential compounds that can be used to develop efficient additives to inhibit methane, without promoting changes in rumen fermentation. This technique can be further used to screen emerging additives prior to larger scale animal trials.

6. Future research and recommendations

Further batch culture evaluation of these four additives with different doses to identify the optimal dose at which their anti-methanogenic properties are enhanced, and any undesirable effects on rumen fermentation and IVDMD are reduced could be considered. Moreover, as the effect of these

additives may change with diet composition, it would be of benefit to evaluate these additives under differing substrate conditions such as those containing some concentrates. The evaluation of new and emerging additives using such a screening method is recommended to determine feasibility to progress to animal level investigations. A thorough economic evaluation of any potential additives must be undertaken to ensure that industry adoption is feasible if desirable anti-methanogenic activity is achieved.

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8. Appendix

Table 4. Effects of additives (%DM) on rumen fermentation parameters, IVDMD and methane over 24 and 48 h of incubation, through three in-vitro batch culture fermentation runs.

Parameter	Control	Commercial additive C			Biochar A			Biochar B			Bovacillus®			Commercial additive A (Powder)			SEM[5]	*P*-value
	0%	0.05%	0.25%	0.50%	2%	5%	8%	1%	3%	5%	0.25%	0.50%	0.75%	0.03%	0.05%	0.10%		
Gas production 24h, mL	37.89	35.89	35.89	35.44	36.28	34.67	34.00	35.67	34.83	32.33	37.11	35.94	37.83	36.22	34.50	36.83	1.781	0.15
Gas production 48h, mL	29.34	28.1	27.97	28.51	27.57	28.83	29.14	26.74	27.41	26.87	27.64	28.88	27.48	27.87	27.69	28.37	1.891	0.35
Cumulative gas production 48h, mL	67.23	63.99	63.86	63.96	63.84	63.50	63.14	62.41	62.24	59.20	64.76	61.79	65.31	64.09	62.19	65.20	2.386	0.43
Cumulative gas production 48h, mL/g DM	127.5	126.22	126.32	126.59	126.56	126.06	124.67	124.42	124.17	117.99	128.26	122.46	128.81	125.93	123.83	129.89	4.862	0.40
CH_4[1] 24h, %	14.74	14.37	14.67	14.27	14.32	12.68†	10.76*	14.38	13.72	12.01*	14.68	15.24	14.92	14.44	14.32	14.74	1.040	<0.01
CH_4 48h, %	15.72	14.91	14.91	15.40	16.18	14.49	13.15*	15.9	15.04	14.61	14.45	15.92	15.81	15.28	15.17	15.54	0.599	<0.01
CH_4 24h, mL	5.59	5.18	5.28	5.04	5.19	4.40	3.68*	5.11	4.76	3.84*	5.47	5.49	5.65	5.22	5.13	5.43	0.361	<0.01
CH_4 48h, mL	4.61	4.21	4.20	4.40	4.47	4.20	3.87	4.25	4.11	3.93	4.01	4.62	4.22	4.29	4.21	4.41	0.388	<0.01
Cumulative CH_4 48h, mL	10.20	9.39	9.48	9.45	9.66	8.61	7.55*	9.36	8.87	7.766*	9.48	10.11	9.69	9.51	9.33	9.85	0.662	<0.01
Cumulative CH_4 48h, mL/g DM	19.32	18.55	18.75	18.70	19.15	17.09	14.91*	18.66	17.70	15.48*	18.77	20.04	19.10	18.7	18.59	19.61	1.303	<0.01
IVDMD[2], %	53.91	54.45	55.00	55.19	57.19	56.28	56.45	55.46	54.48	52.22	55.42	57.16	56.07	53.95	55.82	55.33	1.598	<0.01
pH	6.18	6.20	6.19	6.22	6.23	6.27	6.32*	6.22	6.27	6.31*	6.22	6.17	6.18	6.18	6.16	6.20	0.035	<0.01
Total VFA[3], mM	140.62	143.15	147.09	145.14	153.50	157.36	155.85	151.09	147.93	146.11	147.51	162.29	155.35	156.33	155.99	153.59	6.749	0.01
VFA production (48h-0h), mM	96.52	101.56	102.99	101.04	109.40	113.26	111.75	107.00	103.83	102.01	103.41	118.20	111.25	112.24	111.89	109.49	6.788	<0.01
Acetate (% total VFA)	65.39	65.73	66.37	65.87	66.65	66.59	67.66	66.65	66.64	66.67	65.81	66.82	66.28	66.40	66.32	66.55	0.726	0.11
Propionate (% total VFA)	21.74	21.68	20.78	21.36	21.23	21.23	20.70	21.27	21.30	21.39	21.68	20.91	21.34	21.28	21.40	21.27	0.506	0.88
Butyrate (% total VFA)	8.57	8.40	8.64	8.54	8.07	8.11	7.63	7.95	7.87	7.78	8.35	8.14	8.12	8.18	8.22	8.03	0.315	0.27
BCVFA[4] (% total VFA)	2.27	2.25	2.24	2.24	2.18	2.23	2.22	2.24	2.28	2.23	2.20	2.25	2.31	2.22	2.17	2.24	0.145	0.09
Valerate (% total VFA)	1.80	1.74	1.75	1.77	1.69	1.67†	1.624*	1.70	1.72	1.69	1.75	1.68	1.75	1.71	1.7	1.71	0.080	<0.01
Caproic (% total VFA)	0.23	0.21	0.23	0.22	0.18	0.16	0.16	0.19	0.20	0.24	0.22	0.19	0.20	0.21	0.18	0.21	0.024	0.03
A:P ratio	3.01	3.03	3.19	3.08	3.14	3.14	3.27	3.14	3.13	3.12	3.04	3.20	3.11	3.12	3.10	3.13	0.100	0.74

* means differences (P < 0.05) from control treatment, † means tendency from control treatment (P < 0.10) [1] CH_4, methane. [2] IVDMD, *In vitro* dry matter digestibility.[3] VFA, Volatile fatty acids.[4] BCVFA, Branched- chain fatty acids (iso-valerate and iso-butyrate) [5] SEM, standard error of the means.

Table 5. Effects of additives (% media volume) on rumen fermentation parameters, IVDMD and methane over 24 and 48 h of incubation, through three in-vitro batch culture fermentation runs.

Parameter	Control	Commercial additive A (Liquid)			Sandalwood oil			SEM[5]	*P*-value
	0%	0.25%	0.50%	1%	1%	3%	5%		
Gas production 24h, mL	37.89	36.33	37.61	37.56	36.50	35.44	32.39	1.781	0.15
Gas production 48h, mL	29.34	28.09	28.48	29.39	27.63	26.18	24.24	1.891	0.35
Cumulative gas production 48h, mL	67.23	64.42	66.09	66.94	64.13	61.62	56.63	2.386	0.43
Cumulative gas production 48h, mL/g DM	127.50	123.16	124.61	128.16	122.42	116.16	107.19	4.862	0.4
CH_4[1] 24h, %	14.74	14.43	15.11	14.16	14.67	14.60	14.18	1.040	<0.01
CH_4 48h, %	15.72	15.22	16.25	15.27	14.63	15.04	14.17	0.599	<0.01
CH_4 24h, mL	5.59	5.23	5.63	5.33	5.34	5.20	4.58	0.361	<0.01
CH_4 48h, mL	4.61	4.29	4.64	4.48	4.04	3.95	3.43	0.388	<0.01
Cumulative CH_4 48h, mL	10.20	9.51	10.27	9.77	9.30	9.14	8.01	0.662	<0.01
Cumulative CH_4 48h, mL/g DM	19.32	18.20	19.35	18.61	17.83	17.20	15.15	1.303	<0.01
IVDMD[2], %	53.91	52.69	54.66	53.98	52.89	47.25*	46.75*	1.584	<0.01
pH	6.18	6.21	6.15	6.17	6.22	6.31	6.32	0.035	<0.01
Total VFA[3], mM	140.62	132.97	142.41	152.42	140.18	128.27	126.13	6.749	0.01
VFA production (48h-0h), mM	96.52	88.88	98.32	108.32	96.09	84.18	82.03	6.788	<0.01
Acetate (% total VFA)	65.39	66.66	65.39	65.89	65.48	66.36	66.36	0.726	0.11
Propionate (% total VFA)	21.74	21.61	21.72	21.26	21.58	20.52	21.00	0.506	0.88
Butyrate (% total VFA)	8.57	8.60	8.44	8.71	8.31	8.46	8.76	0.315	0.27
BCVFA[4] (% total VFA)	2.27	2.03	2.32	2.16	2.15	2.28	2.02	0.145	0.09
Valerate (% total VFA)	1.80	1.78	1.84	1.75	1.80	1.85	1.89	0.080	<0.01
Caproic (% total VFA)	0.23	0.24	0.29	0.23	0.25	0.23	0.21	0.024	0.03
A:P ratio	3.01	3.08	3.01	3.10	3.03	3.23	3.16	0.100	0.74

* means differences (P < 0.05) from control treatment, † means tendency from control treatment (P < 0.10) [1] CH_4, methane. [2] IVDMD, *In vitro* dry matter digestibility.[3] VFA, Volatile fatty acids.[4] BCVFA, Branched- chain fatty acids (iso-valerate and iso-butyrate) [5] SEM, standard error of the means.

Table 6. Effects of Citral (%DM) and Commercial additive B (% media volume) doses on rumen fermentation parameters, IVDMD and methane over 24 and 48 h of incubation, through two in-vitro batch culture fermentation runs.

Parameter	Control	Commercial additive B (air exposed prior to incubation)			Commercial additive B (closed prior to incubation)			Citral			SEM[5]	P-value
	0%	1%	3%	5%	1%	3%	5%	0.01%	0.05%	0.10%		
Gas production 24h, mL	38.83	36.42	38.00	36.67	40.67	39.33	38.00	39.50	34.33	28.41*	1.781	<0.01
Gas production 48h, mL	28.83	25.80	24.15†	24.03*	26.05	25.33	24.18†	27.88	26.93	22.45*	1.891	0.03
Cumulative gas production 48h, mL	9.57	8.71	9.62	8.70	10.04	9.30	8.92	10.37	8.65	5.73*	2.386	<0.01
Cumulative gas production 48h, mL/g DM	18.18	17.35	19.16	17.33	19.78	18.49	17.62	20.08	16.61	10.94*	4.862	<0.01
CH_4[1] 24h, %	13.29	13.72	14.96	13.91	14.66	14.38	13.69	15.26	13.47	9.31*	1.040	0.02
CH4 48h, %	15.14	14.38	16.28	14.89	15.58	15.26	15.31	15.54	14.88	13.00	0.599	0.26
CH4 24h, mL	5.20	5.00	5.71	5.12	5.97	5.43	5.21	6.04	4.63	2.69*	0.361	<0.01
CH4 48h, mL	4.37	3.72	3.92	3.58	4.06	3.86	3.70	4.34	4.02	3.05*	0.388	0.01
Cumulative CH4 48h, mL	9.57	8.71	9.62	8.70	10.04	9.30	8.92	10.37	8.65	5.73*	0.662	<0.01
Cumulative CH4 48h, mL/g DM	18.18	17.35	19.16	17.33	19.78	18.49	17.62	20.08	16.61	10.94*	1.303	<0.01
IVDMD[2], %	51.87	52.33	46.96	47.29	54.13	50.55	46.78†	53.26	48.16	42.48*	1.606	<0.01
pH	6.19	6.19	6.22	6.27	6.12	6.21	6.26	6.20	6.31	6.41*	0.035	<0.01
Total VFA[3], mM	142.12	153.01	150.23	150.83	148.03	152.28	148.99	150.19	146.87	156.18	6.749	0.54
VFA production (48h-0h), mM	99.30	110.20	107.41	108.02	105.21	109.47	106.17	107.24	104.06	113.36	6.788	0.60
Acetate (% total VFA)	65.77	67.00	67.68	67.01	67.22	67.34	67.63	67.13	66.25	66.96	0.726	0.31
Propionate (% total VFA)	21.59	20.93	20.31	20.56	20.29	20.61	20.14	20.96	21.38	20.78	0.506	0.35
Butyrate (% total VFA)	8.51	8.09	8.01	8.48	8.44	7.99	8.15	7.90	8.19	8.25	0.315	0.89
BCVFA[4] (% total VFA)	2.16	2.10	2.14	2.07	2.10	2.14	2.13	2.14	2.22	2.11	0.145	0.96
Valerate (% total VFA)	1.73	1.67	1.65	1.65	1.65	1.67	1.68	1.63	1.72	1.67	0.080	0.59
Caproic (% total VFA)	0.23	0.20	0.21	0.24	0.29	0.25	0.27	0.22	0.25	0.23	0.024	0.10
A:P ratio	3.05	3.21	3.33	3.26	3.32	3.27	3.36	3.51	3.11	3.23	0.100	0.23

* means differences ($P < 0.05$) from control treatment, † means tendency from control treatment ($P < 0.10$) [1] CH_4, methane. [2] IVDMD, *In vitro* dry matter digestibility.[3] VFA, Volatile fatty acids.[4] BCVFA, Branched- chain fatty acids (iso-valerate and iso-butyrate) [5] SEM, standard error of the means.

Table 7. Orthogonal analysis of Citral (% media volume) on rumen fermentation parameters, IVDMD and methane over 24 and 48h of incubation

Parameter	Control	Citral			SEM[5]	P-value		
						Treatment	Linear	Quadratic
	0%	0.01%	0.05%	0.10%				
Gas production 24h, mL	38.83	39.50	34.33	28.42*	1.226	<0.01	<0.01	0.62
Gas production 48h, mL	28.83	27.88	26.93	22.45*	1.344	0.09	0.02	0.48
Cumulative gas production 48h, mL	67.67	67.38	61.27*	50.87*	1.842	<0.01	<0.01	0.38
Cumulative gas production 48h, mL/g DM	128.62	130.45	117.79*	97.34*	3.440	<0.01	<0.01	0.26
CH_4[1] 24h, %	13.29	15.26	13.47†	9.31†	1.183	0.09	0.03	0.20
CH_4 48h, %	15.14	15.54	14.88	13.13*	1.027	0.42	0.16	0.56
CH_4 24h, mL	5.20	6.04	4.63	2.69*	0.358	<0.01	<0.01	0.12
CH_4 48h, mL	4.37	4.34	4.02	3.08*	0.251	<0.01	<0.01	0.18
Cumulative $CH_4$48h, mL	9.57	10.37	8.65	5.73*	0.556	<0.01	<0.01	0.12
Cumulative $CH_4$48h, mL/g DM	18.18	20.08	16.61	10.89*	0.959	<0.01	<0.01	0.08
IVDMD[2], %	51.88	53.26	48.17*	42.48*	1.457	<0.01	<0.01	0.51
pH	6.19	6.20	6.31*	6.41*	0.038	<0.01	<0.01	0.88
Total VFA[3], mM	142.12	135.89	146.87	156.18	8.569	0.35	0.10	0.80
VFA production (48h-0h), mM	99.30	93.07	104.06	113.36	7.824	0.33	0.10	0.80
Acetate (% total VFA)	65.77	68.45	66.25	66.96	0.837	0.11	0.94	0.95
Propionate (% total VFA)	21.59	19.94	21.38	20.78	0.617	0.26	0.98	0.85
Butyrate (% total VFA)	8.51	7.42*	8.19	8.25	0.293	0.08	0.55	0.56
BCVFA[4] (% total VFA)	2.16	2.44	2.22	2.11	0.297	0.64	0.50	0.72
Valerate (% total VFA)	1.73	1.54	1.72	1.67	0.065	0.10	0.65	0.88
Caproic (% total VFA)	0.23	0.21	0.25	0.23	0.021	0.35	0.71	0.30
A:P ratio	3.04	3.51	3.11	3.22	0.162	0.22	0.85	0.93

* means differences ($P < 0.05$) from control treatment, † means tendency from control treatment ($P < 0.10$) [1] CH_4, methane. [2] IVDMD, *In vitro* dry matter digestibility.
[3] VFA, Volatile fatty acids. [4] BCVFA, Branched- chain fatty acids (iso-valerate and iso-butyrate) [5] SEM, standard error of the means.

Table 8. Orthogonal analysis of Biochar A (%DM) on rumen fermentation parameters, IVDMD and methane over 24 and 48 h of incubation

Parameter	Control	Biochar A			SEM[5]	P-value		
						Treatment	Linear	Quadratic
	0%	2%	5%	8%				
Gas production 24h, mL	37.89	36.28	34.67	34.00	1.066	0.06	<0.01	0.50
Gas production 48h, mL	29.34	27.57	28.83	29.14	1.667	0.65	0.81	0.43
Cumulative gas production 48h, mL	67.23	63.84	63.50	63.14	2.091	0.46	0.21	0.43
Cumulative gas production 48h, mL/g DM	127.5	126.56	126.06	124.67	3.946	0.95	0.6	0.98
CH_4[1] 24h, %	14.74	14.32	12.69*	10.77*	1.191	<0.01	<0.01	0.22
$CH_4$48h, %	15.72	16.18	14.49*	13.15*	0.650	<0.01	<0.01	0.13
$CH_4$24h, mL	5.59	5.19	4.40*	3.69*	0.425	<0.01	<0.01	0.90
CH4 48h, mL	4.61	4.47	4.20	3.87	0.398	0.13	0.03	0.81
Cumulative $CH_4$48h, mL	10.20	9.66	8.61*	7.56*	0.798	<0.01	<0.01	0.83
Cumulative $CH_4$48h, mL/g DM	19.32	19.15	17.09*	14.91*	1.548	<0.01	<0.01	0.35
IVDMD[2], %	53.91	57.19	56.28	56.45	1.544	0.34	0.32	0.27
pH	6.18	6.23	6.27	6.33	0.041	0.09	0.02	0.94
Total VFA[3], mM	140.62	153.50	157.36	155.85	5.354	0.13	0.06	0.15
VFA production (48h-0h), mM	96.52	109.4	113.26	111.75	5.433	0.14	0.06	0.16
Acetate (% total VFA)	65.39	66.65	66.59	67.66*	0.534	0.05	0.01	0.78
Propionate (% total VFA)	21.74	21.23	21.23	20.70	0.212	0.06	0.02	0.96
Butyrate (% total VFA)	8.57	8.07	8.11	7.63	0.286	0.14	0.04	0.92
BCVFA[4] (% total VFA)	2.27	2.18	2.23	2.22	0.139	0.68	0.71	0.50
Valerate (% total VFA)	1.80	1.69*	1.68*	1.62*	0.074	0.03	<0.01	0.33
Caproic (% total VFA)	0.23	0.18*	0.16*	0.16*	0.017	0.04	0.01	0.11
A:P ratio	3.01	3.14	3.14	3.27*	0.049	0.04	0.01	0.94

* means differences ($P < 0.05$) from control treatment, † means tendency from control treatment ($P < 0.10$) [1] CH_4, methane. [2] IVDMD, *In vitro* dry matter digestibility.
[3] VFA, Volatile fatty acids. [4] BCVFA, Branched- chain fatty acids (iso-valerate and iso-butyrate) [5] SEM, standard error of the means.

Table 9. Orthogonal analysis of Biochar B (%DM) and Sandalwood oil (% of media volume) on rumen fermentation parameters, IVDMD and methane over 24 and 48 h of incubation

Parameter	Control	Biochar B			Sandalwood oil			SEM[5]	P-value			Linear	Quadratic
	0%	1%	3%	5%	1%	3%	5%		Treatment (T)	Concentration (C)	T × C		
Gas production 24h, mL	37.89	35.67	34.83	32.33	36.50	35.44	32.39	1.866	0.64	<0.01	1.00	<0.01	0.81
Gas production 48h, mL	29.34	26.74	27.41	26.87	27.63	26.18	24.24	1.102	0.19	<0.01	0.14	<0.01	0.30
Cumulative gas production 48h, mL	67.23	62.41	62.24	59.20	64.13	61.62	56.63	1.848	0.76	<0.01	0.66	<0.01	0.75
Cumulative gas production 48h, mL/g DM	127.5	124.42	124.17	117.99	122.42	116.16*	107.19*	3.780	0.04	<0.01	0.38	<0.01	0.75
CH_4[1] 24h, %	14.74	14.38	13.72	12.01	14.67	14.60	14.18	1.744	0.06	0.05	0.28	<0.01	0.46
CH_4 48h, %	15.72	15.90	15.04	14.61	14.60	15.04	14.17	0.589	0.12	<0.01	0.29	<0.01	0.93
CH_4 24h, mL	5.59	5.11	4.76	3.84	5.34	5.20	4.58	0.345	0.16	<0.01	0.73	<0.01	0.60
CH_4 48h, mL	4.61	4.25	4.11*	3.93*	4.04*	3.95*	3.43*	0.279	0.03	<0.01	0.34	<0.01	0.32
Cumulative $CH_4$48h, mL	10.2	9.36	8.87	7.77	9.29	9.14	8.01	0.543	0.69	<0.01	0.96	<0.01	0.95
Cumulative $CH_4$48h, mL/g DM	19.32	18.66	17.70	15.48	17.81	17.20	15.15	0.993	0.44	<0.01	0.96	<0.01	0.60
IVDMD[2], %	53.91a	55.46a	54.48a	52.22a	52.89a	47.25b	46.75b	1.482	<0.01	0.01	<0.01	<0.01	0.94
pH	6.18	6.22	6.27	6.32	6.22	6.31	6.32	0.038	0.58	<0.01	0.91	<0.01	0.29
Total VFA[3], mM	140.62	151.09	147.93	146.11	140.18	137.6	126.13*	6.281	<0.01	0.29	0.27	0.22	0.17
VFA production (48h-0h), mM	96.52	107.00	103.83	102.01	96.09	93.52	82.03*	7.449	<0.01	0.29	0.28	0.22	0.17
Acetate (% total VFA)	65.39	66.65	66.64	66.67	66.03	66.30	66.30	0.691	0.22	0.02	0.86	0.02	0.08
Propionate (% total VFA)	21.74	21.27	21.3	21.39	21.49	20.59	21.05	0.417	0.38	0.14	0.52	0.09	0.10
Butyrate (% total VFA)	8.57	7.95	7.87*	7.77*	8.33	8.77	8.44	0.242	<0.01	0.16	0.22	0.13	0.62
BCVFA[4] (% total VFA)	2.27	2.24	2.28	2.23	2.16	2.29	2.11	0.143	0.43	0.44	0.84	0.44	0.51
Valerate (% total VFA)	1.80abc	1.70d	1.72cd	1.70d	1.76bcd	1.85ab	1.88a	0.090	<0.01	0.15	0.04	0.59	0.24
Caproic (% total VFA)	0.23	0.19	0.20	0.24	0.23	0.23	0.24	0.019	0.20	0.45	0.55	0.38	0.18
A:P ratio	3.01	3.13	3.13	3.12	3.07	3.22	3.15	0.177	0.76	0.07	0.43	0.04	0.08

* means differences ($P < 0.05$) from control treatment, † means tendency from control treatment ($P < 0.10$) [1] CH_4, methane. [2] IVDMD, *In vitro* dry matter digestibility.
[3] VFA, Volatile fatty acids. [4] BCVFA, Branched- chain fatty acids (iso-valerate and iso-butyrate) [5] SEM, standard error of the means.