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Article

Evaluation of Rumen Fermentation and Microbial Adaptation to Three Red Seaweeds Using the Rumen Simulation Technique

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Abstract

Several red seaweeds have shown to inhibit enteric CH_4 production; however, adaptation of fermentation parameters to their presence is not well understood. The objective of this study was to examine the effect of three red seaweeds (*Asparargopsis taxiformis, Mazzaella japonica, Palmaria mollis*) on *in vitro* fermentation, CH_4 production, and adaptation using the rumen simulation technique (RUSITEC). The experiment was conducted as a completely randomized design with four treatments, duplicated in two identical RUSITEC apparatus equipped with eight fermenter vessels each. The four treatments included the control (barley straw and barley silage) and the three red seaweeds added to the control diet at 2% diet DM. The experimental period was divided into four phases including a baseline phase (d 0-7; no seaweed included), adaptation phase (d 8-11; seaweed included in treatment vessels), intermediate phase (d 12-16) and a stable phase (d 17-21). The digestibility of organic matter (P = 0.04) and neutral detergent fibre (P = 0.05) was decreased by *A. taxiformis* during the adaptation phase, but returned to control levels in the stable phase. *A. taxiformis* supplementation resulted in a decrease (P < 0.001) in molar proportions of acetate, propionate and total volatile fatty acid (VFA) production, with an increase

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in molar proportions of butyrate, caproate, and valerate; the other seaweeds had no effect ($P > 0.05$) on molar proportions or production of individual VFA. *A. taxiformis* was the only seaweed to suppress CH_4 production ($P < 0.001$), with the suppressive effect increasing ($P < 0.001$) across phases. Similarly, *A. taxiformis* increased ($P < 0.001$) the production of hydrogen (H_2, %, mL/d) across the adaptation, intermediate and stable phases, with the intermediate and stable phases having greater H_2 production than the adaptation phase. In conclusion, *M. japonica* and *P. mollis* did not impact rumen fermentation or inhibit CH_4 production within the RUSITEC. In contrast, we conclude that *A. taxiformis* is an effective CH_4 inhibitor and its introduction to the ruminal environment requires a period of adaptation; however, the large magnitude of CH_4 suppression by *A. taxiformis* inhibits VFA synthesis, which may restrict production performance in vivo.

Keywords: rumen simulation technique; methane production; seaweed; rumen fermentation

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Implications

There is global focus on the search for identifying species of macroalgae that can be fed to livestock as a feedstock, or as a methane mitigant. This study evaluated the effect of three red seaweeds to assess their potential for decreasing ruminant enteric methane production. While only *Asparagopsis taxiformis* was effective at decreasing methane production, this study provides new information on the requirements for adapting animals to seaweed feeding.

Introduction

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Increased greenhouse gas (GHG) concentrations in the atmosphere has resulted in alterations to the ozone layer, consequently raising the global surface temperature (Nema et al., 2012). The agricultural sector contributes to 26.0% of anthropogenic global GHG, mainly CO_2, N_2O and CH_4 (Frank et al., 2017). Enteric CH_4 is produced from the natural fermentation of carbohydrates within the rumen, and contributes approximately 6.0% of global anthropogenic GHG (Beauchemin et al., 2020). While CH_4 has a comparatively shorter half-life in the atmosphere (~ 10 years) than CO_2 which can persist in the atmosphere for over hundreds of years, CH_4 has 28 times the global warming potential of CO_2, making it an attractive target for abatement (IPCC, 2018).

There has been growing interest in the use of macroalgae or seaweed and their associated by-products to reduce enteric CH_4 emissions from ruminants (Abbott et al., 2020). Macroalgae are rich in complex carbohydrates and polysaccharides, including two groups of compounds that are known CH_4 inhibitors. Specific to red seaweeds are the presence of halogenated low molecular weight compounds, including bromoforms and haloforms (Kinley et al., 2020; McCauley et al., 2020). These compounds have been shown to be extremely effective at mitigating enteric CH_4 production with reports of greater than 67% reduction observed with the feeding of *Asparagopsis* species to various ruminants. Secondly, seaweeds contain a variety of phlorotannins that are similar to their terrestrial counterparts (Abbott et al., 2020). Terrestrial tannins have shown to decrease enteric CH_4 production by as much as 30%, with the extent varying dramatically based on tannin profiles (Terry et al., 2020). It is hypothesised that seaweeds with high concentrations of phlorotannins may exhibit a similar antimethanogenic property to terrestrial tannins.

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Rumen adaptation to feed additives such as tannins, fats, and chemical inhibitors, has been well studied; however, knowledge about the ruminal adaptation dynamics to seaweed supplementation is still required. Therefore, the objective of this study was to examine the effect of the three red seaweeds *Asparagopsis taxiformis*, *Mazzaella japonica*, and *Palmaria mollis* on *in vitro* fermentation and gas production, and evaluate the adaptation response of fermentation characteristics to seaweed supplementation within a rumen simulation technique (RUSITEC) system fed barley straw and silage diet.

Material and methods

The experiment was conducted at Agriculture and Agri-Food Canada in Lethbridge, AB, Canada. Donor heifers used in this experiment were cared for in accordance with the guidelines of the Canadian Council on Animal Care (2009).

Seaweed

The three seaweeds used in this experiment included *A. taxiformis*, *M. japonica*, and *P. mollis*. Seaweeds were chosen based on their biomass availability, harvest potential, and biochemical composition. *A. taxiformis* was chosen based on its previously observed characterisation to manipulate rumen fermentation and CH_4 production and was assigned as a positive control. The novel seaweeds *M. japonica* and *P. mollis* are wild harvested off the coast of British Columbia, Canada; *M. japonica* mainly for its carrageenan content and *P. mollis* for human consumption.

Experimental Design and Treatments

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The experiment was conducted as a completely randomized design with four treatments, duplicated in two identical RUSITEC apparatus equipped with eight fermenter vessels each. The four treatments included the control (no added seaweed), and three red seaweeds (*A. taxiformis, M. japonica, P. mollis*) included at 2% diet DM. The substrate consisted of a 50:50 barley straw and barley silage diet (DM basis). The chemical composition of the substrates and seaweeds are shown in Table 1.

The experimental period was divided into four phases; a baseline phase (d 0-7) where fermenters were only fed the diet substrate and measurements were only recorded on d 5-7; the adaptation phase in which seaweed was introduced into the allocated diets and measurements were recorded from d 8-11; the intermediate phase (d 12-16); and the stable phase (d 17-21) to assess changes in rumen fermentation and microbial populations in response to feeding seaweed.

Substrate Processing

Barley straw, barley silage, and seaweeds were ground through a 4 mm screen using a Wiley mill (standard model 4; Arthur H. Thomas Co., Philadelphia, PA, USA). A total of 10 g diet DM was fed to each fermenter daily in bags (10 × 20 cm; 50 ± 10 µ porosity; R1020, ANKOM Technology, Macedon, NY, USA). For the control and during the baseline phase, 5 g DM each of barley straw and barley silage were included in the bags. For seaweed treatments, 0.2 g DM of seaweed replaced equal proportions of barley straw and barley silage.

Inoculum Sampling and Incubation Procedure

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Two RUSITEC apparatuses each fitted with eight fermenters (920 mL) were used for the *in vitro* incubation, so that each treatment was randomly allocated to two fermenters within each apparatus (*n* = 4 vessels/treatment). Each fermenter was fitted with a site for artificial saliva infusion and effluent output. Rumen inoculum was obtained from 3 ruminally cannulated beef heifers previously adapted for two weeks to a barley straw and barley silage diet which included a mineral supplement. Rumen fluid and solid contents were pooled from the 3 heifers, filtered through four layers of cheesecloth, and transported to the laboratory in an insulated thermos. Fermenters were maintained at 39°C by immersion in a water bath. Each fermenter was pre-filled with 180 mL of pre-warmed McDougall's buffer (pH = 8.2; McDougall, 1948) and 720 mL of strained rumen fluid.

One R1020 bag (ANKOM Technology) containing 20 g of mixed solid rumen digesta, and one bag containing 10 g DM of the diet were allocated to each fermenter. After 24 h, the bag containing rumen digesta was replaced by a bag containing the diet. Thereafter, one bag was replaced daily so that each bag remained in the fermenter for 48 h. Bags containing seaweed treatments were introduced into the fermenters on d 8.

The artificial saliva was continually infused into the fermenters using a peristaltic pump set to achieve a dilution rate of 2.9%/h. Effluent was collected in a 1 L flask, and gas was collected in a 2 L gas tight bag (Curity® ; Conviden Ltd., Mansfield, MA, USA) attached to the effluent flask. Feed bag exchange, fermenter pH, gas production, and effluent volume were measured every 24 h at 10 am.

Nutrient Disappearance

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Dry matter digestibility (DMD) was determined from d 3 to 21 after 48 h of fermentation. Feed bags were removed, washed in cold running water for 2 min, and dried at 55°C for 48 h (AOAC, 1995; method 930.15) for determination of DMD. After drying, residues were pooled from d 9 to 11, and d 17 to 19, ground through a 1 mm screen (Wiley mill, standard model 4; Arthur H. Thomas Co., Philadelphia, PA, USA) and analysed for organic matter (OM), NDF, CP, and ether extract (EE) concentrations.

Samples were dried at 550°C for 5 h and OM was calculated as 100 – ash (AOAC, 1995; method 942.05). The NDF content was determined using an ANKOM200 Fibre Analyser based on the procedure described by Van Soest et al., (1991) using sodium sulphite and α-amylase as reagents and expressed exclusive of residual ash. Total N concentration was quantified by flash combustion with gas chromatography and thermal conductivity detection (Carlo Erba Instruments, Milan, Italy (AOAC, 2005); method 990.03). The CP content was calculated as N concentration × 6.25. Fat was determined according to AOAC (2006; method 2003.05) using ether extraction (Extraction Unit E-816 HE; Büchi Labortechnik AG, Flawil, Switzerland).

Gas Production

Total gas production was determined daily using a gas meter (Model DM3A, Alexander-Wright, London, England, UK). A 20 mL sample was collected from the septum of the collection bag using a 26-gauge needle and transferred to a pre-evacuated exetainer (6.8 mL; Labco Ltd., Wycombe, Buckinghamshire, UK). Concentrations of CH_4, O_2, H_2, and CO_2 were determined using a gas chromatograph equipped with a with a

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GS-Carbon-PLOT (30m × 0.32 mm × 3 mm) column and thermal conductivity detector (Agilent Technologies Canada, Inc., Mississauga, ON, Canada) at an isothermal oven temperature of 35°C, with He as the carrier gas (27 cm/s).

Fermentation Variables

Effluent volume and fermenter pH was recorded daily at the time of feed bag exchange. Two, 5 mL effluent samples were placed in vials prefilled with 1 mL of 25% (wt/vol) metaphosphoric acid and 1 mL of 1% (wt/vol) sulphuric acid for analysis of volatile fatty acid (VFA) composition and NH_3 concentration, respectively. Sample vials were kept at -20°C until analysis.

The concentration of VFA were determined by gas chromatography (5890A Series Plus II, Hewlett Packard Co., Palo Alto, CA) equipped with a 30-m Zebron free fatty acid phase fused silica capillary (0.32-mm i.d., 1.0-µm film thickness; Phenomenex, Torrance, CA). The concentration of NH_3 was determined using the phenol-hypochlorite method as described by Broderick and Kang, (1980).

Elemental Analysis

The elemental analysis of seaweeds was conducted by a commercial laboratory (Cumberland Valley Analytical Services, Waynesboro, PA, USA). Phosphorus, Ca, Mg, K, Na, Fe, Mn, Zn, and Cu were determined using AOAC method 985.1 (Association of Official Analytical Chemists, 2000a) with modifications where seaweeds were ashed for 1 h at 535°C, digested in open crucibles for 20 min in 15% HNO_3 on a hot-plate, diluted to 50 mL, and then analysed using inductively coupled plasma. Samples used for Mo analysis were ashed at 480°C for 4 h, digested in an

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open crucible for 20 min in 15% HNO_3 on a hotplate, diluted to 50 mL, and then analysed on axial view inductively coupled plasma. Selenium was analysed using AOAC method 996.16 (Association of Official Analytical Chemists, 2000b). The bromoform concentration of the seaweed was conducted by a commercial laboratory (Bigelow Analytical Services, East Boothbay, ME, USA) using methanol extraction with samples analyzed by GC/MS.

Calculations and Statistical Analysis

The disappearance of DM, OM, NDF, and CP (DMD, OMD, NDFD, and CPD) was calculated as the difference between the nutrient content before and after incubation, and expressed as a percentage. Methane production was expressed as mg per g of DM digested (DMd) and incubated (DMi).

DMd = Total DMi – Total DM remaining after incubation

Data were analysed using the MIXED model procedure of SAS (SAS Inc., Cary, NC, USA). Individual fermenter was considered the experimental unit with the day of sampling treated as a repeated measure. Treatment, phase, and treatment × phase were considered as fixed effects while fermenter within vessel was considered as a random effect. Minimum values of Akaike's information criterion were used to select the covariance structure. Data were tested for normality of variance. Significance was declared at $P \leq 0.05$.

Results

There was a treatment × phase effect ($P \leq 0.05$) on OMD, NDFD, propionate, branched-chain VFA (BCVFA), caproate, valerate, acetate:propionate molar proportions, and total VFA production (Table 3). When averaged across all phases, DMD

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was greatest ($P < 0.01$) for *A. taxiformis* and lowest for *P. mollis*; however, neither were different from the control ($P > 0.05$). There was no effect ($P > 0.05$) of treatment on CPD, pH, or NH_3 production. Effluent output was greater ($P = 0.03$) in *P. mollis* than *A. taxiformis*, although neither were different from the control ($P > 0.05$). Both OMD ($P = 0.04$) and NDFD ($P = 0.05$) were decreased by *A. taxiformis* during the adaptation phase, but returned to control levels in the stable phase (Figure 2).

Molar proportions of acetate were decreased ($P < 0.001$) and butyrate increased ($P < 0.001$) by *A. taxiformis* compared with all other treatments. *A. taxiformis* decreased molar proportions of propionate ($P < 0.001$) during the adaptation, intermediate and stable phases with the decreases being largest in the intermediate and stable phases (Figure 3a). There was no effect of *M. japonica* or *P. mollis* on total VFA across any phase; however *A. taxiformis* had less ($P = 0.01$) total VFA during the intermediate and stable phases compared with the other treatments. The acetate:propionate was higher in *A. taxiformis* than *M. japonica* and *P. mollis* treatments during adaptation, although it was not different from the control during this phase (Figure 3b). *A. taxiformis* increased ($P < 0.001$) molar proportions of caproate, valerate, and BCVFA during the intermediate and stable phase, with no difference detected during the adaptation phase (Figure 3b).

There was no effect ($P \geq 0.34$) of treatment on gas or O_2 production (% or mL; Table 4). *A. taxiformis* decreased ($P < 0.05$) CO_2 (%, mL/d) production compared *to P. mollis*, but neither was different from the control ($P > 0.05$). There was a treatment × phase effect ($P < 0.001$) for CH_4 (%, mL/d, mg/d, mg/g DMd, mg/g DMi) and H_2 (%, mL/d). Control, *M. japonica*, and *P. mollis* had similar CH_4 production across all

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phases (Figure 4a,b), whereas *A. taxiformis* decreased ($P < 0.001$) CH_4 production across adaptation, intermediate and stable phases compared with other treatments. The production of H_2 (%, mL/d) was increased ($P < 0.001$) across the adaptation, intermediate and stable phases by *A. taxiformis*, with the intermediate and stable phases having greater H_2 production than the adaptation phase (Figure 5).

Discussion

This study examined the effect of three red seaweeds on fermentation, nutrient digestibility, and gas production within a RUSITEC fed a roughage based diet. The novelty in this study was the examination of the adaptation of the fermentation system to the introduction of three different seaweeds (*A. taxiformis*, *M. japonica*, *P. palmate*). A 7-d period was allocated for system adaptation as well as examining the fermentation parameters before the introduction of seaweeds. Thereafter, the measurements were divided into the adaptation (d 8-11), intermediate (d 12-16), and stable phases (d 17-21) to evaluate the changes that occurred after administrating the seaweeds until the system and the microbial population became more stabilised.

The lack of difference in DMD between the control and each seaweed demonstrates the ability of the microbial population to effectively degrade seaweed carbohydrates, with all treatments improving in DMD across different phases indicating that the microbial population became more efficient over time. Compared with DMD, OMD and NDFD were more sensitive to the addition of *A. taxiformis* when introduced to the fermenters, given the initial decrease in both variables during the adaptation phase. The disruption to the fermentation of feed due to the introduction of *A. taxiformis* may have been caused by the radical suppression of CH_4, the increase in H_2, and an

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overall shift in the metabolome requiring adaptation of the microbiome. Conversely, the recovery of OMD and NDFD in the stable phase indicated that only a relatively short period of time was required for the rumen microbial community to adapt to the presence of *A. taxiformis*. This short disruption in feed degradation was unique to *A. taxiformis* and was not observed with either *M. japonica* or *P. mollis*.

The suppression of CH_4 production by *A. taxiformis* has been previously documented by both *in vitro* and *in vivo* studies (Machado et al., 2015; Li et al., 2018; Roque et al., 2019b; Roque et al., 2019a; Abbott et al., 2020; Kinley et al., 2020). The present study also observed a rapid drop in CH_4 production with *A. taxiformis* inclusion throughout the adaptation, intermediate, and stable phases compared to the control. The drop in CH_4 production (mL/g DMd) compared to the control was 80.2, 93.7, 95.1% over the adaptation, intermediate and stable phases, respectively. Although not significantly different between these phases, the results suggest that there was an adaptation period to the introduction of *A. taxiformis*, with the CH_4 suppressing effect increasing over time with continued *A. taxiformis* addition. However, within a RUSITEC system it is frequent to observe reductions in certain populations of the microbiota as the length of the experiment increases. For example, Mateos et al., (2017) found that in solid associated samples, protozoal DNA concentration and abundance of *Fibrobacter succinogenes*, *Ruminococcis albus*, and fungi decreased, and the abundance of methanogenic archaea increased over a 14 d period within a RUSITEC, despite relatively stable fermentation variables (Mateos et al., 2017). The changes in microbiota over time may also explain differences observed within the same treatment over time, because although there were significant decreases in CH_4 metrics observed throughout phases for *M. japonica* and *P. mollis*, within each phase

 doi:10.20944/preprints202208.0186.v1

they were not different from the control. The lack of effect of these two species on CH_4 production is reinforced by consistent H_2 production observed across all phases. Similarly, in a companion study in our laboratory using a batch culture technique (unpublished data), we observed that neither *M. japonica* nor *P. mollis* had an effect on *in vitro* fermentation or CH_4 production in forage based diets. The ineffectiveness of these two seaweeds on reducing CH_4 production is likely due to the lack of concentrations of bromoforms in these species. Bromoform has been verified as the effective component in some seaweeds that inhibits enteric CH_4 production (Kinley et al., 2020).

The impact of including *A. taxiformis* within a RUSITEC has been previously evaluated; however, measurements were only taken from immediate addition of the seaweed and at 4, 12 and 24 h intervals each day over a period of 4 days in that study (Roque et al., 2019a). Roque et al., (2019a) observed that 5% OM inclusion rate of *A. taxiformis* decreased CH_4 production by 95%, with the suppression of CH_4 production observed at the first sampling that was conducted 28 h after seaweed introduction into the system. Methane production in that study was almost zero after 76 h of incubation, although no further measurements were observed past this time. In contrast, our experiment showed that the ability of *A. taxiformis* to decrease CH_4 improved over time with some CH_4 production still observed (~1.35 mg/d) during the final phase. The decrease in CH_4 production from *A. taxiformis* in the present study is consistent with an increase in H_2 production over phases, with the greatest CH_4 suppression observed in intermediate and stable phases resulting in more H_2 production than in the adaptation phase.

 doi:10.20944/preprints202208.0186.v1

Although total gas production was not affected by seaweed treatment, *A. taxiformis* caused a reduction in total VFA production in the adaptation, intermediate and stable phases, demonstrating a reduction in microbial degradation of nutrients. Organic matter is degraded by the rumen consortium, generating VFA, the main source of energy provided to ruminants. Iso-acids are products from the degradation of valine, isoleucine, leucine, and proline that are used in the biosynthesis of higher BCVFA (Andries et al., 1987). The BCVFA are required for optimal fibre degradation and efficiency of ruminal fermentation (Firkins, 2021). The increase in these intermediates (butyrate, caproate, BCVFA, valerate) corresponds with the decreased production of acetate and propionate indicating that *A. taxiformis* inhibited major VFA synthesis. Other chemical CH_4 inhibitors that have resulted in large decreases (7-29%) of CH_4 have also been found to result in increased production of valerate and isovalerate (Chung et al., 2011; Martínez-Fernández et al., 2014), consistent with the hypothesis that increased rumen H_2 favours the fermentation pathways that consume H_2, including valerate and caproate (Ungerfeld, 2015; Guyader et al., 2017).

Inhibiting CH_4 production can theoretically increase the availability of H_2 for incorporation into VFA, thereby increasing energy availability to the animal (Johnson and Johnson, 1995; Ungerfeld, 2015). Yet, despite the increase in H_2 production that accompanied the decrease in CH_4 production for the *A. taxiformis* treatment in the present study, total VFA were not increased. Furthermore, an *in vitro* study found that at 1, 2, and 5% OM inclusion of *A. taxiformis*, total VFA were decreased by 16.6, 25.0 and 39.5%, respectively (Machado et al., 2015). Machado et al., (2015) also observed alterations in the molar proportions of VFA with propionate, butyrate, valerate, and isovalerate increasing and acetate and isobutyrate decreasing compared with

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the control. In contrast, Roque et al., (2019a) did not observe a significant decrease in total VFA with *A. taxiformis*, but did find a decrease in the acetate:propionate ratio and valerate production, in comparison with the current study where valerate production increased with the inclusion of *A. taxiformis*. Valerate along with caproate are intermediary VFA and the increase with *A. taxiformis* is related to its lack of incorporation into the three main VFA, indicating an inefficiency of fermentation possibly brought by the decrease in H_2 incorporation into CH_4 (Andries et al., 1987). The increase in BCVFA, valerate and caproate may also indicate that ruminal microbial growth is not optimised in the presence of *A. taxiformis*, as these VFA are essential for cellulolytic bacteria growth, which may contribute towards the reduced NDFD during adaptation.

In conclusion, this study found that *M. japonica* and *P. palamata* did not impact rumen fermentation or exhibit CH_4 suppressing capacity. In contrast, *A. taxiformis* was shown to be an effective CH_4 suppressant with its immediate addition causing negative alterations to fermentation variables, with the large magnitude of CH_4 suppression inhibiting VFA synthesis. These findings may indicate that feeding *A. taxiformis* to ruminants at a dose rate that results in a large decrease in CH_4 production may alter rumen metabolism in a manner that restricts production performance.

Ethics approval

Donor cows used in this experiment were cared for in accordance with the guidelines of the Canadian Council on Animal Care (Canadian Council on Animal Care, 2009).

Data and model availability statement

The data set is not available for public access.

Author contributions

Stephanie Terry: Formal analysis, Writing – Original Draft, Writing – Review and Editing; **Ana Kruger:** Methodology, Investigation, Writing – Review and Editing; **Rob Gruninger:** Methodology, Writing – Review and Editing; **Wade Abbott:** Methodology, Writing – Review and Editing; **Karen Beauchemin:** Conceptualization, Methodology, Writing – Review and Editing, Supervision, Funding acquisition

Declaration of interest

None.

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Figure 1. Outline of experiment dosing and sampling

	Baseline (no seaweed)								Adaptation seaweeds				Intermediate phase					St	
Days	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**1**
Seaweed included									X	X	X	X	X	X	X	X	X	X	X
Gas production, pH, effluent vol.		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
DM disappearance			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
OM, CP, and NDF Disappearance										Samples Pooled								Samples	
VFA, NH_3-N						X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1. Chemical composition of ingredients used in a rumen simulation technique (RUSITEC) to examine the effect of seaweeds on in vitro fermentation and methane production

Ingredient	DM, %	OM, %DM	CP, %DM	NDF, %DM	EE, %DM
Barley silage	92.5	90.9	4.5	45.2	2.91
Barley straw	96.8	91.8	5.04	77.7	1.57
Asparagopsis taxiformis	96.2	52.6	18.4	50.3	0.37
Mazzaella japonica	19.9	71.3	20.7	66.9	0.43
Palmaria mollis	12.4	61.2	21.5	35.6	0.34

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Abbreviations: DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fibre; EE = ether extract

Table 2. Elemental analysis of the seaweeds used in a rumen simulation technique (RUSITEC) to examine the effect of seaweeds on in vitro fermentation and methane production

	Asparagopsis taxiformis	Mazzaella japonica	*Palmaria mollis*
Macro-minerals, mg/kg			
Ca	37397	4486	2626
K	14397	26432	108198
Mg	7012	9815	4143
Na	56641	56936	28389
P	1671	3022	4186
S	19149	77430	8782
Trace elements, mg/kg			
Al	7893.4	57.7	254.3
B	121.9	69.4	254.6
Co	2.5	<1.0	<1.0
Cr	22.8	7.8	4.5
Cu	4.8	1	2.3
Fe	5934	314	588
I	2580	15.7	58.1
Mn	93.6	14.2	13.6
Mo	2.1	1	0.5
Sb	<5.0	<5.0	<5.0
Se	<10.0	<10.0	<10.0
Zn	24.3	23.2	25.9
Toxic heavy metals, mg/kg			
As	16.1	9.7	9.7
Ba	9.2	0.3	0.9
Cd	<0.5	1	2.2
Hg	<10.0	<10.0	<10.0
Pb	<2.5	<2.5	<2.5
Tl	<10.0	<10.0	<10.0

Table 3. Effect of seaweed on in vitro nutrient degradability, pH, volatile fatty acid and ammonia production in a RUSITEC

	Control	*Asparagopsis taxiformis*	*Mazzaella japonica*	*Palmaria mollis*	SEM	Treat	Phase
DMD, %	48.8^{ab}	51.2^{a}	48.8^{ab}	46.9^{b}	0.78	<0.01	<0.001
OMD, %	53.4	50.0	55.3	55.9	1.44	0.05	0.21
CPD, %	54.2	52.8	54.0	56.1	1.06	0.21	0.13
NDFD, %	34.0	28.1	36.8	36.7	2.04	0.03	0.06
Effluent, mL	648.1^{ab}	633.4^{b}	645.1^{ab}	650.4^{a}	4.23	0.03	0.03
pH	6.9	6.9	6.9	6.9	0.01	0.91	<0.001
VFA, mol/100 mol							
Acetate	53.8^{a}	49.3^{b}	53.5^{a}	54.1^{a}	0.60	<0.001	<0.001
Propionate	26.2	21.7	25.9	26.4	0.33	<0.001	<0.001
Butyrate	13.0^{b}	16.8^{a}	13.2^{b}	12.6^{b}	0.56	<0.001	<0.001
BCVFA	1.22	1.63	1.21	1.27	0.021	<0.001	<0.001

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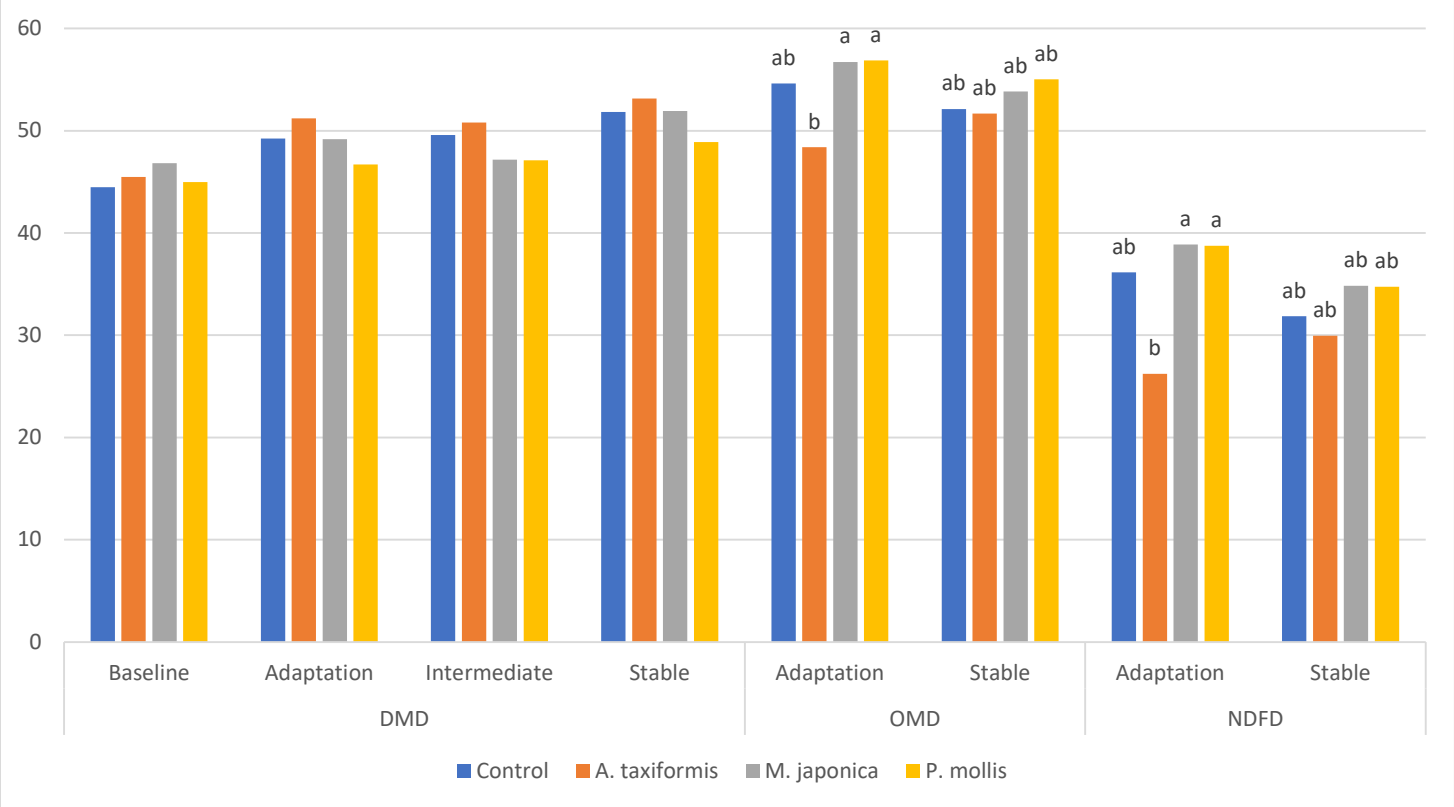
Caproate	0.64	1.75	0.69	0.65	0.080	<0.001	<0.001
Valerate	5.15	7.72	5.24	4.84	0.422	<0.001	<0.001
Total VFA, mmol/d	64.3	59.7	65.5	66.5	2.14	0.16	0.01
A:P	2.08	2.29	2.04	2.06	0.034	<0.001	<0.001
NH_3, mmol/d	3.31	3.16	3.28	3.40	0.087	0.30	<0.001

DMD, dry matter digested; OMD, organic matter digested; CPD, crude protein digested; NDFD, neutral detergent fibre digested; VFA, volatile fatty acids; BCVFA, branched-chain VFA; A:P, acetate:propionate

[a-c]Within a row, treatment means without a common superscript differ ($P \leq 0.05$).

Figure 2. The effect of seaweed and phase on disappearance of dry matter (DMD), organic matter (OMD) and neutral detergent fibre (NDFD) in a RUSITEC



[a-b]Within variable, means without a common superscript differ ($P \leq 0.05$); variables without letters do not have a significant ($P > 0.05$) treatment × phase effect.

Figure 3. The effect of seaweed and phase on the production of a) total volatile fatty acids, propionate, and valerate, and b) caproate, branched-chain volatile fatty acids (BCVFA), and acetate:propionate

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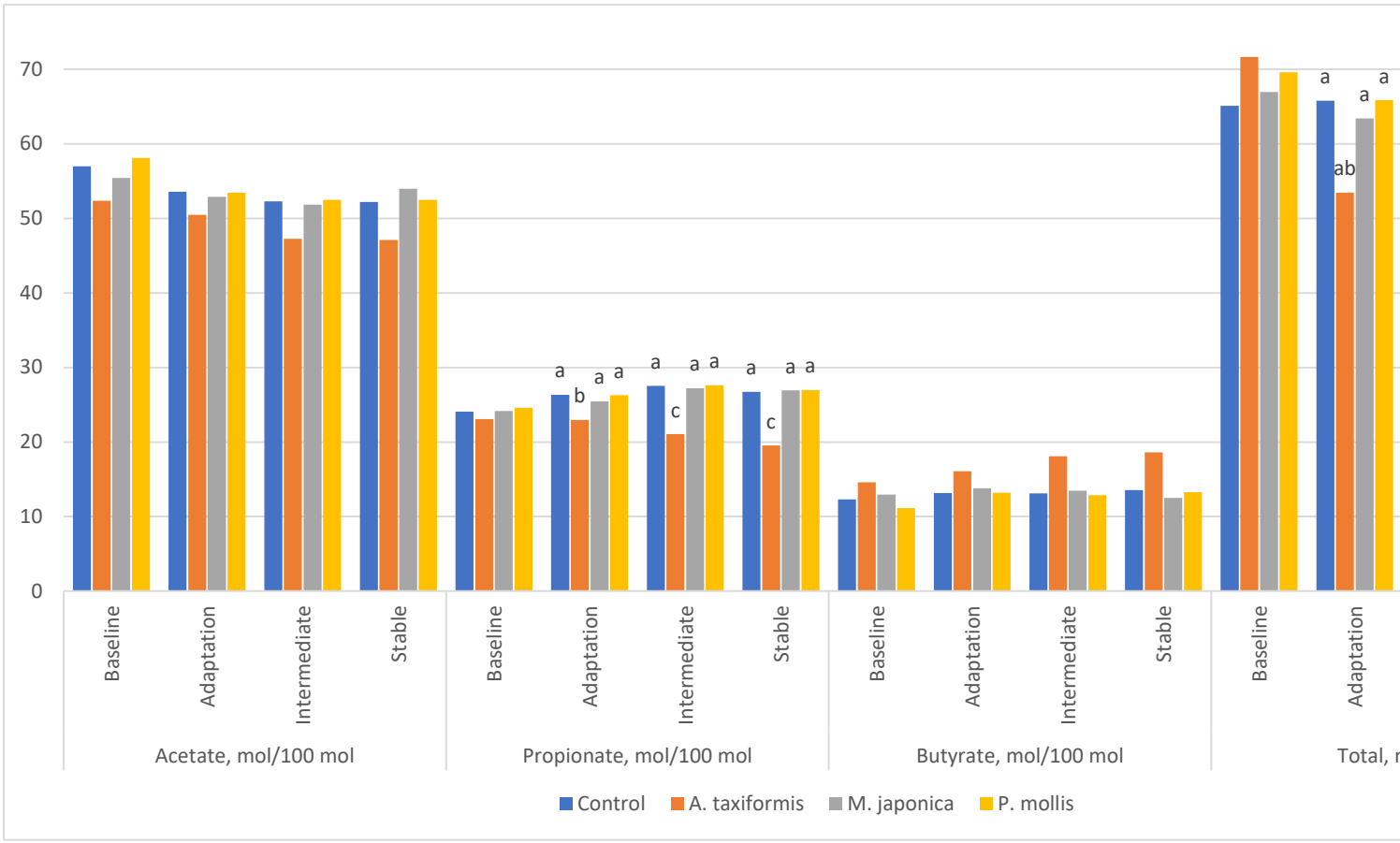

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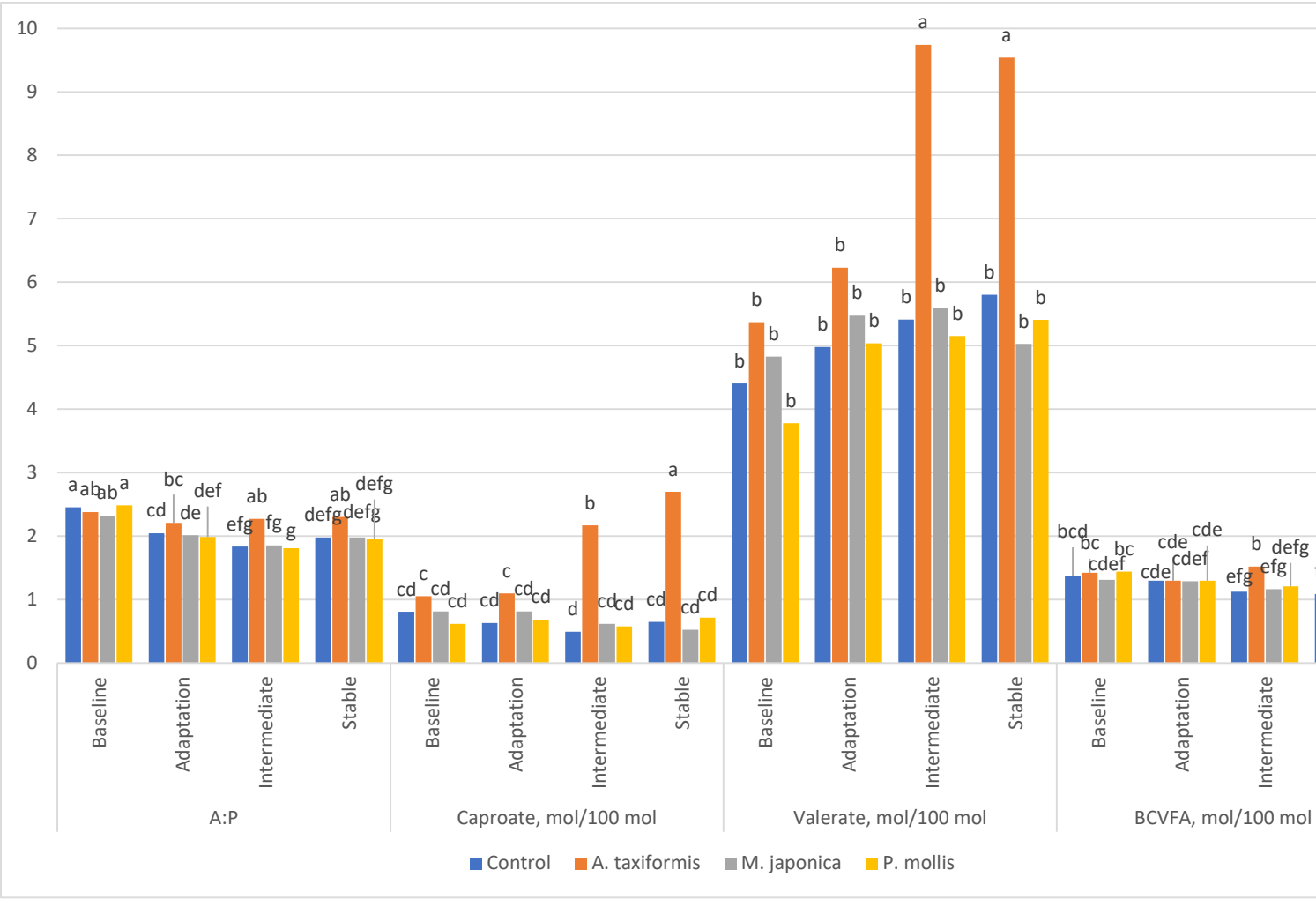

[a-g]Within variable, means without a common superscript differ ($P \leq 0.05$); variables without letters do not have a significant ($P > 0.05$) treatment × phase effect.

Table 4. Effect of seaweed on in vitro gas production in a RUSITEC

	Control	*Asparagopsis taxiformis*	*Mazzaella japonica*	*Palmaria mollis*	SEM	Treat	Phase	T×P
Gas, mL/d	1450.2	1436.2	1523.8	1649.7	115.03	0.55	<0.001	0.61
CH_4, %	3.75	1.45	3.60	4.08	0.238	<0.001	<0.001	<0.001
CH_4, mL/d	59.0	20.2	51.8	65.9	4.59	<0.001	<0.001	<0.001
CH_4, mg/d	41.6	13.9	36.7	45.9	3.06	<0.001	<0.001	<0.001
CH_4, mg/g DMd	8.00	2.80	6.91	8.59	0.450	<0.001	<0.001	<0.001
CH_4, mg/g DMi	4.15	1.39	3.67	4.58	0.306	<0.001	<0.001	<0.001
CO_2, %	27.7^{ab}	25.0^{b}	26.2^{ab}	29.8^{a}	1.12	0.05	<0.001	0.24
H_2, %	0.48	1.90	0.55	0.49	0.053	<0.001	0.06	<0.001
O_2, %	13.7	14.3	14.0	13.2	0.41	0.34	<0.001	0.28
CO_2, mL/d	416.7^{ab}	317.1^{b}	386.3^{ab}	474.8^{a}	26.55	0.01	<0.001	0.50
H_2, mL/d	8.08	24.52	8.32	8.06	1.086	<0.001	0.72	<0.001
O_2, mL/d	203.7	194.6	210.5	210.4	11.10	0.72	0.03	0.39

DMd, dry matter digested; DMi, dry matter incubated

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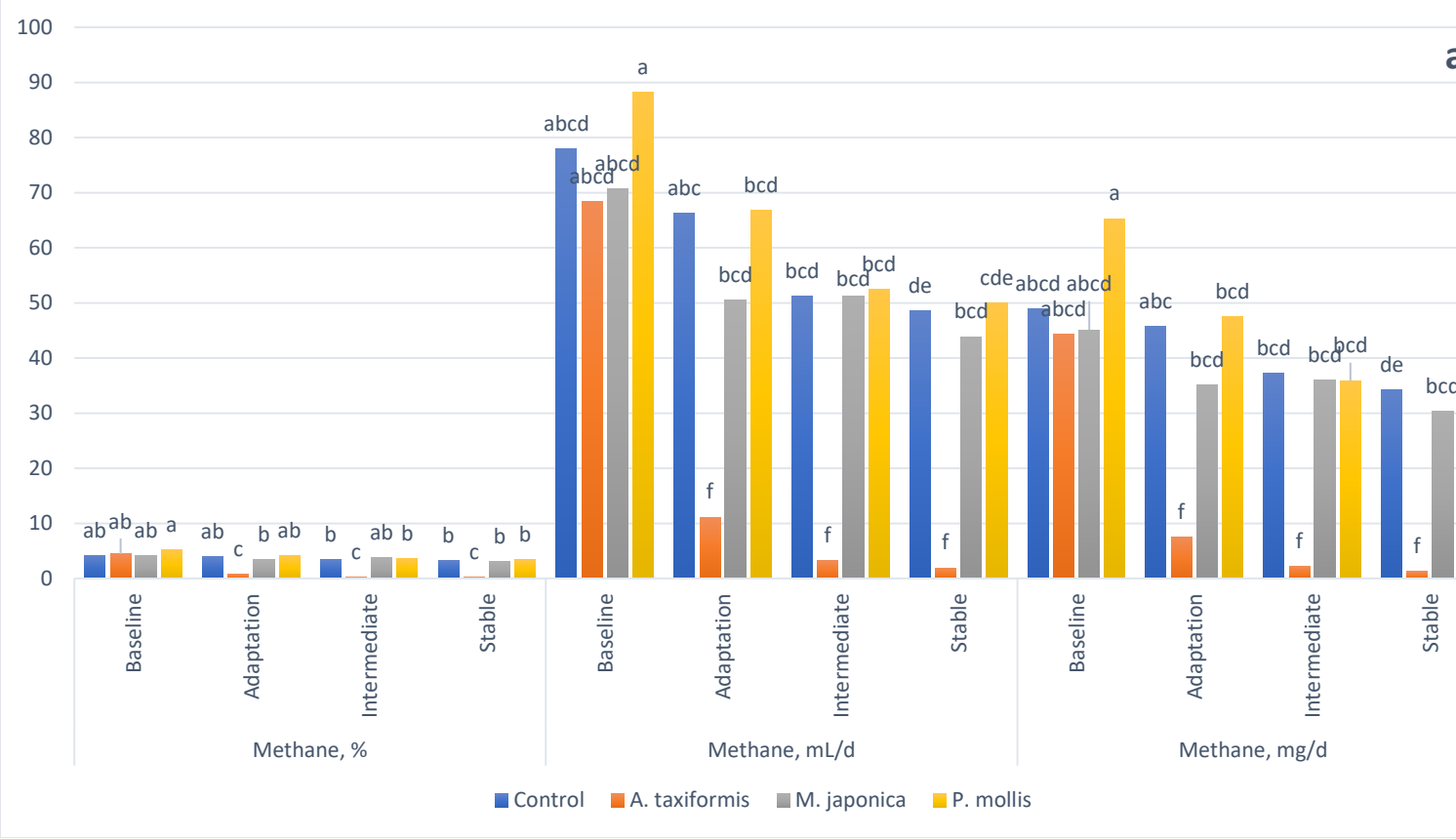
[a-c]Within a row, treatment means without a common superscript differ ($P \leq 0.05$).

Figure 4. The effect of seaweed and phase on the a) percentage of CH_4, and CH_4 production (mL/d, mg/d), and b) CH_4 production on a dry matter disappearance (mg/g DMd) and dry matter incubated basis (mg/g DMi)



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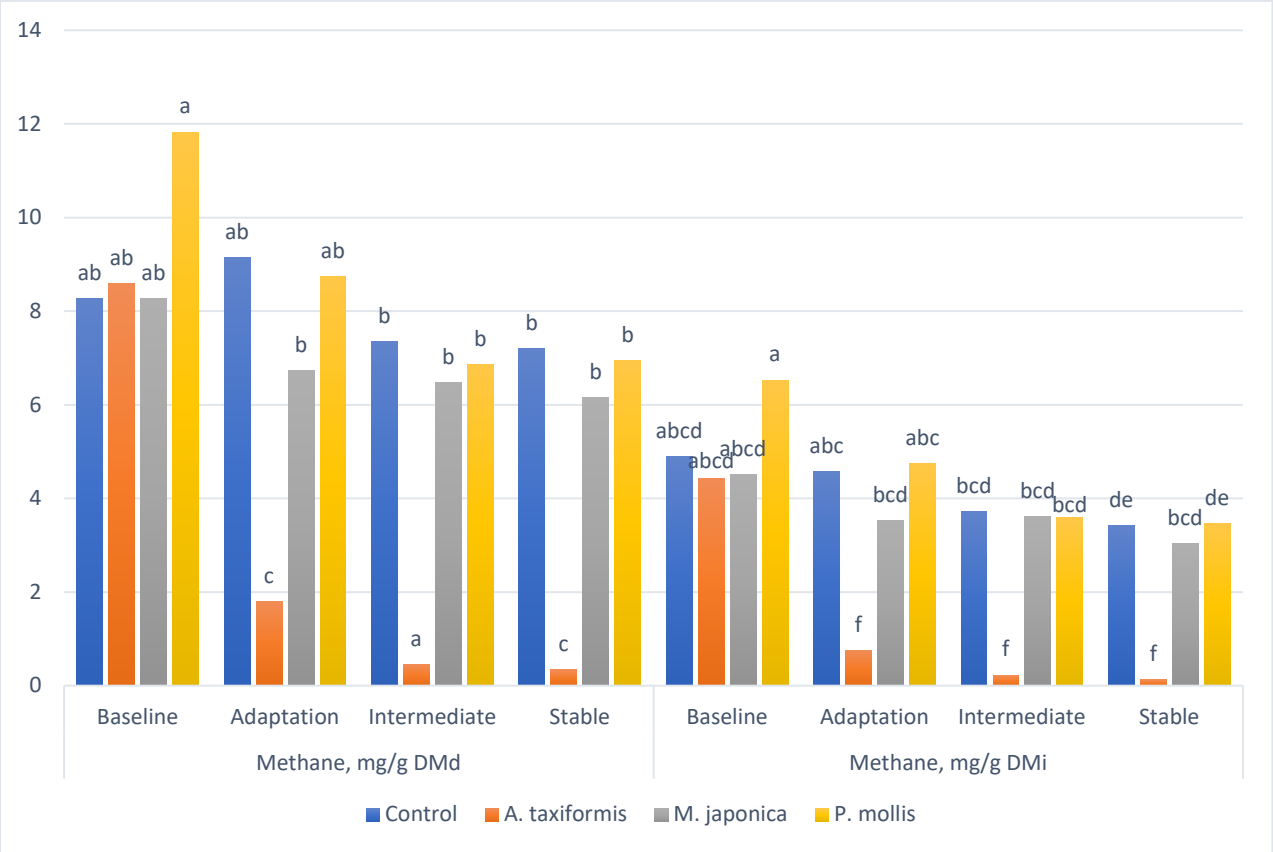

[a-f]Within variable, means without a common superscript differ ($P \leq 0.05$).

Figure 5. The effect of seaweed and phase on the percentage and production of H_2 within a RUSITEC

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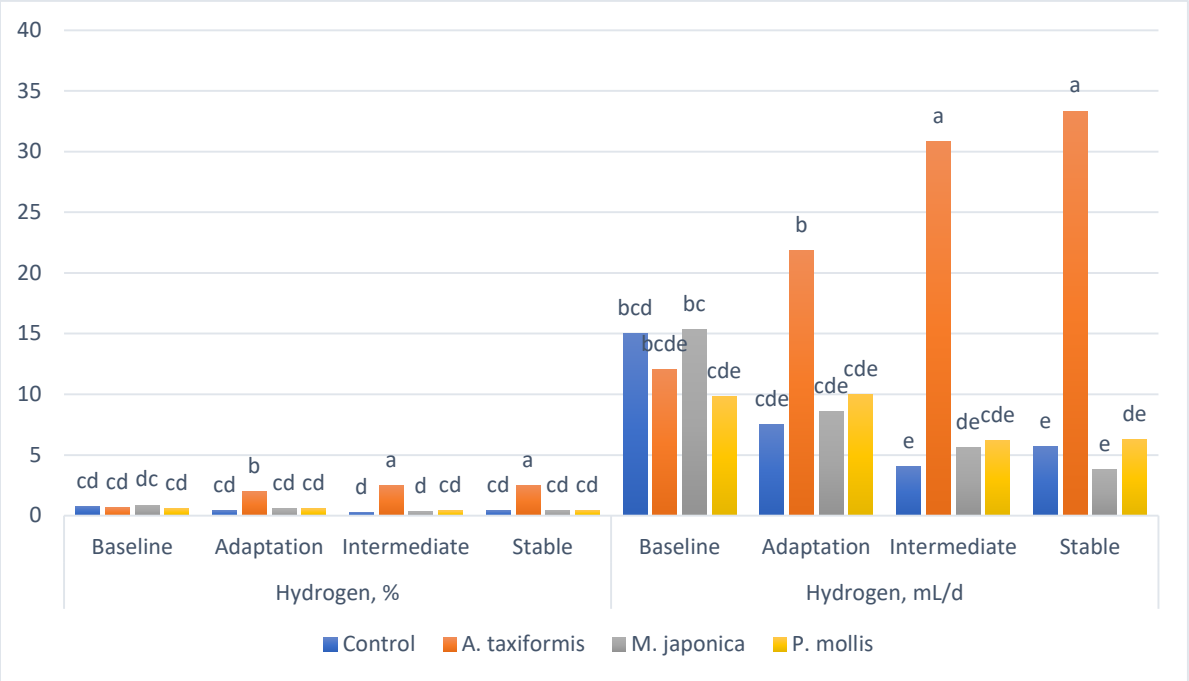

[a-e]Within variable, means without a common superscript differ ($P \leq 0.05$).