

1 **Title: Methane Reduction Potential of Two Pacific Coast Macroalgae During *in-vitro***
2 **Ruminant Fermentation.**

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33

34 **Abstract:**

35 With increasing interest in feed based methane mitigation strategies, fueled by local legal
36 directives aimed at methane production from the agricultural sector in California, identifying
37 local sources of biological feed additives will be critical in keeping the implementation of these
38 strategies affordable. In a recent study, the red alga *Asparagopsis taxiformis* stood out as the
39 most effective species of seaweed to reduce methane production from enteric fermentation. Due
40 to the potential differences in effectiveness based on the location from where *A. taxiformis* is
41 collected and the financial burden of collection and transport, we tested the potential of *A.*
42 *taxiformis*, as well as the brown seaweed *Zonaria farlowii* collected in the nearshore waters off
43 Santa Catalina Island, CA, USA, for their ability to mitigate methane production during *in-vitro*
44 rumen fermentation. At a dose rate of 5% dry matter (DM), *A. taxiformis* reduced methane
45 production by 74% ($p \leq 0.01$) and *Z. farlowii* reduced methane production by 11% ($p \leq 0.04$)
46 after 48 hours and 24 hours of *in-vitro* rumen fermentation respectively. The methane reducing

47 effect of *A. taxiformis* and *Z. farlowii* described here make these local macroalgae promising
48 candidates for biotic methane mitigation strategies in the largest milk producing state in the US.
49 To determine their real potential as methane mitigating feed supplements in the dairy industry,
50 their effect *in-vivo* requires investigation.

51

52 **Key Words:** *Asparagopsis taxiformis*, *Zonaria farlowii* , feed supplementation, greenhouse gas
53 mitigation, *in-vitro* rumen fermentation, macroalgae

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57 **1. Introduction**

58 Methane (CH₄) accounts for more than 10% of the greenhouse gas (GHG) emissions from the
59 US (Myhre, 2013) and enteric fermentation from ruminant animals accounts for approximately
60 25% of the total anthropogenically produced methane (NASEM, 2018). Thus, efficient
61 strategies to lower enteric CH₄ production could result in a significantly reduced carbon footprint
62 from agriculture and animal production more specifically.

63 *In-vitro* studies have demonstrated that some brown and red macroalgae can inhibit
64 microbial methanogenesis (Machado, 2014) and they have been suggested as feed supplements
65 to reduce methanogenesis during enteric fermentation (Machado, 2016; Dubois, 2013; Wang,
66 2008). In addition to its methane reducing affect, utilization of these macroalgae could promote
67 higher growth rates and feed conversion efficiencies in ruminants via the potential net energy
68 yield from the redistribution of energy from the microbial methanogenesis pathway, into more
69 favorable pathways (i.e volatile fatty acids) (Hansen, 2003; Marín, 2009). Therefore, macroalgae

70 feed supplementation may be an effective strategy to simultaneously improve profitability and
71 sustainability of beef and dairy operations.

72 In a recent study (Machado et al. 2014), the red alga *Asparagopsis taxiformis* stood out as
73 the most effective species of seaweed to reduce methane production. In this work, the effect of a
74 large variety of macroalgal species including freshwater, green, red and brown algae on CH₄
75 production during *in-vitro* incubation were compared and the obtained results showed that *A.*
76 *taxiformis* amendment yielded the most significant reduction (~98.9%) of CH₄ production.

77 A major barrier to the implementation of an *A. taxiformis* based methane mitigation
78 strategy is the availability of the seaweed, which has led to the exploration of alternative seaweed
79 species. Previous investigations have collected *A. taxiformis* during diving excursions off the
80 coast of Australia. Due to the potential differences in effectiveness based on the location and
81 growing conditions from which the seaweed is collected and the financial burden of transport,
82 we tested the potential of two different species of subtidal macroalgae (*A. taxiformis* and the
83 brown alga, *Zonaria farlowii*) from Southern California for their ability to mitigate methane
84 production during *in-vitro* rumen fermentation.

85

86 **2. Materials and Methods**

87 **2.1 Experimental Design**

88 To determine the effect of two locally sourced macroalgae species on methane production during
89 *in-vitro* rumen fermentation, *Asparagopsis taxiformis* and *Zonaria farlowii* were supplemented
90 to an *in-vitro* gas production system at a dose rate of 5% DM. Rumen fluid was diluted 3-fold
91 with artificial saliva buffer (Oeztuerk et al., 2015). After homogenization, 200 ml of the mixture
92 was allocated to 300 ml vessels fitted with Ankom head units (Ankom Technology RF Gas

93 Production System, Macedon, NY, USA). Each vessel received 2 g of rumen solids, and 2 g of a
94 basic ration (Super basic ration — SBR, Table 1) commonly used in the dairy industry in
95 California. Rumen solids and SBR were sealed in separate Ankom feed bags and seaweed was
96 included in the respective SBR feed bags (Ankom, Macedon, NY). Vessels were placed in a
97 shaking water bath (39°C) and incubated while mixed at 40 rpm. Foil gas bags (Restek, USA)
98 were connected to the Ankom head units to collect gas at 24 and 48 hours respectively.

99

100 **2.2 Pacific Coast Seaweed Collection and Preparation**

101 *Asparagopsis taxiformis* and *Z. farlowii* were collected from Little Fisherman's Cove on the
102 leeward side of Santa Catalina Island, ~35 km off the coast of Southern California, USA (Figure
103 1). The seaweed was shipped on ice to the University of California, Davis, where it was dried at
104 55°C for 72 hours and ground through a 2 mm Wiley Mill (Thomas Scientific, Swedesboro, NJ).

105

106 **2.3 Rumen Fluid Collection**

107 All animal procedures were performed in accordance with the Institution of Animal Care and
108 Use Committee (IACUC) at University of California, Davis under protocol number 19263.
109 Rumen content was collected from a rumen fistulated Holstein cow, housed at the UC Davis
110 Dairy Research and Teaching Facility Unit. The rumen fluid donor was fed a dry cow total
111 mixed ration (50% wheat hay, 25% alfalfa hay/manger cleanings, 21.4% almond hulls, and 3.6%
112 mineral pellet, Table 1). Two liters of rumen fluid and 30 g of rumen solids were collected 90
113 min after morning feeding. Rumen content was collected via transphonation using a perforated
114 PVC pipe, 500 mL syringe, and Tygon tubing (Saint-Gobain North America, PA, USA). Fluid

115 was strained through a colander and 4 layers of cheesecloth into a 4 L pre-warmed, vacuum
116 insulated container and transported to the laboratory.

117

118 **2.4 Greenhouse Gas Analysis**

119 Methane and CO₂ were measured from gas bags using an SRI Gas Chromatograph (8610C, SRI,
120 Torrance, CA) fitted with a 3'x1/8" stainless steel Haysep D column and a flame ionization
121 detector (FID) with methanizer . The oven temperature was held at 90°C for 5 minutes. Carrier
122 gas was high purity hydrogen at a flow rate of 30 ml/min. The FID was held at 300°C. A 1 mL
123 sample was injected directly onto the column. Calibration curves were developed with Airgas
124 certified CH₄ and CO₂ standard (Airgas, USA).

125

126 **2.5 Statistical Analysis**

127 Differences in CH₄ and CO₂ production were determined using unpaired parametric t-tests with
128 Welch's correction conducted in Graphpad Prism 7 (Graphpad software Inc, La Jolla, CA).
129 Significant differences among treatments were declared at $p \leq 0.05$.

130

Table 1. Composition of dry cow diet and super basic ration (SBR).

Dry Cow Diet		SBR	
Ingredient			
Alfalfa	25%	Alfalfa	70%
Wheat	50%	Dried distillers grain	15%

Almond hulls	21.40%	Rolled corn	15%
Mineral pellets	3.60%		

131

132 **3. Results**

133 **3.1 Gas production profile of *in vitro* fermentation of rumen fluid amended with 5% *A.***

134 *taxiformis*

135 At a dose rate of 5% DM, *A. taxiformis* reduced methane production by 74% after 48 hours of *in-*
136 *vitro* rumen fermentation ($p \leq 0.01$, Figure 2B) and daily methane production remained nearly
137 identical in the presence of *A. taxiformis* on both days (7.1 ± 1.9 ml (g DM)⁻¹ and 6.6 ± 2.5 ml (g
138 DM)⁻¹ after 24 and 48 hours respectively). Methane production in the control vessels increased
139 by 76% after 48 hours of incubation (20.3 ± 11 ml (g DM)⁻¹ and 35.5 ± 8.5 ml (g DM)⁻¹ at 24
140 and 48 hours respectively).

141 While methane production varied with 5% DM inclusion of *A. taxiformis*, CO₂
142 production remained similar between treatment (41.9 ± 6.2 ml (g DM)⁻¹ and 65.23 ± 9.1 ml (g
143 DM)⁻¹ at 24 and 48 hours respectively) and control vessels (47.4 ± 13.4 ml (g DM)⁻¹ and
144 69.0 ± 15.9 ml (g DM)⁻¹ at 24 and 48 hours respectively).

145

146 **3.2 Gas production profile of *in vitro* fermentation of rumen fluid amended with 5% *Z.***

147 *farlowii*

148 At a dose rate of 5% DM, *Z. farlowii* reduced methane production by 11% after 24 hours of *in*
149 *vitro* rumen fermentation ($p \leq 0.04$, Figure 3A). Daily methane production decreased slightly at
150 48 hours compared to 24 hours of incubation for both the control and treatment vessels (Control

151 = 62.5 ± 3.3 ml (g DM)⁻¹ and 51.4 ± 2.9 ml (g DM)⁻¹ CH₄, at 24 and 48 hours respectively;
152 treatment = 55.3 ± 2.7 and 45.9 ± 3.7 ml (g DM)⁻¹ CH₄, at 24 and 48 hours respectively).

153 While methane production decreased slightly for all vessels at 48 hours, CO₂ production
154 nearly doubled (Control = 74.1 ± 7.7 ml (g DM)⁻¹ and 117.9 ± 14.6 ml (g DM)⁻¹ CO₂, at 24
155 and 48 hours respectively; treatment = 67.6 ± 4.1 ml (g DM)⁻¹ and 114.2 ± 6.0 ml (g DM)⁻¹
156 CO₂, at 24 and 48 hours respectively). Carbon dioxide production from vessels amended with
157 5% DM of *Z. farlowii* did not differ from the control vessels at 24 or 48 hours ($p \leq 0.27$ and $p \leq$
158 0.70 respectively).

159

160 **4. Discussion**

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

165 The data presented here suggest that subtidal macroalgae from Santa Catalina Island,
166 Southern California reduced the *in-vitro* production of CH₄ when added to rumen content from
167 California dairy cattle, suggesting that California seaweed might represent a viable option for use
168 in feed based methane mitigation strategies. In addition to demonstrating the potential of the
169 local *A. taxiformis* for methane mitigation during enteric fermentation, we also demonstrated
170 significant methane reduction in the brown alga *Z. farlowii*, a species of seaweed commonly
171 found along the Southern California Bight, without obvious impact on CO₂ production (Figures 2
172 and 3, panels A and B).

173 The effectiveness of a macroalgae in reducing methane production during rumen
174 incubation has been linked to the concentration of halogenated bioactives including bromoform
175 and di-bromochloromethane (Machado, 2016). However, in contrast to *A. taxiformis*, which has
176 been shown to produce several halomethane compounds, *Z. farlowii* amendment only reduced
177 methane on a short time scale. These findings suggest that either the bioactives in *Z. farlowii* are
178 more bioavailable but less effective or concentrated, or methane reduction is occurring via a
179 different compound or a different mode of action. Previous studies have identified multiple
180 phenolic lipids produced by *Z. farlowii* from Southern California waters as possessing
181 antimicrobial activity (Gerwick and Fenical, 1981). However, the reduction of methane in
182 vessels amended with *Z. farlowii* was modest compared to those amended with *A. taxiformis*.
183 *Zonaria farlowii* is commonly found along the Southern California Bight, which makes it a
184 potential candidate for non-terrestrial farming operations along the Southern California Coast. A
185 more in-depth nutrient analysis of *Z. farlowii* along with *in-vitro* assays will be essential to help
186 determine its value for future methane mitigation strategies and to determine its potential for use
187 in dairy operations.

188

189 **5. Conclusion**

190 *Asparagopsis taxiformis* and *Z. farlowii* collected off Santa Catalina Island were evaluated for
191 their ability to reduce methane production from dairy cattle fed a mixed ration widely utilized in
192 California. The methane reducing effect of the *A. taxiformis* and *Z. farlowii* described in this
193 study makes these macroalgae promising candidates for biotic methane mitigation strategies in
194 the largest milk producing state in the US. With expected growth in livestock production, it is

195 necessary to investigate and confirm the effect of these macroalgae *in-vivo*, in order to ensure
196 that farmers have sufficient incentive to implement such strategies.

197

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241 **Figure 1. Map showing the location of Santa Catalina Island relative to the Southern**
242 **California mainland.** Inset: The red alga *Asparagopsis taxiformis* (A) and the brown alga
243 *Zonaria farlowii* (B) were collected (2-5 m depth) in Little Fisherman's Cove, located ~0.6 km
244 from the USC Wrigley Marine Science Center.

245

246 **Figure 2. Methane and CO₂ production during *in-vitro* fermentation of rumen fluid**
247 **amended with *A. taxiformis*.** Production of CH₄ [ml (g DM)⁻¹] and CO₂ [ml (g DM)⁻¹]
248 from vessels without (n=4) and with 5% (n=4) *A. taxiformis* as additive. Methane and CO₂ were
249 measured at 24 h (A & B respectively) and 48 h (C & D respectively). “*” indicate significant
250 difference (p value ≤ 0.05), “ns” indicates not significant. Error bars represent the standard error
251 from the mean.

252

253 **Figure 3. Methane and CO₂ production during *in-vitro* fermentation of rumen fluid**
254 **amended with *Z. farlowii*.** Production of CH₄ [ml (g DM)⁻¹] and CO₂ [ml (g DM)⁻¹] from
255 vessels without (n=3) and with 5% (n=3) *Z. farlowii* as additive. Methane and CO₂ were
256 measured at 24 h (A & B respectively) and 48 h (C & D respectively). “*” indicate significant
257 difference (p value ≤ 0.05), “ns” indicates not significant. Error bars represent the standard error
258 from the mean.

259

118° 30' W

SAN PEDRO

SOUTHERN CALIFORNIA MAINLAND



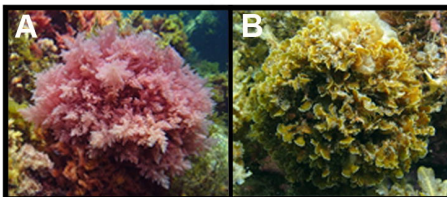
California



30° 30' N



SANTA CATALINA IS.



Little Fisherman's Cove

USC Wrigley Marine Science Center

5 km 10 km



