

Influence of *Kappaphycus Alvarezii* and *Gracilaria Salicornia* Supplementation on *in Vitro* Fermentation Pattern, Total Gas and Methane Production of Mixed Substrates

Vithal K. Munde

Indian Veterinary Research Institute

Asit Das (✉ drasitdas@rediffmail.com)

National Dairy Research Institute <https://orcid.org/0000-0001-8316-8834>

Putan Singh

ICAR-Indian Veterinary Research Institute: Indian Veterinary Research Institute

Ashok K. Verma

ICAR-Indian Veterinary Research Institute: Indian Veterinary Research Institute

Nirmala Muwel

ICAR-Indian Veterinary Research Institute: Indian Veterinary Research Institute

Alok Mishra

ICAR-Indian Veterinary Research Institute: Indian Veterinary Research Institute

Rajib Deb

National Dairy Research Institute

Kanti Raje

ICAR-Indian Veterinary Research Institute: Indian Veterinary Research Institute

Research Article

Keywords: Red seaweed, in vitro fermentation, in vitro gas production,

Posted Date: October 20th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-601180/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

This experiment was conducted to study the effect of supplementation of *Kappaphycus alvarezii* (KA) and *Gracilaria salicornia* (GS) *in vitro* fermentation pattern, total gas and methane production of mixed substrates. Basal substrate comprising of concentrates and wheat straw (50:50) was supplemented with either 0% (control), 1 (KA₁), 2 (KA₂), 4 (KA₄), 6 (KA₆), and 8 % (KA₈) of *Kappaphycus*; and, 1 (GS₁), 2 (GS₂), 4 (GS₄), 6 (GS₆), and 8 (GS₈) of *Gracilaria*, respectively. Asymptote, rate constant of gas production and t-half, concentration of total volatile fatty acids (TVFA), and *in vitro* dry matter digestibility (IVDMD) was not affected up to 2% level KA supplementation, beyond which asymptote, and rate constant of gas production, TVFA, and IVDMD decreased and t-half increased (P<0.001). Asymptote, rate constant of gas production, TVFA and IVDMD was not affected at 1% level of inclusion, beyond which a steady decline in these parameters was observed (P<0.001). Methane production (ml/g DM) was higher (P<0.001) in CON, followed by KA₁ and KA₂, and lower values were observed in by KA₄, KA₆ and KA₈. Methane production (ml/kg DM) declined (P<0.001) steadily with increased level of GS *in* the substrates. From the results it was concluded that inclusion of *Kappaphycus alvarezii* and *Gracilaria salicornia* at 2 and 1%, respectively in the fermentation substrate can reduce *in vitro* methane production without any adverse impact on total gas production and *in vitro* dry matter digestibility.

Introduction

Seaweeds are renewable natural resource of macroscopic marine algae found growing in large quantities along the coasts of India. Mainly they are used as sources of phycocolloids, fodder, fertilizers and for direct human consumption. Generally, seaweeds are markedly rich in minerals, complex carbohydrates, proteins and low molecular weight nitrogenous compounds, lipids, vitamins, volatile compounds, polyphenols and pigments. However, only limited data are available on the effect of seaweeds on ruminant production (Makkar *et al.*, 2016). Li *et al.*, (2016) reported that supplementation of *Asparagopsis* in Merino cross sheep fed with high fibre diet resulted in dose dependent reduction in methane emission. Dietary supplementation of *A. taxiformis* reduced enteric CH₄ emissions in steers without any adverse impact on production performance (Roque *et al.*, 2021). Feeding of tropical seaweed based formulation (*Kappaphycus alvarezii*, *Gracilaria salicornia* and *Trbinaria conoides* at 1:1:1) improved antioxidants, immunity and milk yield in lactating Murrah buffaloes (Maheswari *et al.*, 2021). As compared to *in vivo* experiments there are many more *in vitro* experiments that demonstrated the effect of seaweeds supplementation on fermentation characteristics. Supplementation of five different brown seaweed extracts reduced methane emission *in vitro*, but the response varied depending upon the species (Choi *et al.*, 2021). Machado *et al.* (2014b) reported that supplementary feeding of *Asparagopsis* inhibited total gas production by 61.8% and CH₄ production by 98.9%. Maia *et al.*, (2016) reported that on incubation with meadow hay, *Ulva* sp., *Gigartina* sp. and *G. vermiculophylla* decreased methane production, but with corn silage, methane production was only decreased by *G. vermiculophylla*. Thus, it is evident that the capability of macroalgae to alter fermentation characteristics depend on species, active component present, and level of inclusion (Molina-Alcaide *et al.*, 2017). *Kappaphycus alvarezii* and *Gracilaria salicornia*, are the two most abundantly available cultivated species of red seaweeds of India. To, the best of our knowledge the influence of these two species

on rumen fermentation parameters is not yet tested. It was hypothesized that both the tropical seaweeds, *Kappaphycus alvarezii* and *Gracilaria salicornia*, have anti-methanogenic activity, that may differ in magnitude, and if supplemented in an appropriate amount would reduce enteric methane emission without any adverse impact on rumen fermentation. Taking all these points into account this research work was carried out to study the effect of graded levels of *Kappaphycus alvarezii* and *Gracilaria salicornia* supplementation of mixed substrates on *in vitro* total gas and methane production, dry matter digestibility, total volatile fatty acid production, fractions of VFA and NH₃-N production.

Materials And Methods

All protocols and procedures followed in this study were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of Ministry of Environment, Forests and Climate Change, Government of India, New Delhi.

2.1. Preparation of substrates with Red Sea weeds

Mixed substrate comprising of concentrate to roughage ratio of 50:50 on dry matter (DM) basis was used as control (CON). The anti-methanogenic potentials of *Kappaphycus alvarezii* and *Gracilaria salicornia* (GS) were evaluated using *in vitro* gas production system. Mixed substrates were supplemented at 0% (control), 1% (KA₁), 2 (KA₂), 4 (KA₄), 6 (KA₆), and 8 (KA₈) of *Kappaphycus alvarezii*; and, 1 (GS₁), 2 (GS₂), 4 (GS₄), 6 (GS₆), and 8 % (GS₈) of *Gracilaria salicornia*, respectively. Each treatment was comprising of 6 replicates.

Rumen liquor sampling

Four rumen fistulated animals kept on standard diets was used as donor animals. Rumen liquor was collected before feeding in the morning. After collection rumen liquor samples were screened with 4 layers of muslin cloth in a preheated thermos at 39⁰ C and were transported to laboratory for inoculation of substrates. *In vitro* gas production was studied as per the procedure of Menke and Steingass (1988). Accurately weighed 200 mg of each substrates in triplicate was put into the graduated 100 ml calibrated glass syringe (Haberle Labortechnik, Lonsee-Ettenchie, Germany). The medium consisting of buffer and rumen liquor in 2:1 ratio (Menke and Steingass, 1988) was dispensed (30 ml) into the syringes by an automatic dispenser (OPTIFIX, Walter Graf & Co., Wertheim, Germany). Fermentation syringes were then kept in an incubator at 39°C with periodical shaking. Thus, total numbers of samples incubated were 2 (seaweeds)×6 (treatments) 7 (incubations)×3 (triplicate)=252. Besides, four blank syringes containing no substrate were also run during each incubation. The fermentation was terminated after 96 h of incubation.

Proximate and nutrient composition

Samples of wheat straw, fodder maize, *Kappaphycus alvarezii* and substrates were analyzed for proximate principles, fibre constituents and minerals. Proximate analysis (dry matter (DM), organic matter (OM), crude protein (CP), crude fiber (CF), and nitrogen free extract (NFE)) of the samples were estimated as per AOAC (2005) and cell wall constituents (neutral detergent fiber (NDF), acid detergent fiber (ADF), hemicellulose and cellulose) were estimated as per Van Soest *et al.* (1991). For determination of the concentration of minerals

(calcium, magnesium, iron, copper, zinc, manganese and iodine), samples of feedstuffs and seaweeds were subjected to wet digestion (HNO_3 : HClO_4 : H_2SO_4 - 3:2:1) using Kelplus- KES 12L R system till it became clear and the digested samples were diluted with double distilled water (DDW) and passed through Whatman filter paper No. 42 and final volume was made to 25 ml. The contents of different minerals in the feed sample were analyzed using atomic absorption spectrophotometer (Hitachi- 5000 series). The calibration curve for determination of different minerals was prepared using a blank and working standard solution of different minerals. The calibration was periodically verified by analyzing a standard at the frequency of 20 readings. If the recovery was outside the limits, the analysis was stopped and the system was recalibrated. Content of P in feedstuff and seaweed was determined by using a colorimetric method (AOAC, 2005)

Measurement of total gas and methane production

For each substrate, cumulative gas production (CGP) was calculated as the amount of gas production (ml) from the substrate minus gas production from blank divided by the weight of substrate. Gas production (ml/g DM) after 6, 12, 24, 48, 72 and 96 h of incubation was recorded by piston displacement. For methane estimation, 100 μl of gas sampled from headspace (after 24h of incubation) of the syringe was injected into a Nucon-5765 gas chromatograph equipped with Porapak Q column and flame ionization detector (Agarwal *et al.*, 2008). A mixture of 50% carbon dioxide and 50% methane (Spancan; Spantech Products Ltd, Godstone, UK) was used as standard.

2.3. Estimation of volatile fatty acids (VFA)

Estimation of volatile fatty acids was done by using Nucon-5765 gas chromatograph (AIMIL, New Delhi, India) equipped with a double flame ionization detector and the glass column (4 ft length and 1/8 inch diameter) packed with chromosorb 101 as per method described by Cottyn and Boucque (1968) and later modified by Agarwal *et al.* (2008). The gas flows for nitrogen, hydrogen and air were 30, 30 and 320 ml/min, respectively. Temperature of injector oven, column oven and detector was 270⁰C, 172⁰C and 270⁰C, respectively. Standard VFA mixture was prepared by mixing stock solutions (each of 25 mg/ml concentration) of standard VFAs and water (acetic acid, 1.68 ml; propionic acid, 0.48 ml; butyric acid, 0.24 ml; distilled water, 7.24 ml) to obtain final concentration of acetic acid, 7.0; propionic acid, 1.62; butyric acid, 0.68 mM/100 ml. Fermentation liquor samples were prepared by adding 0.2 ml of 25 % metaphosphoric acid per ml of liquor, allowing it to stand for 2 h followed by centrifugation at 7000 \times g for 10 min. Supernatant was used for estimation of VFA.

2.4. Ammonia nitrogen estimation

Ammonia nitrogen was estimated by the method of Weatherburn (1967). Briefly, to the sample (suitable quantity), 5.0 ml of solution A (1 g phenol and 5 mg sodium nitroprusside) was added to which 5.0 ml of solution B (0.5 g sodium hydroxide 0.84 ml sodium hypochlorite) was added and mixed thoroughly. The tubes were incubated at 39⁰C for 15 min for colour development. Samples were then read spectrophotometrically at 625 nm against a reagent blank. In a similar way standard tubes (ammonia nitrogen concentration ranging from 1.0 to 10 $\mu\text{g}/\text{ml}$) were processed and a calibration curve was plotted. Concentration of the unknown sample was calculated by the standard curve.

2.5. *In vitro* true digestibility (IVTD) of substrates

After 24 h of incubation, the content of the syringes was transferred to spout-less beaker by repeated washings with 100 ml of neutral detergent solution. The flask content was refluxed for 1 h and filtered through pre-weighed Gooch crucibles (Grade 1). The dry matter content of the residue was weighed and *in vitro* true digestibility of feed was calculated as follows:

$$\text{(Initial DM of feed taken for incubation - DM residue)}$$

$$\text{True digestibility (TD \%)} = \frac{\text{-----}}{\text{(Initial DM of feed taken for incubation)}} \times 100$$

$$\text{(Initial DM of feed taken for incubation)}$$

2.6. *Statistical analysis*

Cumulative gas production recorded at different hours of incubation were fitted to the following model to determine gas production kinetics: $Y = b \times (1 - e^{-c \cdot t})$; where 'y' is the cumulative volume (ml) of gas produced at time 't' (h), 'b' the asymptotic gas volume (ml) and 'c' the rate constant of gas production ($\% \text{ h}^{-1}$). The constants b and c were determined using the nonlinear procedure of SAS (2001). Substrate specific times were defined by the half time ($t^{-1/2}$) of asymptotic gas production. Halftime (h) of gas production ($t^{-1/2}$) [i.e., the time (h) when half of the asymptotic gas volume (b; ml) was produced] was calculated as: $t_{1/2} = \ln 2/c$.

Data of the study were subjected to analysis of variance using the General Linear Model (GLM) procedure of the Statistical Software Package (SPSS for windows, V21.0; Inc., Chicago, IL, USA). The effect of green fodder replacement with corn silage on blood biomarker was tested using the following model:

$$Y_{ijk} = \mu + S_i + D_j + (S \times D)_{ij} + e_{ijk}$$

Where; Y_{ijk} is the dependent variable, μ is the overall mean of the population, S_i is the mean effect of the source of seaweed, D_j is the mean effect of dose of seaweed, $(S \times D)_{ij}$ is the effect of the interaction between source and dose, and e_{ijk} is the unexplained residual element assumed to be independent and normally distributed. Individual animals were used as the experimental unit for all data. The pair-wise comparison of means was carried out using "Tukey's honest significant difference (HSD) test". Significance was determined at $P < 0.05$ and the values are presented in the tables. Treatment means were separated using Duncan's Multiple range Test and were considered significant at $P < 0.05$.

Results

3.1. *Chemical composition of feed ingredients and substrates*

Chemical composition of wheat straw, fodder maize, *Kappaphycus alvarezii* and concentrate mixture are presented in Table 1. Nutritional composition of wheat straw, maize fodder and concentrate mixture was

within the normal range reported for these feed ingredients (Ranjhan, 1988). Both the seaweeds were characterized by higher acid soluble and insoluble ash content. The OM component of the seaweeds comprised mostly of NFE and cell content with lower CP, CF and EE content.

3.2. *In vitro* gas production kinetics

Data pertaining to *in vitro* gas production (IVGP) at different hours of incubation are presented in Figure 1. *In vitro* cumulative gas production was affected by both hour of incubation ($P < 0.001$) and level of inclusion of seaweed ($P < 0.001$). The interaction between period x treatment was significant ($P < 0.001$) for *Gracilaria salicornia* but not for *Kappaphycus alvarezii*. This would imply that dose response of inclusion of *Gracilaria salicornia* differed at different hours of incubation. Rate constant of gas production decreased and t-half increased when *Kappaphycus alvarezii* was included at a rate higher than 2%. Asymptote, IVGP ($P < 0.001$) and rate constant of gas production decreased and t-half increased when *Gracilaria salicornia* was included at a rate higher than 1%. Interestingly, at lower level of inclusion (up to 2%), rate constant of gas production increased and t-half decreased in KA₁ and KA₂ as compared to control (Table 2).

3.3. *In vitro* gas and methane production

In vitro gas production (IVGP) and methane production at 24 h of incubation of substrates containing various proportions of *Kappaphycus alvarezii* and *Gracilaria salicornia* has been presented in the Table 3. Proportion of methane in gas was highest ($P < 0.001$) in CON, followed by KA, KA₁ and was lowest in KA₂, KA₄ and KA₄. Similar trend was also observed when substrates containing different proportions of *Gracilaria salicornia*. In the case of *Kappaphycus alvarezii* based substrates methane production (ml/g DM) was higher ($P < 0.001$) in CON, KA₁, and KA₂ as compared to other 3 groups. Methane production (ml/kg DM) differed ($P < 0.001$) among all *Gracilaria salicornia* containing substrates (ml /kgDM or DOM) declined steadily with increased level of *Gracilaria salicornia* in the substrates (Figure 2).

3.4. *In vitro* dry matter digestibility

Data pertaining to the *in vitro* true dry matter digestibility at 24 h of incubation is presented in Table 4. There was improvement ($P < 0.001$ in true dry matter digestibility in KA₁ and KA₂ as in case of *Gracilaria salicornia* treatment *in vitro* true dry matter digestibility was similar in control and GS₁ while it was found significantly lower in all other treatment groups (GS₂, GS₃ and GS₄).

Discussion

Cumulative gas production (CGP), asymptote and rate constants of IVGP decreased at higher levels of inclusion of seaweeds in the substrate. These findings are in general agreement with those available in literature which indicate an overall decrease in fermentation and CGP. Kinley *et al* (2016b) reported that inclusion of *Asparagopsis* in the substrate reduced IVGP. In an earlier experiment, linear reduction in IVGP was attributed to phlorotannins from *Ascophyllum nodosum* (Wang *et al.*, 2008). In this experiment, we did not measure the phlorotannin content of the substrates, but from the literature it is known that both the seaweeds are rich sources of phenolics (Ganesan *et al.*, 2008) that may show inhibitory effect on overall

fermentation (Machado *et al.*, 2016a; Paul *et al.*, 2006). Dietary phenolics decrease nutrient utilization by inhibiting microbial activity (Blunt *et al.*, 2013) and by forming insoluble complexes with nutrients (Targett and Arnold, 1998). Lower IVGP in substrates having higher doses of *Kappaphycus alvarezii* and *Gracilaria salicornia* are thus explicable. An interesting observation was that at lower level of inclusion (upto 2%) of *Kappaphycus alvarezii* in the substrata resulted increased IVGP, IVDMD and TVFA production (Table 5 and 6). Considering the stimulation of fermentation at lower level of inclusion, it is advocated to explore the additive as a mean to improve efficiency of rumen function. Earlier research indicates that 9 out 20 algal extract improved overall fermentation over controls *in vitro* (Dubois *et al.*, 2013). The positive interaction that we have observed between doses and seaweed sources imply that dose response of the seaweeds are not similar. At higher levels of Inclusion of both the seaweed products resulted in reduction of IVGP, however, the effect of *Kappaphycus alvarezii* on IVGP was less pronounced as compared to that of *Gracilaria salicornia*. This could be due to higher content of phenolics in *Gracilaria salicornia* than *Kappaphycus alvarezii*. Total phenolics content (mg gallic acid equivalent) was 2.7 times (1.5 vs 4.1 mg/g) higher in *Gracilaria salicornia* as compared to *Kappaphycus alvarezii* (Ganesan *et al.*, 2008). The significant interaction that was observed between seaweed sources and doses imply that inhibitory effect of seaweed on IVGP was observed at different doses for *Kappaphycus alvarezii* and *Gracilaria salicornia*. Inclusion of *Kappaphycus alvarezii* and *Gracilaria salicornia* at 2 and 1% respectively did not show any adverse impact on IVGP and rate constants of gas production. This is evident that inclusion of these seaweeds beyond the aforesaid levels may hamper the microbial activity in the rumen and utilization of nutrients.

Anti-methanogenic property of brown seaweeds have been reported earlier (Wang *et al.* 2008; Dubois *et al.* 2013; Kinley and Fredeen 2015). Here, in this experiment it is demonstrated that supplementation of *Kappaphycus alvarezii* and *Gracilaria salicornia* can reduce the enteric methane emission. Our results corroborate well with those of Machado *et al.* (2014b) who have reported methane reducing activity of Asparagopsis. It was reported that inclusion of Asparagopsis in the fermentation substrate resulted in near elimination of methane emission (Kinley *et al.*, 2016b). In another experiment it was reported that Dictylata and Asparagopsis reduced methane emission by 92 and 98%, respectively (Machado *et al.*, 2014a). In this experiment any such dramatic response was absent. However, methane production decreased by 46.5 and 18.4%, while *Gracilaria salicornia* and *Kappaphycus alvarezii* contributed 8% of the substrates. This could be attributed to doses of seaweed. In the previous experiment, level of inclusion was 16% (0.2 g microalga in 1.0 g substrates), whereas, in this experiment maximum level of inclusion was 8%. Both the seaweeds are rich sources of halogenated low molecular weight compounds, in particular brominated and chlorinated haloformes (Paul *et al.*, 2006), that inhibits methyl transfer reactions essential for methanogenesis (Liu *et al.*, 2011). In addition, they are rich sources of plant secondary metabolites (PSM) (Wang *et al.* 2008; Kinley and Fredeen 2015). These substances are known for their anti-methanogenic activity (Machado *et al.*, 2016a; Machado *et al.*, 2016b). Considering the multitude of functional components having antimethanogenic activity that are present in *Kappaphycus alvarezii* and *Gracilaria salicornia* (Ganesan *et al.*, 2008), it is not possible to attribute the anti-methanogenic response observed in this experiment to a singular component. A comparison between the two seaweeds revealed that substrates containing *Gracilaria salicornia* were more effective in reducing methane emission as compared to substrates containing *Kappaphycus alvarezii*. This observation could be linked to higher phenolics and PSM content

of *Gracilaria salicornia* (Ganesan *et al.*, 2008). Data pertaining to dose response reveal that supplementation of both *Kappaphycus alvarezii* and *Gracilaria salicornia* are effective in reducing methane emission at a level as low as 1% of the substrates. This level is still lower than the level of 2% of *Asparagopsis* in the fermentation substrate that was effective in reducing methane emission (Kinley *et al.*, 2016b). This is remarkable because these two seaweeds show anti-methanogenic activity at a very low level of inclusion, thus making them more suitable for use as an anti-methanogenic agent without compromising the nutritional quality of the feed.

In vitro fermentation pattern at 24 h of incubation.

Ruminants derive most of their energy through metabolism of volatile fatty acids (VFA) that are produced as an end-product of microbial fermentation. Any reduction in production of VFA could be seen in a negative context. Considering the vast anti-microbial agents present in red seaweed (Paul *et al.*, 2006), it was suspected that inclusion of red seaweeds may decrease the TVFA production at higher levels of inclusion. The results of this experiment indeed testify this assumption. Both *Gracilaria salicornia* and *Kappaphycus alvarezii* reduced *in vitro* VFA production. This finding corroborates well with that of Machado *et al.* (2016a) who observed a significant reduction in TVFA production due to supplementation of red seaweed at a rate between 1-2% to a low quality Rhode grass based substrate. It was suggested that supplementation of red seaweed is more responsive to poor quality roughages because of higher fibre and lower protein content. In this experiment, we used standard mixed substrata having roughage: concentrates of 50:50. Yet at higher level of inclusion, both *Kappaphycus alvarezii* and *Gracilaria salicornia* reduced VFA production. However, inclusion level up to 2 and 1 % respectively for *Kappaphycus alvarezii* and *Gracilaria salicornia* showed no adverse impact on TVFA production. This finding is in agreement with that of Kinley *et al.* (2016a) who reported that inclusion of *Asparagopsis* between 1 to 2 % of the DM did not decrease VFA production. However, VFA production decreased consistently at higher level of inclusion of both *Kappaphycus alvarezii* and *Gracilaria salicornia*. This could be linked to overall decrease in fermentation activity as was also reflected in lower IVGP and rate constant of gas production due to presence of antimicrobial activity in red seaweeds (Amorim *et al.*, 2012). Even though proportion of propionate was higher when seaweeds were added at 8% in the substrate, no definite trend could be established because values varied between the treatments. This finding are not in agreement with those of Kinley *et al.* (2016b) who reported an increase in propionate at an expense of acetate in response to supplementation of red seaweed extract. Results of this experiment suggests that changes in A:P ratio in response to different doses of *Kappaphycus alvarezii* and *Gracilaria salicornia* are minor. Thus, it is unlikely that the anti-methanogenic activity of *Kappaphycus alvarezii* and *Gracilaria salicornia* are mediated through a major shift in partitioning of hydrogen among VFAs. Our finding corroborates well with those of Wang *et al.* (2008). *In vitro* concentration of NH₃-N reflects the balance between NH₃-N produced and it's utilization for microbial protein synthesis. As stated before both *Kappaphycus alvarezii* and *Gracilaria salicornia* are rich sources of phenolics (Ganesan *et al.*, 2008) that may hamper microbial degradation of plant protein (Martinez *et al.*, 2006). In a previous report it was demonstrated that crude seaweed extract of *Ascophyllum nodosum* at a very low level (125 µg phlorotannin /ml) reduced *in vitro* NH₃-N production (Wang *et al.*, 2008). A similar response was also observed in this experiment with respect *Gracilaria salicornia*. The response with respect to *Kappaphycus alvarezii*, however,

was very weak and a significant reduction of NH₃-N was observed only at 6 and 8% level of inclusion. The differences in response between *Kappaphycus alvarezii* and *Gracilaria salicornia* could be explained on the basis of their phenolics content. As stated earlier, *Gracilaria salicornia* contained 2.7 times more phenolics as compared to *Kappaphycus alvarezii*.

The anti-methanogenic activity that we have observed in this experiment could be of use only if there is no compromise on the nutritional quality of the substrates. It is evident that at lower level of inclusion *Kappaphycus alvarezii* stimulates rumen fermentation *in vitro*. This promising response needs to be ascertained through *in vivo* experimentation. At higher level of inclusion both *Kappaphycus alvarezii* and *Gracilaria salicornia* reduced IVDMD. This corroborates well with our finding of reduced IVGP as both these parameters are strongly correlated (Menke and Steingass, 1988). Further, results are also in agreement with previous report (Wang *et al.*, 2008). Such reduction in IVDMD was often due to decrease in digestibility of fibre components and the responses were more pronounced while substrates comprising of poor quality roughages were used (Wang *et al.*, 2008). Results of this experiment demonstrate that at higher levels of inclusion both can reduce the IVDMD of mixed substrates that are typical of ruminant feeding. A comparison between *Kappaphycus alvarezii* and *Gracilaria salicornia* revealed that responses are more pronounced while GS was used as supplement. Thus, more caution is required while *Gracilaria salicornia* was used as animal feed additive. However, the inhibitory effect of the seaweeds on IVDMD was not pronounced while the level of inclusion was restricted to 1 and 2% for *Gracilaria salicornia* and *Kappaphycus alvarezii*, respectively. Thus, it is evident that these two seaweeds may not be included in the ruminant diet at dose rate higher than that mentioned above. Present *in vitro* research on the effect of Red seaweeds on enteric methane emission, setting the stage for further *in vivo* studies to proof them as promising candidates.

Conclusion

Inclusion of *Kappaphycus alvarezii* and *Gracilaria salicornia* at 2 and 1% respectively in the fermentation substrate reduced methane production *in vitro* without any adverse impact on total gas production and *in vitro* dry matter digestibility. Tropical red seaweed *Gracilaria salicornia* exhibited more robust anti-methanogenic activity than *Kappaphycus alvarezii*.

Abbreviations

DM Dry matter

CON Control

CM Concentrate mixture

IVGP *In vitro* gas production

TVFA Total volatile fatty acids

NH₃-N Ammonia nitrogen

IVDMD *In vitro* dry matter digestibility

DOM Digestible organic matter

VFA Volatile fatty acid

IVTD *In vitro* true digestibility

TD True digestibility

NDF Neutral detergent fibre

ADF Acid detergent fibre

CF Crude fibre

ASA Acid soluble ash

OM Organic matter

CP Crude protein

NFE Nitrogen free extract

BIS Bureau of Indian standards

CGP Cumulative gas production

h hour

A/P Acetate/propionate

KA Kappaphycus alvarezii

GSGracilaria salicorniai

Declarations

Funding (information that explains whether and by whom the research was supported)

This project was funded by the CSIR-NMITLI programme (Project title: *Kapaphycus alvarezii* and Red sea weed based formulations for improving productivity and health of dairy animal and poultry).

Conflicts of interest/Competing interests (include appropriate disclosures)

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Availability of data and material (data transparency)

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability (software application or custom code)

Not applicable

Authors' contributions (optional: please review the submission guidelines from the journal whether statements are mandatory)

V.K. Munde conceptualized the project, actually conducted the experiment and analysed the data. Asit Das conceptualized the project, supervised laboratory analysis, made the first draft. AK Verma, and Putan Singh provided laboratory support, edited the draft manuscript, provided critical inputs. Nirmal Muwel, Kanti Raje and Alok Mishra, did the laboratory analysis, and tabulation. Rajib Deb made contribution in making the figure, formatting the paper and editing the manuscript

Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals

Not applicable

Ethics approval (include appropriate approvals or waivers)

All protocols and procedures followed in this study were approved by the Committee for the 104 Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of Ministry of 105 Environment, Forests and Climate Change, Government of India, New Delhi. Kappaphycus 106 alvarezii and Gracilaria salicornia meals (thrashed by-products after extraction of carrageenans) 107 were procured from Aquagri Processing Private Limited, New Delhi

Consent to participate (include appropriate statements)

Not applicable

Consent for publication (include appropriate statements)

All authors provide their consent to publish the manuscript

Acknowledgements

This project was funded by the CSIR-NMITLI programme (Project title: Kapaphycus alvarezii and Red sea weed based formulations for improving productivity and health of dairy animal and poultry). Authors

gratefully acknowledge the financial support. The authors wish to thank the Director, Indian Veterinary Research Institute, Patna, Bareilly (U.P.), India for providing necessary facilities to conduct the present study. First author acknowledges the financial support and necessary permission granted by the Vice-Chancellor, MAFSU, Nagpur during this programme.

References

1. Agarwal, N., Kamra, D.N., Chatterjee, P.N., Kumar, R. and Chaudhary, L. C. 2008. In vitro methanogenesis, microbial profile and fermentation of green forages with buffalo rumen liquor as influenced by 2-bromoethanesulphonic acid. *Asian Australasian Journal of Animal Sciences*, **21**, 818-823.
2. Amorim, R.D.N.D.S., Rodrigues, J.A.G., Holanda, M.L., Quinderé, A.L.G., Paula, R.C.M.D., Melo, V.M.M. and Benevides, N.M.B. 2012. Antimicrobial effect of a crude sulfated polysaccharide from the red seaweed *Gracilaria ornata*. *Brazilian Archives of Biology and Technology*, **55**, 171-181.
3. AOAC. 2005. Official Methods of Analysis. 18th ed. Association of Official Analytical Chemist, Washington, DC
4. Blunt, J.W., Copp, B.R., Keyzers, R.A., Munro, M.H. and Prinsep, M.R. 2013. Marine Natural Products. *Natural Product Reports*, **30**, 237-323.
5. Choi, Y.Y., Shin, N.H., Lee, S.J., Lee, Y.J., Kim, H.S., Eom, J.S., Lee, S.S., Kim, E.T. and Lee, S.S. 2021. In vitro five brown algae extracts for efficiency of ruminal fermentation and methane yield. *Journal of Applied Phycology*, **33**, 1253-1262.
6. Cottyn, B.G. and Boucque, C.V. 1968. Rapid method for the gas-chromatographic determination of volatile fatty acids in rumen fluid. *Journal of Agricultural and Food Chemistry*. **16**, 105-107.
7. Dubois, B., Tomkins, N.W., Kinley, R.D, Bai, M., Seymour, S., Paul, N.A and de Nys, R. 2013. Effect of tropical algae as additives on rumen in vitro gas production and fermentation characteristics. *American Journal of Plant Sciences*, **4**, 34–43.
8. Ganesan, P, Kumar, C.S. and Bhaskar, N. 2008. Antioxidant properties of methanol extract and its solvent fractions obtained from selected Indian red seaweeds. *Bioresource Technology*, **99**, 2717-2723.
9. Kinley, R. and Fredeen, A. 2015. In vitro evaluation of feeding North Atlantic stormtoss seaweeds on ruminal digestion. *Journal of Applied Phycology*, **27**, 2387–2393.
10. Kinley, R.D., de Nys, R., Vucko, M.J., Machado, L. and Tomkins, N.W. 2016b. The Red Macroalgae *Asparagopsis taxiformis* is a Potent Natural Antimethanogenic That Reduces Methane Production during in Vitro Fermentation with Rumen Fluid. *Animal Production Science*, **56**, 282-289.
11. Kinley, R.D., Vucko, M.J., Machado, L. and Tomkins, N.W. 2016a. In vitro evaluation of the antimethanogenic potency and effects on fermentation of individual and combinations of marine macroalgae. *American Journal of Plant Science*, **7**, 2038-2054.
12. Li, X., Norman, H.C., Kinley, R.D. and Laurence, M. 2016. *Asparagopsis taxiformis* decreases enteric methane production from sheep. *Animal Production Science*, **58**, 681-688.
13. Liu, H., Wang, J., Wang, A. and Chen, J. 2011. Chemical inhibitors of methanogenesis and putative applications. *Applied Microbiology and Biotechnology*, **89**:1333–1340.

14. Machado, L., Magnusson, M., Paul, N. A., de Nys, R. and Tomkins, N. 2014. Effects of marine and freshwater macroalgae on in vitro total gas and methane production. *PLoS One*, 9, e85289.
15. Machado, L., Magnusson, M., Paul, N.A., Kinley, R., de Nys, R. and Tomkins, N. 2016. Identification of bioactives from the red seaweed *Asparagopsis taxiformis* that promote antimethanogenic activity in vitro. *Journal of Applied Phycology*, 28, 3117-3126.
16. Machado, L., Magnusson, M., Paul, N.A., Kinley, R., de Nys, R. and Tomkins, N. 2016a. Dose- response effects of *Asparagopsis taxiformis* and *Oedogonium* sp. on in vitro fermentation and methane production. *Journal of Applied Phycology*, 28, 1443-1452.
17. Machado, M.P., Silva, A.L. L.D., Biasi, L.A., Deschamps, C., Bessalho Filho, J.C. and Zanette, F. 2014a. Influence of calcium content of tissue on hyperhydricity and shoot-tip necrosis of in vitro regenerated shoots of *Lavandula angustifolia* Mill. *Brazilian Archives of Biology and Technology*, 57, 636-643.
18. Maia, M.R., Fonseca, A.J., Oliveira, H.M., Mendonça, C. and Cabrita, A.R. 2016. The potential role of seaweeds in the natural manipulation of rumen fermentation and methane production. *Scientific Reports*, 6, 32321.
19. Makkar, H.P.S., Tran, G., Heuze, V., Giger-Reverdin, S., Lessire, M., Lebas, F. and Ankers,
20. 2016. Seaweeds for livestock diets: a review. *Animal Feed Science and Technology*, 212, 1-17.
21. Maheswari, M., Das, A., Datta, M. and Tyagi, A. K. 2021. Supplementation of tropical seaweed-based formulations improves antioxidant status, immunity and milk production in lactating Murrah buffaloes. *Journal of Applied Phycology*, 33, 2629–2643.
22. Martinez, T., McAllister, T.A., Wang, Y. and Reuter, T. 2006. Effects of tannic acid and quebracho tannins on in vitro ruminal fermentation of wheat and corn grain. *Journal of the Science of Food and Agriculture*, 86, 1244–1256.
23. Menke, K.H. and Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Animal Research Development*, 28,7-55.
24. Molina-Alcaide, E., Carro, M. D., Roleda, M. Y., Weisbjerg, M. R., Lind, V. and Novoa-Garrido, M. 2017. In vitro ruminal fermentation and methane production of different seaweed species. *Animal Feed Science Technology* 228, 1-12.
25. Paul, N., de Nys, R. and Steinberg, P. 2006. Chemical defence against bacteria in the red alga *Asparagopsis armata*: linking structure with function. *Marine Ecology Progress Series*, 306, 87–101.
26. Ranjhan, S.K. 1998. *Nutrient Requirements for Livestock and Poultry*, 2nd ed. Indian Council for Agricultural Research, New Delhi, India.
27. Roque, B. M., Venegas, M., Kinley, R. D., de Nys, R., Duarte, T. L., Yang, X. and Kebreab, E. 2021. Red seaweed (*Asparagopsis taxiformis*) supplementation reduces enteric methane by over 80 percent in beef steers. *Plos one*, 16, e0247820.
28. SAS Institute Inc, 2001. SAS for Windows, version 8.2.
29. SPSS. 2012. *Statistical packages for Social Sciences*, Version 20, SPSS Inc., Illinois, USA.
30. Targett, N.M. and Arnold, T.M. 1998. Mini review predicting the effects of brown algal phlorotannins on marine herbivores in tropical and temperate oceans. *Journal of Phycology*, 36, 195-205.

31. Van Soest, P. J., Robertson, J. B. and Lewis, B. A. (1991). Symposium: carbohydrate methodology, metabolism, and nutritional implications in dairy cattle. *Journal of Dairy Science*, 74, 3583-3597.
32. Wang Y., Xu, Z., Bach, S.J. and McAllister, T.A. 2008. Effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on in vitro ruminal digestion of mixed forage or barley grain. *Animal Feed Science Technology*, 145,375-395.
33. Weatherburn, M.W. 1967. Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry*, 39, 971-974.

Tables

Table 1. Chemical composition of concentrate mixture (CM), fodder maize (GM), wheat straw (WS), red seaweeds *Kappaphycus alvarezii* (KA) and *Gracilaria salicornia* (GS)

	Feed stuff				
	CM	GM	WS	KA	GS
Dry matter	88.7	19	92.56	99.74	99.56
On % dry matter basis					
Ash	7.49	10.2	11.20	43.98	41.77
Acid soluble ash	5.69	9.00	3.52	34.82	36.68
Organic matter	91.5	89.8	88.80	56.02	58.23
Crude protein	20.4	9.85	2.97	5.20	6.97
Ether extract	2.23	1.07	1.00	0.38	0.35
Crude fibre	6.68	26.4	38.50	10.60	8.20
Nitrogen free extract	62.1	52.5	46.33	39.84	42.71
Neutral detergent fibre	25.4	58.4	76.51	23.47	20.67
Cell content	71.7	41.6	23.49	76.53	79.33
Acid detergent fibre	7.91	37.9	51.15	21.93	19.61
Lignin	1.44	6.96	7.48	5.14	4.90
Hemicellulose	17.4	20.5	25.36	1.54	1.06
Cellulose	6.47	30.9	43.67	16.79	14.71

Table 2. Kinetics of *In vitro* gas production of substrates containing graded levels of *Kappaphycus alvarezii* and *Gracilaria salicornia*

Treatments	b	c	t-half
<i>Kappaphycus alvarezii</i>			
CON	53.33 ^a ±0.37	0.047 ^{ab} ±0.001	14.72 ^{ab} ±0.25
KA ₁	56.71 ^b ±0.81	0.052 ^b ±0.002	13.38 ^a ±0.45
KA ₂	56.53 ^b ±0.72	0.052 ^b ±0.002	13.25 ^a ±0.62
KA ₄	57.11 ^b ±0.82	0.047 ^{ab} ±0.002	14.84 ^{ab} ±1.03
KA ₆	56.13 ^b ±0.62	0.044 ^a ±0.001	15.75 ^b ±0.90
KA ₈	52.11 ^a ±0.54	0.046 ^a ±0.001	15.04 ^{ab} ±0.75
<i>Gracilaria salicornia</i>			
CON	54.11 ^c ±0.39	0.045 ^b ±0.001	15.27 ^a ±0.32
GS ₁	50.74 ^{bc} ±0.42	0.048 ^b ±0.001	14.5 ^a ±0.52
GS ₂	49.94 ^{bc} ±1.91	0.033 ^a ±0.003	21.33 ^b ±1.10
GS ₄	48.29 ^b ±1.72	0.032 ^a ±0.003	21.73 ^b ±0.68
GS ₆	45.48 ^{ab} ±2.21	0.031 ^a ±0.004	22 ^b ±0.91
GS ₈	41.89 ^a ±2.09	0.029 ^a ±0.004	23.42 ^b ±1.25
Effect			
Dose	0.001	0.001	0.001
Source	0.001	0.001	0.001
Dose x source	0.001	0.006	0.001

b, potential gas production (ml/200 mg substrate); c, rate constant (%/h); t-half is the time at which 50% of b was produced.

Table 3. In vitro gas and methane production of substrates containing graded levels of *Kappaphycus alvarezii* and *Gracilaria salicornia* at 24 h of incubation

Treatment†	IVGP (ml/g)	Methane (% total gas)	Methane (ml/g DM)	Methane (ml/g DOM)
<i>Kappaphycus alvarezii</i>				
CON	179.2 ^{ab} ±3.75	28.4 ^c ±0.25	50.8 ^b ±0.68	95.4 ^c ±0.40
KA ₁	190.8 ^{cd} ±2.39	26.1 ^b ±0.24	49.7 ^b ±0.88	90.5 ^b ±1.09
KA ₂	196.7 ^d ±2.11	25.3 ^b ±0.39	49.8 ^b ±0.67	89.4 ^b ±1.22
KA ₄	180.8 ^{abc} ±3.75	23.9 ^a ±0.40	44.6 ^a ±1.59	83.0 ^a ±2.11
KA ₆	180.8 ^{abc} ±4.55	23.6 ^a ±0.39	42.6 ^a ±0.98	81.2 ^a ±1.28
KA ₈	174.2 ^a ±4.36	23.8 ^a ±0.22	41.5 ^a ±1.06	81.2 ^a ±1.10
<i>Gracilaria salicornia</i>				
GS ₁	173 ^d ±2.1	26.0 ^b ±0.26	45.1 ^d ±0.79	86.5 ^d ±1.10
GS ₂	143 ^c ±2.8	25.4 ^b ±0.36	36.2 ^c ±0.96	76.8 ^c ±1.46
GS ₄	134 ^{bc} ±5.5	23.6 ^a ±0.46	31.7 ^b ±1.18	69.4 ^b ±1.49
GS ₆	128 ^b ±4.4	23.4 ^a ±0.77	29.7 ^b ±0.76	67.2 ^{ab} ±1.63
GS ₈	113 ^a ±3.8	24.1 ^a ±0.35	27.1 ^a ±0.70	64.9 ^a ±0.88
Effects				
Dose	0.0001	0.0001	0.0001	0.0001
Source	0.0001	0.923	0.0001	0.0001
Dose x source	0.0001	0.985	0.0001	0.0001

a,b,c, d, e Mean(±SE) with different superscript in a column vary significantly (P<0.001)

SEM, standard error of mean; DOM, digestible organic matter

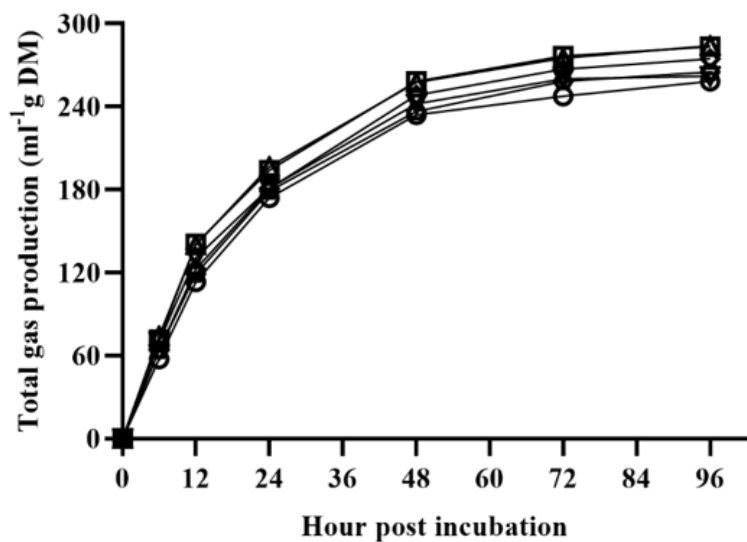
Table 4. *In vitro* dry matter digestibility (IVDMD) and fermentation characteristics at 24 h of incubation of substrates containing various proportions of *Kappaphycus alvarezii* and *Gracilaria salicornia*

Treatment	Parameters					
	IVDMD (%)	TVFA (mmol/l)	%Acetate	% Propionate	% butyrate	NH ₃ -N (mg/dl)
<i>Kappaphycus alvarezii</i>						
CON	55.5 ^{ab} ±0.63	23.8 ^c ±0.81	67.5 ^c ±0.42	22.2 ^a ±0.37	10.3 ^a ±0.23	9.89 ^{ab} ±0.400
KA ₁	57.4 ^{cd} ±0.40	24.6 ^c ±0.25	68.0 ^c ±0.68	22.4 ^{ab} ±0.48	9.6 ^a ±0.25	11.0 ^{bc} ±0.32
KA ₂	58.4 ^d ±0.35	25.4 ^c ±0.40	66.6 ^{bc} ±0.19	23.4 ^{ab} ±0.27	10.1 ^a ±0.14	11.3 ^c ±0.50
KA ₄	55.7 ^{abc} ±0.63	22.0 ^b ±0.46	65.4 ^{ab} ±0.71	22.6 ^{ab} ±0.55	12.0 ^b ±0.36	10.2 ^{bc} ±0.62
KA ₆	55.7 ^{abc} ±0.76	19.2 ^a ±0.41	64.1 ^a ±0.58	23.7 ^{ab} ±0.66	12.1 ^b ±0.20	8.87 ^a ±0.118
KA ₈	54.6 ^a ±0.73	18.2 ^a ±0.72	64.8 ^a ±0.55	23.8 ^b ±0.48	11.4 ^b ±0.30	8.87 ^a ±0.257
<i>Gracilaria salicornia</i>						
GS ₁	54.5 ^d ±0.35	24.3 ^e ±0.59	68.0 ^b ±0.34	22.9 ^c ±0.25	9.13 ^b ±0.191	10.5 ^c ±0.30
GS ₂	49.3 ^c ±0.47	20.8 ^d ±0.63	68.6 ^b ±0.48	18.9 ^{ab} ±0.70	12.5 ^d ±0.517	9.01 ^b ±0.168
GS ₄	47.9 ^{bc} ±0.93	16.5 ^c ±0.56	72.1 ^c ±0.37	17.3 ^a ±0.40	10.5 ^c ±0.415	9.10 ^b ±0.577
GS ₆	46.8 ^b ±0.74	14.3 ^b ±0.79	71.1 ^c ±1.20	20.1 ^b ±0.67	8.75 ^b ±0.540	7.61 ^a ±0.442
GS ₈	44.3 ^a ±0.64	9.2 ^a ±0.35	64.8 ^a ±0.27	28.2 ^d ±0.55	6.99 ^a ±0.412	7.00 ^a ±0.261
Effects						
Dose	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Source	0.0001	0.923	0.0001	0.0001	0.0001	0.0001
Dose x source	0.0001	0.985	0.0001	0.0001	0.0001	0.0036

^{a,b,c,d,e} Mean(±SE) with different superscript in a column vary significantly (P<0.001)

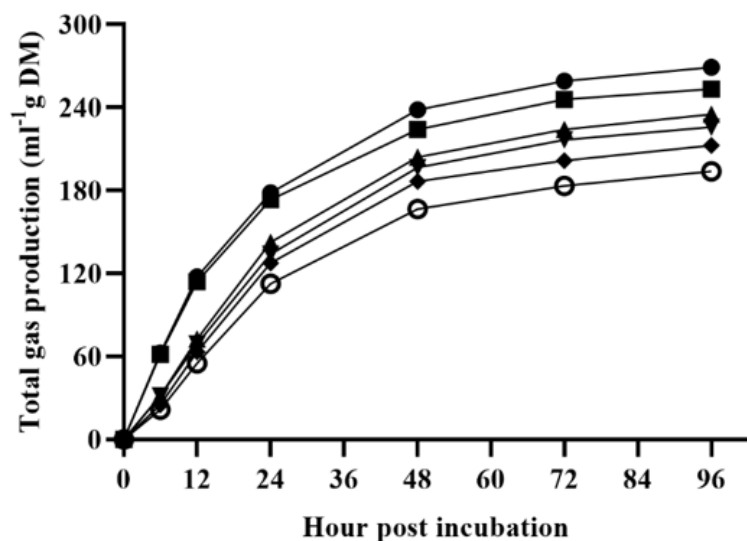
Figures

a) *Kappaphycus alvarezii*



● CON ■ KA₁ ▲ KA₂ ▼ KA₄ ◆ KA₆ ○ KA₈

b) *Gracilaria salicornia*

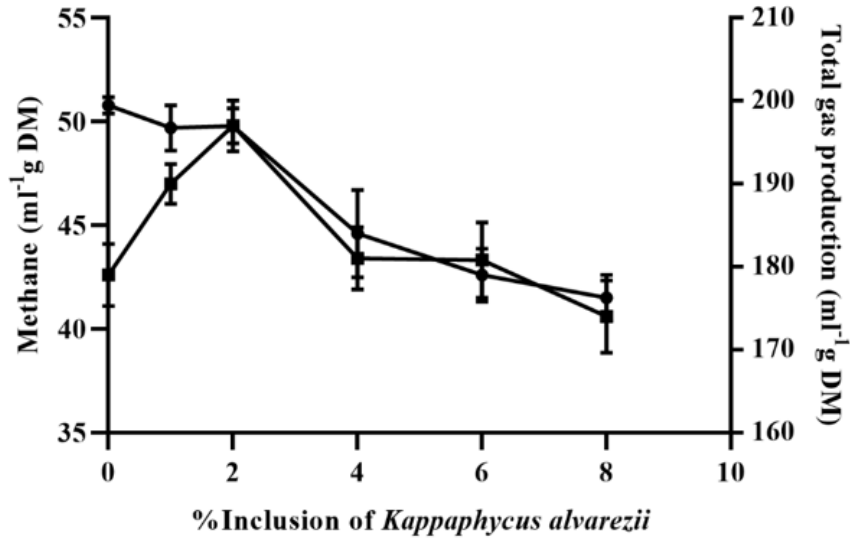


● CON ■ GS₁ ▲ GS₂ ▼ GS₄ ◆ GS₆ ○ GS₈

Figure 1

In vitro cumulative gas production of incubation of substrates containing various proportions of *Kappaphycus alvarezii* and *Gracilaria salicornia*

a) *Kappaphycus alvarezii*



■- Total gas production (ml⁻¹g DM) ●- Methane (ml⁻¹g DM)

b) *Gracilaria salicornia*

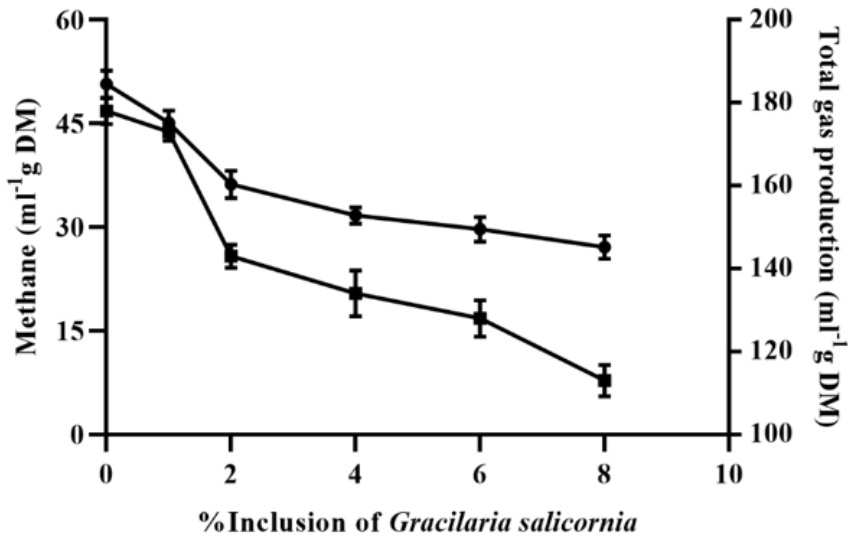


Figure 2

In vitro total gas and methane production from incubation of substrates containing various proportions of *Kappaphycus alvarezii* and *Gracilaria salicornia*