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Methane Reduction Potential of Two Pacific Coast Macroalgae During *in vitro* Ruminant Fermentation

Charles G. Brooke[1†], Breanna M. Roque[1], Claire Shaw[1†], Negeen Najafi[1], Maria Gonzalez[1], Abigail Pfefferlen[1], Vannesa De Anda[1], David W. Ginsburg[2], Maddelyn C. Harden[2], Sergey V. Nuzhdin[2], Joan King Salwen[3], Ermias Kebreab[1] and Matthias Hess[1†]*

[1] Department of Animal Science, University of California, Davis, Davis, CA, United States, [2] Dornsife College of Letters, Arts and Sciences, University of Southern California, Los Angeles, CA, United States, [3] Blue Ocean Barns Inc., Redwood City, CA, United States

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***Correspondence:**
Matthias Hess
mhess@ucdavis.edu

†ORCID:
Charles G. Brooke
0000-0002-2612-6141
Claire Shaw
0000-0002-2495-0612
Matthias Hess
0000-0003-0321-0380

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With increasing interest in feed-based methane mitigation strategies and regional legal directives aimed at methane production from the agricultural sector, identifying local sources of biological feed additives will be critical for rendering these strategies affordable. In a recent study, the red alga *Asparagopsis taxiformis* harvested offshore Australia was identified as highly effective for reducing methane production from enteric fermentation. Due to potential difference in methane-reduction potential and the financial burden associated with transporting the harvested seaweed over long distances, we examined locally sourced red seaweed *A. taxiformis* and brown seaweed *Zonaria farlowii* for their ability to mitigate methane production when added to feed widely used in the Californian dairy industry. At a dose rate of 5% dry matter (DM), California-sourced *A. taxiformis* and *Z. farlowii* reduced methane production by up to 74% ($p < 0.05$) and 11% ($p < 0.05$) during *in vitro* rumen fermentation, respectively. No effect on CO_2 production was observed for either seaweed. The measured decrease in methane production induced by *A. taxiformis* and *Z. farlowii* amendment, suggest that these local macroalgae are indeed promising candidates for biotic methane mitigation strategies in California, the largest milk producing state in the United States. To determine their real potential as methane mitigating feed supplements in the dairy industry, their effect *in vivo* will need to be investigated.

Keywords: *Asparagopsis taxiformis*, feed supplementation, macroalgae, methane mitigation, *in vitro* rumen fermentation, *Zonaria farlowii*

INTRODUCTION

Methane (CH_4), produced by human activity, accounts for more than 10% of the total greenhouse gases emitted in the United States (EPA, 2020) and enteric fermentation from ruminant animals accounts for approximately 25% of the total anthropogenic methane produced (NASEM, 2018). Thus, efficient strategies to lower enteric CH_4 production would enable a significantly reduced carbon footprint of agriculture and more specifically of animal production.

In vitro studies have demonstrated that some brown and red macroalgae can inhibit microbial methanogenesis (Machado et al., 2014; Roque et al., 2019) and they have been suggested as

feed supplements to reduce methanogenesis during enteric fermentation (Wang et al., 2008; Dubois et al., 2013; Machado et al., 2016). Utilization of these macroalgae could also promote higher growth rates and feed conversion efficiencies in ruminants via the potential net energy yield from the redistribution of energy from the microbial methanogenesis pathway into more favorable pathways for the animal (i.e., volatile fatty acids) (Hansen et al., 2003; Marin et al., 2009). Therefore, macroalgae feed supplementation may be an effective strategy to simultaneously improve profitability and sustainability of beef and dairy operations.

In a recent study (Machado et al., 2014), the red macroalgae *Asparagopsis taxiformis* stood out as the most effective species from a panel of tested seaweed to reduce methane production. In this screening effort, the effect of a variety of macroalgal species including freshwater, green, red, and brown algae on CH_4 production during *in vitro* incubation were compared, and results showed that *A. taxiformis* amendment yielded the most significant reduction (∼98.9%) of CH_4 production.

A major challenge in the implementation of an *A. taxiformis*-based methane mitigation strategy is the availability of this bioactive seaweed, which has led to the exploration of alternative seaweed species. Given that California, which is the largest milk producing state in the United States, has a shoreline of 1,350 km, it is imperative that local seaweeds are tested for methane mitigation properties in order to reduce the financial burden associated with harvesting the seaweed at locations that require long storage and transportation. Besides the economical aspect, the identification of local seaweed is of particular importance since the key halogenated compound responsible for macroalgae's methane mitigation abilities, bromoform, differs in concentration across both location and season (Carpenter and Liss, 2000). Here, we tested the potential of two different species of subtidal macroalgae (*A. taxiformis* and the brown alga, *Zonaria farlowii*) collected from offshore Southern California for their ability to mitigate methane production during *in vitro* rumen fermentation.

MATERIALS AND METHODS

Experimental Design

To determine the effect of two locally sourced macroalgae species on methane production during *in vitro* rumen fermentation, *A. taxiformis* and *Z. farlowii* were supplemented to an *in vitro* gas production system at a dose rate of 5% DM. This dose rate showed significant methane reduction during *in vitro* rumen fermentation for Australia-sourced *A. taxiformis* (Kinley et al., 2016; Roque et al., 2019). Rumen fluid was diluted threefold with artificial saliva buffer (Oeztuerk et al., 2005). After homogenization, 200 mL of the mixture was allocated to 300 mL vessels fitted with Ankom head units (Ankom Technology RF Gas Production System, Macedon, NY, United States). Each vessel received 2 g of rumen solids, and 2 g of a basic ration (Super Basic Ration; SBR; 70% alfalfa, 15% dried distillers' grain, and 15% rolled corn) commonly used in the dairy industry in California. Rumen solids and SBR were sealed in separate Ankom 5 cm × 5 cm concentrate feed bags and seaweed was included in the respective SBR feed bags (Ankom, Macedon, NY, United States). Control vessels were run in parallel with each seaweed treatment. Vessels were placed and incubated in a temperature controlled shaking water bath (39°C; 40 rpm). Foil gas bags (Restek, United States) were connected to the Ankom head units to collect gas at 24 and 48 h, respectively. The experimental design of this study is summarized in **Figure 1**.

Pacific Coast Seaweed Collection and Preparation, and Biochemical Analysis

Adult (gametophyte) stages of *A. taxiformis* and *Z. farlowii* were collected on May 14, 2018 from Little Fisherman's Cove (33°26′37″ N, 118°29′26″ W) on the leeward side of Santa Catalina Island, ∼35 km off the coast of Southern California, United States (**Figure 2**). The seaweed was shipped on ice to the University of California, Davis, where it was dried at 55°C for 72 h and ground through a 2 mm Wiley Mill (Thomas Fisher Scientific, Swedesboro, NJ, United States) before being placed into Ankom concentrate feed bags. Biochemical analyses of the seaweeds were performed by Cumberland Valley Analytical Services to determine the biochemical composition of the seaweeds used in this study.

Rumen Fluid Collection

All animal procedures were performed in accordance with the Institution of Animal Care and Use Committee (IACUC) at the University of California, Davis, under protocol number 19263. Rumen content was collected from a rumen fistulated Holstein cow, housed at the UC Davis Dairy Research and Teaching Facility Unit. The rumen content donor was fed a dry cow total mixed ration (50% wheat hay, 25% alfalfa hay/manger cleanings, 21.4% almond hulls, and 3.6% mineral pellet). Two liters of rumen fluid and 30 g of rumen solids were collected 90 min after morning feeding. Rumen content was collected via transphonation using a perforated PVC pipe, 500 mL syringe, and Tygon tubing (Saint-Gobain North America, PA, United States). Fluid was strained through a colander and four layers of cheesecloth into a 4 L pre-warmed, vacuum insulated container and transported to the laboratory. Rumen fluid and solids were collected on May 29, 2018 and June 19, 2018 for the *in vitro* trial to determine methane-mitigation potential of *A. taxiformis* and *Z. Farlowii*, respectively. Trials were started on the day of rumen fluid collection.

Greenhouse Gas Analysis

Methane and CO_2 were measured from gas bags using an SRI Gas Chromatograph (8610C, SRI, Torrance, CA, United States) fitted with a 3′ × 1/8″ stainless steel Haysep D column and a flame ionization detector (FID) with a methanizer. The oven temperature was held at 90°C for 5 min. Carrier gas was high purity hydrogen at a flow rate of 30 mL/min. The FID was held at 300°C. A 1 mL sample was injected directly onto the column. Calibration curves were developed with Airgas certified CH_4 and CO_2 standard (Airgas, United States).

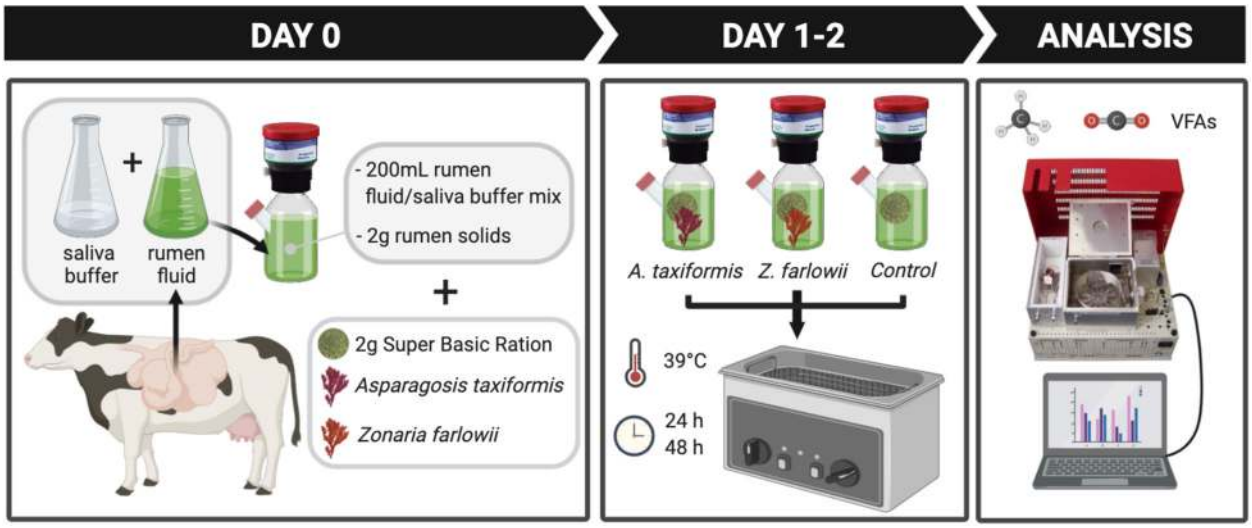

FIGURE 1 | Experimental design. Rumen content was collected from a rumen fistulated Holstein cow and rumen fluid was diluted threefold with saliva buffer after rumen solids and fluid were separated. Fluid/buffer mixture was amended with rumen solids and Super Basic Ration. To test effect of *Asparagopsis taxiformis* and *Zonaria farlowii*, fermentation vessels were supplemented with 5% dry matter (DM) of the corresponding seaweed. Carbon dioxide (CO_2), methane (CH_4) production and volatile acid profiles were determined after 24 and 48 h.

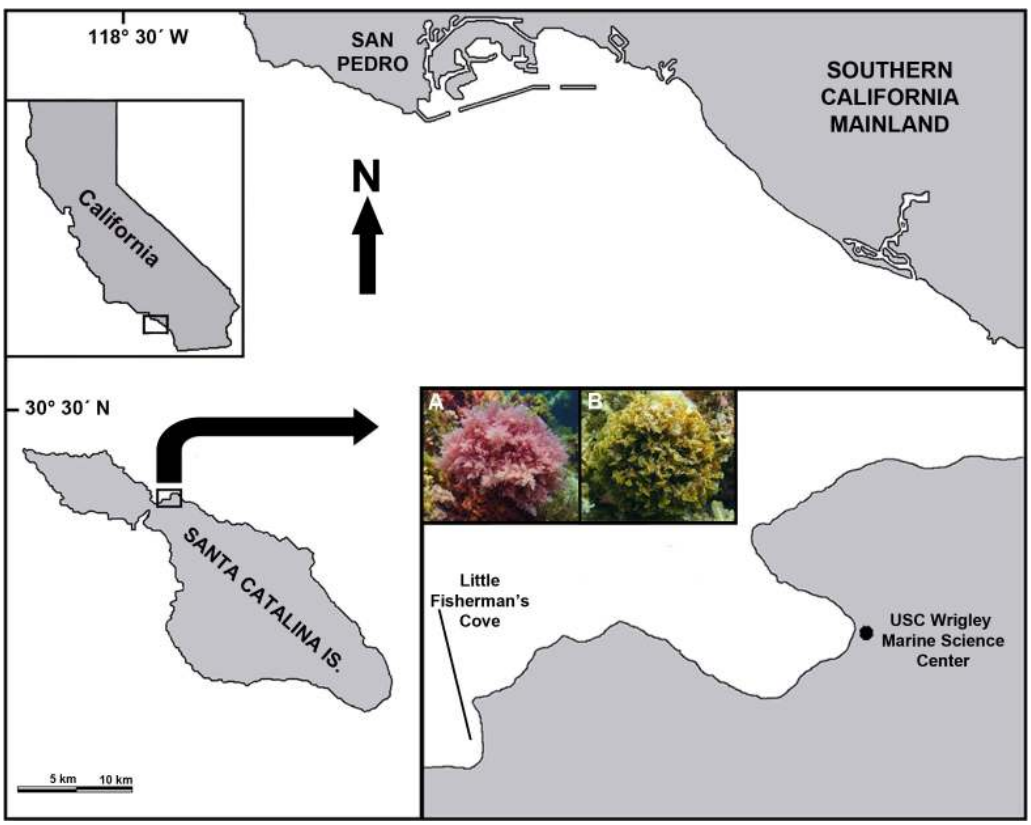

FIGURE 2 | Sampling sites. Map showing the location of Santa Catalina Island relative to the Southern California mainland. Inset on right: the red alga *Asparagopsis taxiformis* **(A)** and the brown alga *Zonaria farlowii* **(B)**. Algae were collected (2–5 m depth) in Little Fisherman's Cove, located ∼0.6 km from the USC Wrigley Marine Science Center.

Statistical Analysis

Differences in CH_4 and CO_2 production between sampling time points for each seaweed were determined using unpaired parametric *t*-tests with Welch's correction conducted in Graphpad Prism 7 (Graphpad Software Inc., La Jolla, CA, United States). Significant differences among treatments were declared at $p < 0.05$.

RESULTS

Biochemical Analysis of *Asparagopsis taxiformis* and *Zonaria farlowii*

Biochemical analysis of the two Pacific Coast seaweeds revealed a stark difference in their bromoform composition (2,305 $\mu g\ g^{-1}$ dry weight for *A. taxiformis* and 35 $\mu g\ g^{-1}$ dry weight for *Z. farlowii*) as well as in their nutritional composition (**Table 1**). Additionally, crude protein and starch content was significantly (1.9- and 8-fold, respectively) higher, whereas crude fat and non-fiber carbohydrates were (2.2- and 2.6-fold, respectively) lower in *A. taxiformis* compared to *Z. farlowii*. Concentrations of iron were almost twice as high in *Z. farlowii* (1,765 ppm) compared to *A. taxiformis* (939 ppm).

Gas Production Profile of *in vitro* Fermentation of Rumen Fluid Amended With 5% *A. taxiformis*

Amendment of feed with 5% DM of *A. taxiformis* reduced total gas production by ∼28% (from 142.8 ± 42 mL to 103.4 ± 13.9 mL) and ∼30% (from 214.3 ± 40.3 mL to 151.4 ± 16.4 mL) during 24 and 48 h of rumen incubation. Additionally, the total amount of methane produced remained somewhat similar in *A. taxiformis* vessels, during both 24 and 48 h of *in vitro* rumen fermentation [7.1 ± 1.9 mL $(g\ DM)^{-1}$ and 6.6 ± 2.5 mL $(g\ DM)^{-1}$, respectively), the amount of methane increased over the same time period in the absence of *A. taxiformis* (control vessels). Methane in the control vessels increased from 20.3 ± 11 mL $(g\ DM)^{-1}$ to 35.5 ± 8.5 mL $(g\ DM)^{-1}$ during 24 and 48 h of *in vitro* rumen fermentation, respectively (**Figures 3A,C**). At a dose rate of 5% DM, *A. taxiformis* reduced methane production by 65% over 24 h and 74% over 48 h during *in vitro* rumen fermentation ($p < 0.05$, **Figure 3C**). While methane production varied with 5% DM inclusion of *A. taxiformis,* CO_2 production remained similar between treatment [41.9 ± 6.2 mL $(g\ DM)^{-1}$ and 65.23 ± 9.1 mL $(g\ DM)^{-1}$ at 24 and 48 h, respectively] and control vessels [47.4 ± 13.4 mL $(g\ DM)^{-1}$ and 69.0 ± 15.9 mL $(g\ DM)^{-1}$ at 24 and 48 h, respectively] (**Figures 3B,D**).

TABLE 1 | Biochemical composition of *Asparagopsis taxiformis* and *Zonaria farlowii*.

	Asparagopsis taxiformis	*Zonaria farlowii*
$\mu g\ g^{-1}$ Dry weight		
Bromoform	2,305	35
% Dry matter		
Organic matter	53.0	71.1
Crude protein	18.2	9.40
Neutral Detergent Fiber	25.7	41.7
Starch	0.80	0.10
Crude fat	0.55	1.22
Calcium	1.98	3.70
Phosphorus	0.25	0.14
Magnesium	1.35	0.83
Potassium	2.02	2.30
Sodium	12.3	3.27
ppm		
Iron	939	1,765
Manganese	26.0	31.0
Zinc	37.0	54.0
Copper	6.00	5.00
Non-fiber carbohydrate	9.01	23.7

Gas Production Profile of *in vitro* Fermentation of Rumen Fluid Amended With 5% *Z. farlowii*

Amendment of feed with 5% DM of *Z. farlowii* did not reduce total gas production beyond a slight reduction of 5% (from 274.2 ± 16.4 mL to 261.8 ± 25.2 mL) during 24 h of rumen incubation. Over 48 h total gas production increased by 2% (from 336.9 ± 23.3 mL to 343.8 ± 23.9 mL). At a dose rate of 5% DM, *Z. farlowii* reduced methane production by 11.5% after 24 h and 10.7% after 48 h of *in vitro* rumen fermentation ($p < 0.05$, **Figure 4A**). Daily methane production decreased slightly at 48 h compared to 24 h of incubation for both the control and treatment vessels [Control = 62.5 ± 3.3 mL $(g\ DM)^{-1}$ and 51.4 ± 2.9 mL $(g\ DM)^{-1}$ CH_4, at 24 and 48 h, respectively; treatment = 55.3 ± 2.7 and 45.9 ± 3.7 mL $(g\ DM)^{-1}$ CH_4, at 24 and 48 h, respectively (**Figures 4A,C**)].

While methane production decreased slightly for all vessels at 48 h, CO_2 production nearly doubled [Control = 74.1 ± 7.7 mL $(g\ DM)^{-1}$ and 117.9 ± 14.6 mL $(g\ DM)^{-1}$ CO_2, at 24 and 48 h, respectively; treatment = 67.6 ± 4.1 mL $(g\ DM)^{-1}$ and 114.2 ± 6.0 mL $(g\ DM)^{-1}$ CO_2, at 24 and 48 h, respectively]. Carbon dioxide production from vessels amended with 5% DM of *Z. farlowii* did not differ from the control vessels at 24 or 48 h ($p = 0.27$ and $p = 0.70$, respectively; **Figures 4B,D**).

DISCUSSION

With increasing interest in feed-based methane mitigation strategies, fueled by legal directives aimed at reducing methane production from the agricultural sector, identification of local biotic feed-supplements will be critical to render large-scale methane mitigation strategies economical.

Our data indicate that subtidal macroalgae from Santa Catalina Island, Southern California reduced the *in vitro* production of CH_4 when added to rumen content from California dairy cattle. The addition of *A. taxiformis* reduced microbial methane production by up to ∼78%, which is slightly lower but similar to the reduction (∼95%) of enteric methane

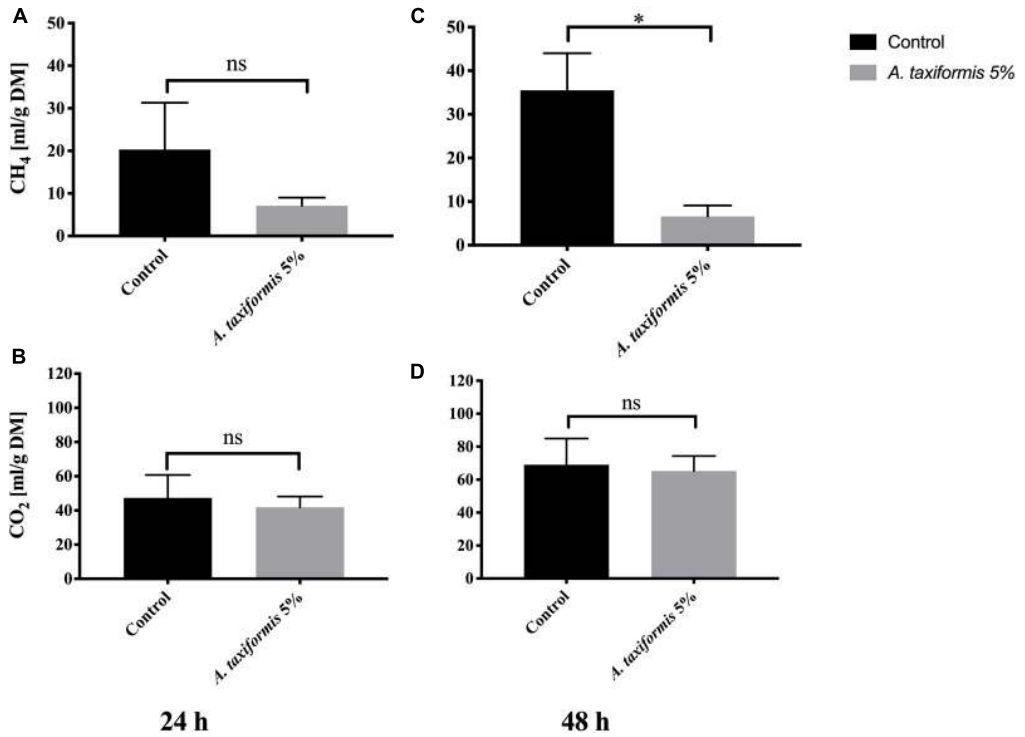

FIGURE 3 | Methane and CO_2 production during *in vitro* fermentation of rumen fluid amended with *A. taxiformis*. Production of CH_4 mL (g DM)$^{-1}$ and CO_2 mL (g DM)$^{-1}$ from vessels without (n = 4) and with 5% (n = 4) *A. taxiformis* as additive. Methane and CO_2 were measured at 24 h (**A,B**, respectively) and 48 h (**C,D**, respectively). "*" indicates significant difference (p-value < 0.05), "ns" indicates not significant. Error bars represent the standard error from the mean.

we reported previously when the effect of Australian sourced *A. taxiformis* on 96 h of *in vitro* rumen fermentation was determined (Roque et al., 2019). Taking the variations in microbiome composition and function as well as potential experimental variations into consideration a direct comparison of these two different seaweeds would be certainly be of great value to evaluate the real and explicit potential of these promising feed amendments. However, since *in vitro* assays are only a rough proxy of the *in vivo* response, such comparison might be better suited for an *in vivo* follow-up experiment. The effects of adding Pacific *Z. farlowii* on methane production was significantly less prominent: a reduction of only 11% was measured when *Z. farlowii* was added at 5% DM. This difference in methane mitigation is not surprising when considering the amount of bromoform concentration associated with the different seaweeds we tested (2,305 μg g^{-1} dry weight) for *A. taxiformis* and 35 μg g^{-1} dry weight for *Z. farlowii*). In fact, bromoform concentrations for *A. taxiformis* were maintained throughout our harvesting and sample preparation process at levels similar to the highest values that had been reported for the Australian *A. taxiformis* (Vucko et al., 2017). Organobromine compounds such as bromomethane (methyl bromide; CH_3Br) and bromoform ($CHBr_3$) are abundantly found in marine algae (Gribble, 2000) and have been identified as inhibiting microbial methanogenesis (Tomkins et al., 2009). It is not surprising that California-sourced seaweed, specifically *A. taxiformis* with its elevated level of bromoform, reduced methanogenesis during *in vitro* rumen fermentation. Based on these findings, we therefore hypothesize that California-sourced seaweeds might represent a viable option for use in feed-based methane mitigation strategies, especially when considering the significantly reduced carbon footprint associated with utilizing local natural resources.

In addition to demonstrating the potential of the local *A. taxiformis* for methane mitigation during enteric fermentation, we were also able to demonstrate methane reduction with no obvious impact on total gas and CO_2 production (**Figures 3A,B**, **4A,B**) by the brown alga *Z. farlowii,* a species of seaweed commonly found along the Southern California Bight.

Released CO_2 can be used as a highly sensitive proxy capable of detecting changes in the overall metabolic carbon respiration and growth of a microbial community within an ecosystem (Kaschuk et al., 2010; Zheng et al., 2019). Changes in CO_2 profiles would therefore indicate significant perturbation and dysbiosis of the healthy rumen microbiome with potentially significant changes in the overall function of the rumen ecosystem as observed for example during acidosis when animals are transition to quickly to feed that contains high level of rapidly digestible carbohydrate such as barley and other cereals and which poses a life-threatening condition to the ruminant animal (Navarre et al., 2012; Eger et al., 2018). The lack of changes of observed CO_2 profiles suggest that addition of neither *A. taxiformis* nor *Z. farlowii* had an effect on overall microbiome metabolism

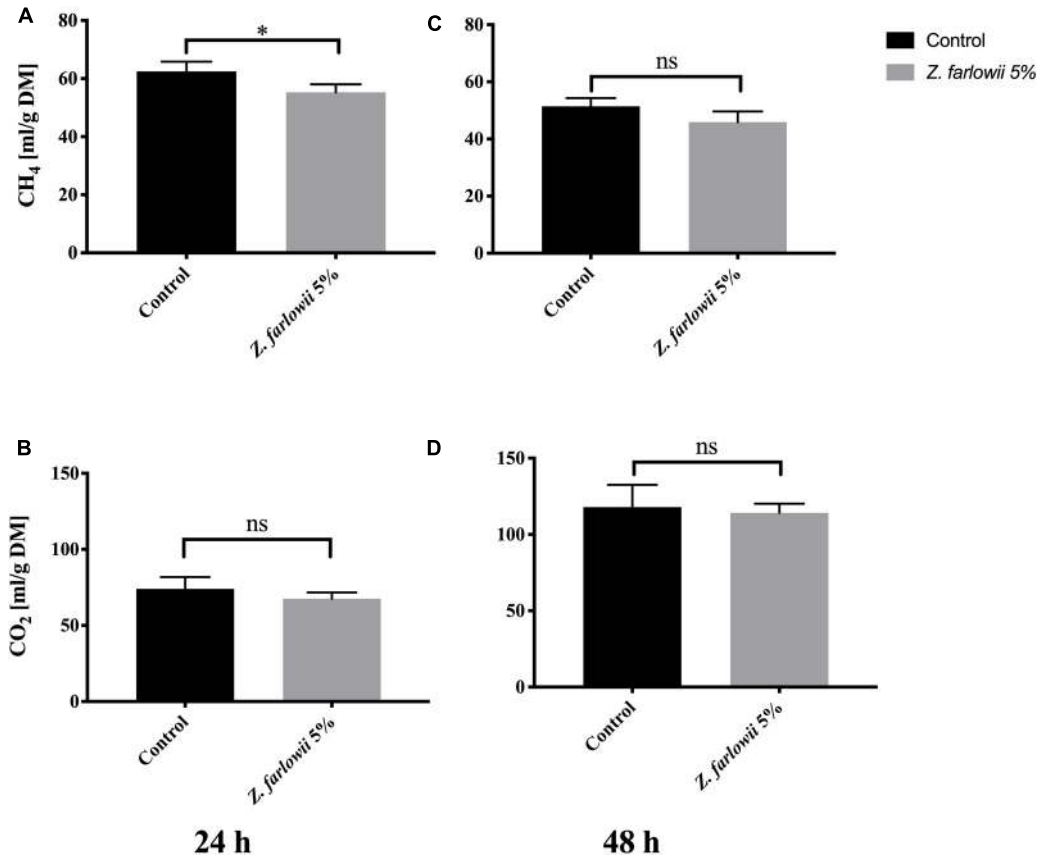

FIGURE 4 | Methane and CO_2 production during *in vitro* fermentation of rumen fluid amended with *Z. farlowii*. Production of CH_4 mL (g DM)$^{-1}$ and CO_2 mL (g DM)$^{-1}$ from vessels without (n = 3) and with 5% (n = 3) *Z. farlowii* as additive. Methane and CO_2 were measured at 24 h (**A,B**, respectively) and 48 h (**C,D**, respectively). "*" indicates significant difference (p-value < 0.05), "ns" indicates not significant. Error bars represent the standard error from the mean.

and healthy function was maintained. To get a more detailed understanding of potential metabolic changes at the sub-microbiome level, which might not be detected by measuring CO_2 production and release, more detailed microbiome analyses should be performed before these seaweeds are applied at large scale to ruminant animals.

The effectiveness of macroalgae in reducing methane production during rumen incubation has been linked to the concentration of halogenated bioactives including bromoform and di-bromochloromethane (Machado et al., 2016). In contrast to *A. taxiformis*, which has been shown to produce several halomethane compounds, *Z. farlowii* only caused a modest reduction of methanogenesis and consequently higher amounts of emitted enteric methane during *in vitro* rumen fermentation. These findings suggest that either the bioactives in *Z. farlowii* are more bioavailable but less effective or concentrated, or methane reduction mediated by *Z. farlowii* amendment occurs via a different compound or mode of action. *Z. farlowii* is commonly found along the Southern California Bight, which makes it a potential candidate for non-terrestrial farming operations along the Southern California Coast. In order to understand the molecular mechanism by which *Z. farlowii* reduces methane production from the rumen microbiota, as well as the nutritional value of *Z. farlowii* more detailed, follow-up *in vitro* and *in vivo* studies involving *Z. farlowii* as feed additive are required. Only with these data in hand will it be possible to determine *Z. farlowii*'s value for future methane mitigation strategies and to determine its potential for use in dairy operations.

CONCLUSION

Asparagopsis taxiformis and *Z. farlowii* collected off Santa Catalina Island were evaluated for their ability to reduce methane production from dairy cattle fed a mixed ration widely utilized in California operations. The methane reducing effect of *A. taxiformis* and *Z. farlowii* described in this study make these macroalgae promising candidates for biotic methane mitigation strategies in the largest milk producing state in the United States, though further follow-up studies will be required to both substantiate methane mitigation ability and elucidate the molecular mechanism by which these macroalgae reduce methane production in livestock. With expected growth in livestock production, it is necessary to investigate and confirm the effect of these macroalgae *in vivo* in order to ensure that farmers have sufficient incentive to implement such strategies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by all animal procedures were performed in accordance with the Institution of Animal Care and Use Committee (IACUC) at the University of California, Davis, under protocol number 19263.

AUTHOR CONTRIBUTIONS

CB, BR, and MH conceived and designed the experiment. DG, MCH, and SN collected seaweed. CB, BR, CS, NN, MG, AP, VD, and MH performed experiments and analyzed data. CB, BR, and MH wrote major part of the manuscript. All authors were involved in writing the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.00561/full#supplementary-material

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Conflict of Interest: JS was employed by Blue Ocean Barns Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.