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In vitro evaluation of utilisable crude protein and methane production for a diet in which grass silage was replaced by different levels and fractions of extracted seaweed proteins

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ABSTRACT

Utilisable crude protein (uCP), methane (CH_4) production and other fermentation parameters were analysed *in vitro* for a diet in which grass silage was replaced by different levels of seaweed protein fractions prepared from three seaweed species: *Saccharina latissima*, *Alaria esculenta* and *Palmaria palmata*. Ten fractions from these three species in which the protein content had been increased and the salt content reduced by simple processing were tested, with inclusion levels in the diet based on the nitrogen content of the fractions. Following an extraction procedure, four fractions from *Saccharina latissima*, three from *Alaria esculenta* and one from *Palmaria palmata*, were incrementally included in the diet by replacing high quality silage with approximately 0, 0.15, 0.30 and 0.45 g/g DM, while two high-protein fractions of *Palmaria palmata* were tested at replacement levels of 0, 0.075, 0.15 and 0.225 g/g DM. To estimate fermentation parameters, 500 mg of each diet were incubated in bottles with 60 mL buffered rumen fluid. Estimated uCP increased linearly with increasing replacement rate of grass silage with seaweed protein fractions (from 158 g/kg DM to 206 g/kg DM on average for all fractions). Increasing protein fraction from the brown seaweed *Saccharina latissima* in the diet significantly increased true organic matter digestibility (OMD) (from on average 0.786 to 0.821). Organic matter digestibility decreased with increasing level of *Alaria esculenta* fractions (from on average 0.785 to 0.733), which also gave a linear decrease in CH_4 production (from on average 45.3 to 38.5 mL/g organic matter). As a result of decreased CH_4 production and OMD, total volatile fatty acid concentration decreased with increasing level of *Alaria esculenta* fractions (from on average 69.5 to 63.0 mmol/L). Thus, positive and species-specific effects of seaweed on estimated uCP and fermentation parameters were observed *in vitro* when protein fractions remaining after an extraction procedure on seaweed partly replaced grass silage in the feed ration.

Abbreviations: CP, crude protein; CH_4, methane; DM, dry matter; NH_3N, ammonia; MCP, microbial crude protein; N, nitrogen; OMD, organic matter digestibility; uCP, utilisable crude protein; RUP, rumen undegradable protein; VFA, volatile fatty acids

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1. Introduction

A large and diverse seaweed flora exists world-wide, but only a few seaweed species have been tested as animal feeds (Makkar et al., 2016). However, seaweeds were used as livestock feed thousands of years ago in Ancient Greece (Makkar et al., 2016). Seaweed silage was first reported in the 1900s, as a way of preserving the biomass, and used to feed cattle, sheep and other ruminants during winter (Evans and Critchley, 2014). The use of seaweeds to feed livestock, especially among farmers in coastal areas, has recently gained momentum in relation to seaweed aquaculture and the use of marine resources as alternative protein sources (Skjermo et al., 2014). Seaweeds are rich in carbohydrates and minerals, while the protein content varies between the species (Bjarnadóttir et al., 2018). The varying biochemical composition has to be taken into account when using seaweed as feed for ruminants (Makkar et al., 2016; Tayyab et al., 2016; Gaillard et al., 2018). The increased demand for food world-wide has intensified the search for alternative protein sources and, since some seaweed species are rich in protein, they could act as an alternative protein source in livestock production (Lamminen et al., 2018). However, before seaweeds can be widely used in animal nutrition, more knowledge is needed about their potential for improving animal health and for reducing the environmental impacts of livestock production. For example, it is important to know the available and utilisable crude protein (uCP) content in different seaweed species. Moreover, seaweeds in the feed may be able to inhibit methane (CH_4)-producing microbes and thus reduce CH_4 emissions from ruminant production, which would be a major benefit considering the significant amounts of CH_4 produced by ruminants as part of their normal digestion (Kinley et al., 2016).

Use of unprocessed or raw seaweed biomass, singly or as a mixture of different species, as feed for ruminants has been studied extensively (*e.g.* Ventura and Castañón, 1998; Tayyab et al., 2016; Molina-Alcaide et al., 2017; Gaillard et al., 2018). However, the large brown algae (kelps) have low protein content (50–150 g/kg DM) (Angell et al., 2016) and high salt content, which exceeds 40% of dry weight when harvested in spring (Schiener et al., 2015). This may limit the acceptable inclusion levels in animal feed. We therefore included a simple processing step to increase the protein concentration and reduce the salt content in seaweed biomass.

To our knowledge, use of protein fractions remaining after extraction procedures on seaweeds as a feed component in the diet of ruminants has only been reported in one previous study (Özkan Gülzari et al., 2019), a companion paper based on the work reported here.

In vitro techniques are widely used in animal nutrition research, since *in vivo* experiments are very expensive, time-consuming and laborious. Many *in vitro* techniques have been developed to study ruminant nutrition and fermentation processes and to estimate uCP content and CH_4 production (Edmunds et al., 2012; Ramin and Huhtanen, 2012; Molina-Alcaide et al., 2017). Utilisable crude protein is an estimate of metabolisable protein of both feed and microbial origin. The objective of this study was to evaluate the effects on estimated uCP and other fermentation parameters of replacing grass silage with different levels of seaweed protein fractions. The hypothesis tested was that uCP and CH_4 production are dependent on seaweed species, degree of processing, protein content of the fractions and inclusion level in the diet.

2. Materials and methods

2.1. Sample preparation

Three seaweed species, the red *Palmaria palmata* and the brown *Saccharina latissima* and *Alaria esculenta*, hereafter referred to as *Palmaria*, *Saccharina* and *Alaria*, respectively, were used in this study. Wild *Palmaria* biomass was harvested in Bodø, Norway, while cultivated *Saccharina* and *Alaria* biomass was harvested off the coast of Trøndelag, Norway. For *Palmaria*, collected biomass was processed by removing epiphytes and associated species, both flora and fauna, and then briefly rinsing with fresh water to remove surface salts. After draining off surface water, the damp biomass was vacuum-packed and frozen at −20 °C until processing. For the brown seaweeds (*Saccharina* and *Alaria*), harvested biomass was drained of surface seawater and stored in plastic bags at −20 °C until processing. The processing steps applied to seaweed biomass to obtain the different fractions are illustrated in Fig. 1. In brief, frozen *Palmaria* biomass was milled and added to a stirred tank containing temperate water at 30 °C, in a ratio of one part wet biomass to three parts water. Xylanase (Sigma X2629) was used to help solubilise the biomass and release soluble compounds. After incubation at pH 4.5–5.0 and 30 °C for 5 h, the biomass slurry was centrifuged in a continuous centrifuge. Frozen brown seaweed biomass (*Saccharina and Alaria*) was milled and heat-treated (70–80 °C, 10 min) before transfer to a stirred tank. The heat treatment was included in order to prevent bacterial growth during overnight incubation. Cold water was added until the temperature reached 27 °C (wet biomass:water 1:1) and the pH was adjusted to 7.6. An alginate lyase was added to partly hydrolyse alginate and thereby facilitate solid-liquid separation by centrifugation. After incubation for 15 h at 22–25 °C, the biomass slurry was centrifuged in a continuous centrifuge.

The centrifuge sludges from all three biomass types were collected and air-dried (25–30 °C) to give washed biomass fractions of *Palmaria*, *Saccharina* and *Alaria* (P2, S2 and A2, respectively). For *Saccharina*, dried (at 60–70 °C), unprocessed biomass was also included (fraction S1). Unprocessed biomass and parts of the processed biomass (P2, S2 and A2) were further treated with a protease (Alcalase®, Novozymes) in order to solubilise more of the protein, giving fractions P5, S5 and A5 from unprocessed biomass and S6, A6 and P6 from processed fractions. The moisture content in the fractions was determined gravimetrically after drying at 105 °C until constant weight of fractions was achieved (typically 24 h). Ash content was determined after heating dry fractions at 600 °C for 12 h. Total nitrogen (N) was determined by the CHNS-O elemental combustion system (Costech Instruments ECS 4010) as described by Stévant et al. (2018). Laminaran and mannitol content were determined by high performance liquid chromatography, according to Sandbakken et al. (2018). The polyphenolic content of algal extracts was determined colorimetrically using Folin-Ciocalteu reagent

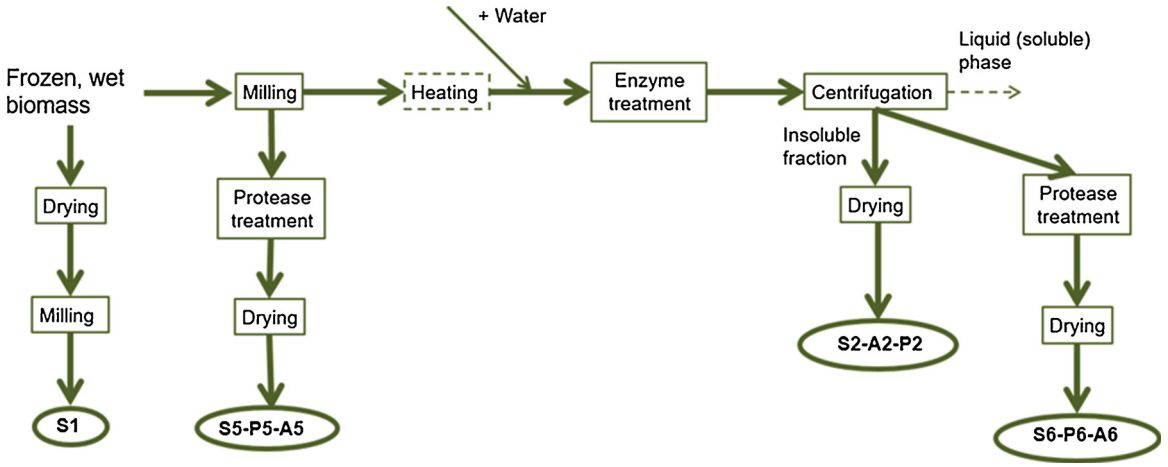

Fig. 1. Process flow used to obtain different protein-rich fractions from seaweed biomass. Seaweed species and fractions are *Palmaria palmata* P2, P5 and P6; *Saccharina latissima* S1, S2, S5 and S6; and *Alaria esculenta* A2, A5 and A6. Enzyme treatments are xylanase and alginate lyase for *P. palmata, and S. latissima* and *A. esculenta*, respectively.

according to the method of Ragan and Glombitza (1986). The chemical composition of the fractions is shown in Table 1. The level of seaweed fraction inclusion in the diet was based on the N content of the fractions (Table 1). Ten protein fractions from the three seaweed species were tested. Four fractions from *Saccharina* (S1, S2, S5 and S6), three from *Alaria* (A2, A5 and A6) and one from *Palmaria* (P5) were used to replace high-quality grass silage in a diet at a rate of 0, 0.15, 0.30 and 0.45 g/g dry matter (DM) (approximately 15, 30 and 45%). Two high-protein fractions of *Palmaria* (P2 and P6) were tested at a replacement level of 0, 0.075, 0.15 and 0.225 g/g DM (approximately 7.5, 15 and 22.5%). Due to differences in N concentration between the fractions, the DM proportion in the different diets differed slightly.

2.2. *In vitro* gas production measurements

The *in vitro* analyses were performed at the Swedish University of Agricultural Sciences, Umeå, Sweden. All handling of animals was approved by the Swedish Ethical Committee on Animal Research, represented by the Court of Appeal for Northern Norrland in Umeå. Three dairy cows of the Swedish Red breed, fed a total mixed ration (grass silage/concentrate ratio 600/400 g/kg on DM basis), were used as donor animals of rumen inoculum. Rumen fluid was collected 2 h after the morning feeding. Rumen fluid from each cow was strained separately through a double layer of cheesecloth into pre-warmed thermos flasks that had previously been flushed with carbon dioxide (CO_2). The rumen fluid was then strained through four layers of cheesecloth and mixed with buffered mineral solution supplemented with peptone (pancreatic digested casein) at 39 °C under constant stirring and continuous flushing with CO_2. Prior to *in vitro* incubation, 500 mg of substrate (OM incubated) were weighed into serum bottles and flushed with CO_2. Then 60 mL of previously prepared buffered rumen fluid were added to each bottle using a dispenser and the bottles were placed in a water bath and continuously agitated at 39 °C for 48 h. The procedure was replicated in three consecutive runs on three different days.

2.3. Methane production and estimated utilisable crude protein

The method of Ramin and Huhtanen (2012) was used to measure CH_4 production. In brief, at the end of the fermentation (48 h) process, 200 µL of gas were withdrawn from the headspace of each serum bottle with a gas-tight syringe and injected into a gas chromatograph. Samples of liquid were taken at 8, 16, 24 and 30 h during incubation in order to determine ammonia concentration, information needed in estimating uCP (sum of feed undegraded protein and microbial cells) at 16 h according to the method of Edmunds et al. (2012):

$$\mathrm{uCP}\left(\frac{\mathrm{g}}{\mathrm{kg}}\mathit{of\ DM}\right) = \frac{(\mathrm{NH3\ Nblank} + \mathrm{Nsample} - \mathrm{NH3Nsample})}{\text{sample weight (mg of DM)}} \times 6.25 \times 1000$$

where NH_3Nblank is the average amount (mg) of NH_3-N in the blanks at each time point, Nsample is the amount (mg) of nitrogen in the original substrate and NH_3Nsample is the amount (mg) of NH_3-N in the liquid phase in the bottle at each time point.

After 48 h (end of incubation), liquid samples were taken for volatile fatty acid (VFA) analysis. Fermentation was terminated by removing all bottles from water baths and placing them on ice. The residues in each bottle were then filtered through 11-µm nylon bags (Saatifil PES; Saatitech S.p.A., Veniano, Como, Italy) and later used for analysis of true organic matter digestibility (OMD), according to Mertens (2002). Blank corrections were also made for *in vitro* OMD within runs, by subtracting blank *in vitro* OMD values from the values obtained for samples. Organic matter digestibility was determined by taking into account the OM of individual feeds

Table 1
Chemical composition of the grass silage and fractions of each seaweed species used in the *in vitro* diet.

Feed/Fraction	Description	Dry matter, g/kg	Nitrogen,g/kg DM	Organic matter, g/kg DM	Laminaran[1] g/kg DM	Mannitol g/kg DM	Polyphenols[2] g/kg DM
Grass silage	Fermented timothy grass	897	25.1	841	–	–	–
Alaria esculenta (**A2**)	Pellet after enzyme (alginate lyase) treatment and centrifugation	937	31.0	789	6.5	5.0	5.3
Alaria esculenta (**A5**)	Protease-treated biomass, freeze-dried	927	25.9	731	11.5	9.9	5.1
Alaria esculenta (**A6**)	Protease-treated A2 biomass, freeze-dried	960	29.0	800	7.6	5.2	5.0
Palmaria palmata (**P2**)	Pellet after enzyme (xylanase) treatment and centrifugation	956	77.0	911	–	–	3.8
Palmaria palmata (**P5**)	Protease-treated biomass, freeze-dried	963	41.0	829	–	–	7.6
Palmaria palmata (**P6**)	Protease-treated P2 biomass, freeze-dried	955	78.1	913	–	–	9.1
Saccharina latissima (**S1**)	Dried and milled biomass	917	23.0	581	1.3	10.7	5.3
Saccharina latissima (**S2**)	Pellet after enzyme (alginate lyase) treatment and centrifugation	973	45.9	714	2.1	0.22	3.5
Saccharina latissima (**S5**)	Protease-treated biomass, freeze-dried	963	21.9	595	2.2	10.1	8.4
Saccharina latissima (**S6**)	Protease-treated S2 biomass, freeze-dried	970	35.0	761	0.6	2.6	9.7

[1] Measured as glucose.
[2] Concentration expressed as phloroglucinol equivalent for *Alaria esculenta and Saccharina latissima* and as gallic acid equivalent for *Palmaria palmata*.

and the residues left after incubation.

2.4. Statistical analysis

The data were analysed using the GLIMMIX procedure of SAS 9.4 (SAS Institute, Cary, NC). Seaweed fraction and the level of the fraction in the diet (g/g DM) were treated as fixed effects and test run (1–3) as random effect. As the DM proportion of seaweed fractions in the diet varied, it was treated as a continuous variable in order to test for differences between fractions at different levels of inclusion. In addition, the quadratic effect of the dietary proportion and the interactions between fraction and linear and quadratic effects of dietary proportion were included as a fixed effect in the model if significant. For uCP, the model was also run with the N concentration in the diet as a continuous fixed effect instead of DM proportion. Least square (LS-) means were used to obtain estimated means at DM inclusion levels used in the trial, *i.e.* from 0 to 0.5 g/g DM, and Tukey-Kramer *post hoc* test comparison was used to test for differences between the estimated means.

3. Results

3.1. Chemical composition

The red seaweed *Palmaria* had a higher N concentration than the brown seaweeds and its protein-enriched fractions (P2 and P6) had a higher N concentration (77.0 and 78.1 g/kg DM, respectively) and lower ash content than the other fractions (Table 1). The *Saccharina* S5 fraction had the lowest N concentration (21.9 g/kg DM) of all fractions, including the grass silage (Table 1). The *Saccharina* fractions had the highest ash values, as can be observed from their low organic matter content (581–761 g/kg DM) (Table 1).

3.2. Effects on uCP and fermentation parameters

A significant overall quadratic increase in estimated uCP for all inclusion levels was observed when the different seaweed protein fractions were used to replace grass silage in the diet (Table 2, Fig. 2). The highest value was observed for the *Saccharina* and *Palmaria* fractions (Table 2). The strongest effect on uCP of increasing dietary N was observed for the *Palmaria* P5 fraction, while the responses were similar for the *Saccharina* fraction S2 and the *Palmaria* fractions P2 and P6 (Fig. 2).

On average, there was no linear effect on OMD of increasing level of seaweed protein fraction in the diet, but inclusion of some fractions increased OMD linearly. This was evident as a significant fraction × level interaction (Table 3). For example, digestibility increased linearly with increasing level of *Saccharina* fractions (Table 3), whereas digestibility decreased linearly with increasing level of *Alaria* fractions (Table 3). The relationship between diet OMD and CH_4 production is shown in Fig. 3. When *Saccharina* fractions were used to replace grass silage in the diet, OMD tended to increase while CH_4 production decreased (Fig. 3). The relative CH_4 production was lower from *Saccharina* than from *Palmaria* (Fig. 3). On average, total gas production increased linearly with increasing level of seaweed fraction (Table 4). Increasing level of *Alaria* fractions tended to lower CH_4 production, whereas increasing level of other fractions did not show any inhibitory effect (Table 5). Methane production decreased slightly with increasing proportion of *Saccharina* fractions (Table 5). Total VFA content tended to decrease linearly with increasing level of all seaweed species and fractions in the diet (Table 6). However, the decrease was more pronounced with increasing level of the *Alaria* fractions (Table 6). A significant linear trend was obtained for acetate and propionate, indicating an increase or decrease in these two VFA proportions

Table 2
Effect of increasing levels of different seaweed fractions in a grass silage-based diet (g/g dry matter (DM)) on estimated utilisable crude protein (uCP, g/kg DM). The fractions are defined in Table 1.

Fraction	Level												P-value[1]				
	0		0.1		0.2		0.3		0.4		0.5						
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE[2]	F	L	F × L	Q	F × Q
A2	157	9.7	153	9.2	158^{b}	9.4	171^{abc}	9.3	194^{bc}	9.3	225^{a}	10.2	0.014	< 0.01	0.15	< 0.01	0.01
A5	158	9.7	150	9.3	150^{b}	9.4	158^{abc}	9.4	173^{cde}	9.2	194^{bc}	9.9					
A6	157	9.7	153	9.2	156^{b}	9.4	167^{abc}	9.3	186^{bcd}	9.3	213^{b}	10.3					
P2	157	9.7	177	9.4	238^{a}	9.4	–		–		–						
P5	157	9.7	159	9.2	169^{b}	9.4	186^{ab}	9.3	212^{ab}	9.3	246^{a}	10.5					
P6	157	9.7	174	9.4	231^{a}	9.4	–		–		–						
S1	158	9.7	150	9.3	147^{b}	9.4	150^{c}	9.4	158^{de}	9.2	171^{bc}	9.3					
S2	158	9.7	162	9.3	172^{b}	9.4	190^{a}	9.4	216^{ab}	9.2	248^{a}	9.8					
S5	158	9.7	145	9.3	139^{b}	9.4	138^{c}	9.4	144^{e}	9.2	155^{c}	9.4					
S6	157	9.7	149	9.2	149^{b}	9.4	157^{bc}	9.3	173^{cd}	9.2	198^{b}	10.0					

[abcd]Different superscript letters within columns indicate significant differences between estimates with Tukey's adjustment.
[1] F: Fraction; L: Linear; Q: Quadratic.
[2] SE: standard error.

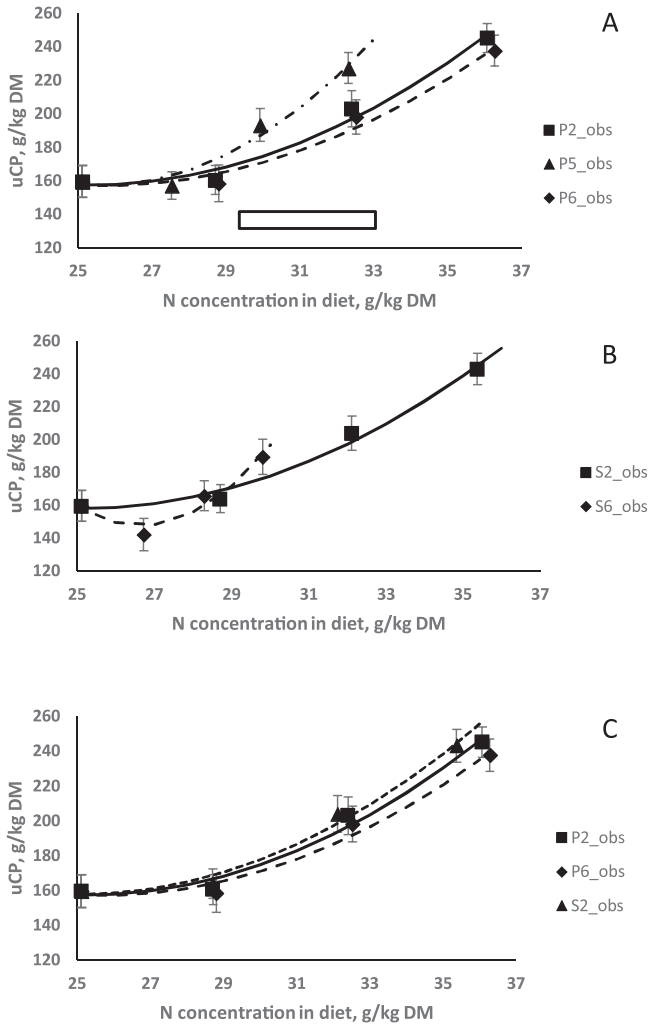

Fig. 2. Predicted utilisable crude protein (uCP) content (g/kg dry matter (DM)) with increasing nitrogen (N) concentration (g/kg DM) in the diet for fractions of different seaweed species: (A) *Palmaria palmata* fractions (P2, P5 and P6); (B) *Saccharina latissima* fractions (S2 and S6); and (C) *Palmaria palmata* fractions P2 and P6 compared with *Saccharina latissima* fraction S2. Observed (obs) mean values, bars indicate standard error. The horizontal box in (A) indicates the range of level in the diet at which P5 differed significantly ($P < 0.05$) from P2, P6 and S2. S2, P2 and P6 did not differ significantly. Different fractions are defined in Table 1.

with increasing level of seaweed fraction (Tables 7 and 8). In most cases, acetate increased, while the proportion of propionate decreased, with increasing level of seaweed fraction in the diet (Tables 7 and 8). On average, there was no linear effect on the proportion of butyrate with increasing level of seaweed fraction (Table 9). The effect on pH was very small and in most cases an increasing level of seaweed fractions did not influence the final pH (Table 10).

4. Discussion

This study evaluated the effects on uCP and other *in vitro* fermentation parameters of replacing grass silage in a ruminant diet with protein-enriched fractions from seaweeds. Inclusion of extracted protein fractions from seaweeds improved digestibility and to some extent reduced CH_4 production. The majority of the 10 fractions tested from three different seaweed species showed increased uCP when grass silage was replaced with increasing level of extracted protein fraction in the *in vitro* diet.

Use of raw seaweeds in animal diets has been investigated in previous *in vitro* and *in situ* experiments (Tayyab et al., 2016; Molina-Alcaide et al., 2017; Gaillard et al., 2018). The degradability of protein in intact seaweeds differs, with some seaweeds containing considerably higher amounts of rumen undegradable protein, but showing higher digestibility in the intestine.

The red seaweed species have a higher protein content, as demonstrated by Tayyab et al. (2016). However, individuals of these species, *e.g. Palmaria*, have relatively small structural morphology, making collection of biomass challenging. Moreover, red seaweeds are not cultivated or harvested on industrial scale for feed production in Europe. On the other hand, the brown seaweed species, known as kelps, are structurally large and are currently cultivated around the European coast and harvested on an industrial scale for

Table 3
Effect of increasing levels of different seaweed fractions in a grass silage-based diet (g/g dry matter) on true organic matter digestibility (OMD). The fractions are defined in Table 1.

Fraction	Level												P-value[1]				
	0		0.1		0.2		0.3		0.4		0.5						
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE[2]	F	L	F × L	Q	F × Q
A2	0.783	0.009	0.771	0.0078	0.759^{c}	0.0072	0.747^{c}	0.0073	0.734^{b}	0.0082	0.722^{c}	0.0096	< 0.01	0.85	< 0.01	ns	ns
A5	0.784	0.009	0.778	0.0079	0.772^{bc}	0.0072	0.766^{bc}	0.0073	0.761^{b}	0.0080	0.755^{bc}	0.0093					
A6	0.788	0.009	0.775	0.0078	0.761^{c}	0.0072	0.748^{c}	0.0073	0.734^{b}	0.0082	0.721^{c}	0.0097					
P2	0.785	0.009	0.782	0.0072	0.778^{abc}	0.0087	–	–	–		–						
P5	0.789	0.009	0.798	0.0078	0.807^{a}	0.0072	0.816^{a}	0.0074	0.825^{a}	0.0083	0.834^{a}	0.0098					
P6	0.790	0.009	0.790	0.0072	0.790^{abc}	0.0087	–	–	–		–						
S1	0.786	0.009	0.795	0.0081	0.804^{a}	0.0073	0.813^{a}	0.0072	0.821^{a}	0.0076	0.830^{a}	0.0084					
S2	0.779	0.009	0.786	0.0079	0.793^{ab}	0.0072	0.800^{a}	0.0072	0.807^{a}	0.0079	0.814^{a}	0.0092					
S5	0.789	0.009	0.796	0.0081	0.803^{a}	0.0073	0.810^{a}	0.0072	0.817^{a}	0.0076	0.823^{a}	0.0085					
S6	0.776	0.009	0.781	0.0079	0.787^{abc}	0.0072	0.792^{ab}	0.0073	0.798^{a}	0.0081	0.803^{ab}	0.0094					

^{abcd}Different letters within columns indicate significant differences between estimates with Tukey's adjustment.
[1] F: Fraction; L: Linear; Q: Quadratic.
[2] SE: standard error.

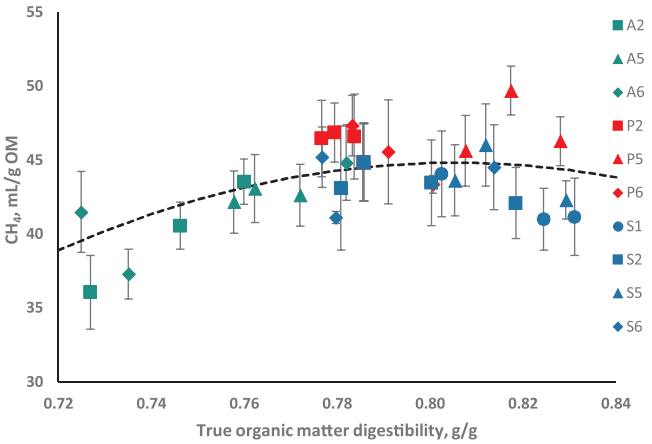

Fig. 3. Relationship between diet true organic matter digestibility (OMD) and methane (CH_4) production (mL/g organic matter): $CH_4 = -482$ (SE = 100.3) + 1309.1 (SE = 256.4, P < 0.001) × OMD – 812.8 (SE = 163.95, P < 0.001) × OMD × OMD. Symbols are observed mean values, horizontal bars are standard error. Different fractions are defined in Table 1. (Symbol colours can be seen in the online version of the diagram).

food, feed and other applications. However, they have low concentrations of protein.

In a recent study conducted *in situ* in dairy cows, it was shown that the red seaweed *Palmaria* can supply the rumen with high amounts of rumen-degradable protein and that another red seaweed, *Porphyra* sp., and the green seaweed *Ulva* sp. can be used as sources of bypass protein (Tayyab et al., 2016). On the other hand, the brown seaweed *Pelvetia canaliculata* showed low degradability in that study and is not recommended for feeding dairy cows (Tayyab et al., 2016). In another recent study, Gaillard et al. (2018) showed that *Palmaria* is a potential source of amino acids for ruminants, based on total amino acid concentration and degradability.

In the present study, we evaluated whether elevated protein content and reduced salt content in seaweeds improved their feed value. The two brown algae, *Saccharina* and *Alaria*, were heat-treated during processing, which could have affected uCP negatively by denaturation of soluble protein. Heat treatment has also been shown to increase the bypass of protein from the rumen in green protein (Chowdhury et al., 2018). However, on comparing the fractions S5 and S1 with their heat-treated, protein-enriched counterparts S6 and S2, respectively, it is evident that the processing had a positive impact on uCP. Likewise, the processing of *Palmaria* without heating (comparing the fractions P2 and P5), resulted in the highest relative increase in uCP. This indicates that it was the processing, and not the heat treatment, that increased uCP. Some fractions were also treated with a protease to solubilise more of the protein. This had no effect on uCP, as can be seen by comparing A6 and A2, P6 and P2, S6 and S2, and S5 and S1.

Protein evaluation systems aim to optimise the feed protein content and accurately predict the protein requirements of the animal (Schwab et al., 2005). The method used in the present study for estimation of uCP has been evaluated previously in a study by Gidlund et al. (2018), which showed that estimated uCP accurately ranked the diets in terms of omasal CP flow. In that study, the relationship between observed flow of CP and estimated uCP was relatively high ($R^2 = 0.87$), indicating that reliable estimates of uCP can be obtained using the gas *in vitro* system. Except for the P2 fraction, all other fractions tested here showed a substantial increase in

Table 4
Effect of increasing levels of different seaweed fractions in a grass silage-based diet (g/g dry matter) on total gas production (mL/g organic matter). The fractions are defined in Table 1.

Fraction	Level												P-value[1]				
	0		0.1		0.2		0.3		0.4		0.5						
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE[2]	F	L	F × L	Q	F × Q
A2	248	15.1	245	14.7	237	14.5	226[b]	14.6	211[b]	14.8	191[b]	15.4	0.014	< 0.01	< 0.01	< 0.01	ns
A5	245	15.1	251	14.7	252	14.6	249[ab]	14.6	243[ab]	14.7	232[ab]	15.2					
A6	251	15.1	249	14.7	243	14.5	233[ab]	14.6	219 [ab]	14.8	201 [ab]	15.4					
P2	247	15.1	256	14.5	260	15.0	–		–		–						
P5	247	15.2	258	14.7	265	14.6	269[a]	14.7	268[a]	15.1	264[a]	15.9					
P6	245	16.9	265	15.2	281	16.6	–		–		–						
S1	243	15.2	250	14.8	253	14.6	252[ab]	14.6	247 [ab]	14.6	238 [ab]	14.9					
S2	245	15.1	250	14.7	252	14.6	249[ab]	14.6	243 [ab]	14.7	232 [ab]	15.2					
S5	245	15.2	254	14.8	258	14.6	258[ab]	14.6	255 [a]	14.6	247 [ab]	14.9					
S6	244	15.1	248	14.7	249	14.5	245[ab]	14.6	238 [ab]	14.8	226 [ab]	15.3					

[abcd]Different superscript letters within columns indicate significant differences between estimates with Tukey's adjustment.
[1] F: Fraction; L: Linear; Q: Quadratic.
[2] SE: standard error.

Table 5
Effect of increasing levels of different seaweed fractions in a grass silage-based diet (g/g dry matter) on methane (CH_4) production (mL/g organic matter). The fractions are defined in Table 1.

Fraction	Level												P-value[1]				
	0		0.1		0.2		0.3		0.4		0.5						
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE[2]	F	L	F × L	Q	F × Q
A2	45.6	2.39	43.8	2.30	41.9	2.26	40.0	2.27	38.1	2.32	36.2	2.43	0.011	< 0.01	< 0.01	ns	ns
A5	44.3	2.39	43.8	2.30	43.4	2.26	42.9	2.26	42.4	2.31	41.9	2.40					
A6	46.0	2.39	44.3	2.30	42.6	2.26	41.0	2.27	39.3	2.33	37.6	2.43					
P2	45.3	2.39	46.1	2.26	47.0	2.36	–		–		–						
P5	45.4	2.41	45.8	2.31	46.2	2.27	46.7	2.30	47.1	2.38	47.5	2.52					
P6	44.3	2.39	45.2	2.26	46.1	2.36	–		–		–						
S1	44.9	2.40	44.2	2.32	43.4	2.27	42.6	2.26	41.8	2.28	41.0	2.34					
S2	44.5	2.39	44.1	2.31	43.6	2.26	43.1	2.26	42.6	2.31	42.1	2.39					
S5	45.7	2.40	45.1	2.32	44.6	2.27	44.1	2.26	43.5	2.28	43.0	2.35					
S6	44.9	2.39	44.7	2.31	44.6	2.27	44.4	2.27	44.3	2.33	44.1	2.43					

[1] F: Fraction; L: Linear; Q: Quadratic.
[2] SE: standard error.

Table 6
Effect of increasing levels of different seaweed fractions in a grass silage-based diet (g/g dry matter) on total volatile fatty acid concentration (mmol/L). The fractions are defined in Table 1.

Fraction	Level												P-value[1]				
	0		0.1		0.2		0.3		0.4		0.5						
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE[2]	F	L	F × L	Q	F × Q
A2	69.1	2.06	68.9	1.91	67.9	1.85	66.1	1.86	63.5	1.94	60.1	2.14	< 0.01	0.015	0.016	< 0.01	ns
A5	69.2	2.07	70.4	1.91	70.7	1.86	70.3	1.86	69.0	1.92	67.0	2.09					
A6	70.4	2.06	70.4	1.90	69.5	1.85	67.8	1.86	65.3	1.95	62.0	2.15					
P2	69.9	2.05	68.8	1.83	66.9	2.01	–		–		–						
P5	70.1	2.06	71.5	1.90	72.1	1.85	71.9	1.86	70.9	1.96	69.1	2.18					
P6	69.0	2.05	70.6	1.84	71.4	2.09	–		–		–						
S1	68.8	2.08	70.0	1.93	70.4	1.88	70.0	1.86	68.8	1.88	66.8	1.97					
S2	69.3	2.07	70.1	1.91	70.0	1.86	69.1	1.86	67.4	1.91	64.9	2.08					
S5	70.7	2.08	71.9	1.93	72.3	1.87	71.9	1.86	70.6	1.88	68.6	1.98					
S6	70.2	2.06	70.8	1.91	70.6	1.85	69.6	1.86	67.8	1.93	65.2	2.12					

[1] F: Fraction; L: Linear; Q: Quadratic.
[2] SE: standard error.

Table 7
Effect of increasing levels of different seaweed fractions in a grass silage-based diet (g/g dry matter) on proportion of acetate in total volatile fatty acids (mmol/mol). The fractions are defined in Table 1.

Fraction	Level												P-value[1]				
	0		0.1		0.2		0.3		0.4		0.5						
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE[2]	F	L	F × L	Q	F × Q
A2	683	4.2	687	4.2	690^{a}	4.1	692^{ab}	4.1	695^{ab}	4.2	697^{ab}	4.3	0.01	< 0.01	< 0.01	0.04	ns
A5	684	4.2	685	4.2	685^{a}	4.1	685^{bc}	4.1	685^{bc}	4.2	684^{bc}	4.3					
A6	683	4.2	686	4.2	689^{ab}	4.1	691^{ab}	4.1	693^{ab}	4.2	694^{ab}	4.3					
P2	683	4.2	687	4.1	690^{ab}	4.2	–		–		–						
P5	683	4.2	682	4.2	680^{b}	4.1	678^{c}	4.1	676^{c}	4.2	673^{c}	4.3					
P6	683	4.2	687	4.1	689^{ab}	4.3	–		–		–						
S1	683	4.3	687	4.2	690^{a}	4.1	693^{ab}	4.1	695^{ab}	4.1	697^{ab}	4.2					
S2	682	4.2	687	4.2	692^{a}	4.1	696^{a}	4.1	700^{a}	4.2	703^{a}	4.2					
S5	683	4.2	685	4.2	687$_{ab}$	4.1	689^{ab}	4.1	690^{ab}	4.1	691^{ab}	4.2					
S6	684	4.2	688	4.2	692^{a}	4.1	695^{a}	4.1	699^{a}	4.2	701^{a}	4.3					

^{abcd}Different superscript letters within columns indicate significant differences between estimates ($P < 0.05$) determined by Tukey's multiple comparison.
[1] F: Fraction; L: Linear; Q: Quadratic.
[2] SE: standard error.

Table 8
Effect of increasing levels of different seaweed fractions in a grass silage-based diet (g/g dry matter) on proportion of propionate in total volatile fatty acids (mmol/mol). The fractions are defined in Table 1.

Fraction	Level												P-value[1]				
	0		0.1		0.2		0.3		0.4		0.5						
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE[2]	F	L	F × L	Q	F × Q
A2	212	1.4	208^{ab}	1.3	205^{b}	1.2	202^{bc}	1.2	198^{bcd}	1.3	195^{bcd}	1.5	< 0.01	< 0.01	< 0.01	ns	ns
A5	211	1.4	209^{ab}	1.3	207^{b}	1.2	206^{b}	1.2	204^{b}	1.3	202^{b}	1.5					
A6	212	1.4	209 ^{ab}	1.3	206^{b}	1.2	202^{bc}	1.2	199^{bcd}	1.3	196^{bcd}	1.5					
P2	211	1.4	207^{b}	1.2	203^{b}	1.4	–		–		–						
P5	212	1.4	212^{a}	1.3	213^{a}	1.2	214^{a}	1.2	214^{a}	1.3	215^{a}	1.5					
P6	211	1.4	208^{ab}	1.2	204^{b}	1.5	–		–		–						
S1	213	1.5	210^{ab}	1.3	207^{b}	1.2	205^{bc}	1.2	202^{bcd}	1.2	199^{bcd}	1.3					
S2	212	1.4	208^{ab}	1.3	205^{b}	1.2	201^{bc}	1.2	198^{cd}	1.3	194^{cd}	1.4					
S5	212	1.5	210^{ab}	1.3	207^{b}	1.2	205^{b}	1.2	203^{bc}	1.2	200^{bc}	1.4					
S6	211	1.4	208^{ab}	1.3	204^{b}	1.2	200^{c}	1.2	194^{d}	1.3	193^{d}	1.5					

^{abcd}Different superscript letters within columns indicate significant differences between estimates ($P < 0.05$) determined by Tukey's multiple comparison.
[1] F: Fraction; L: Linear; Q: Quadratic.
[2] SE: standard error.

uCP relative to the increase in dietary N concentration, which indicates that the fractions tested are a potential source of good feed protein. Unfortunately, we did not have any information about the digestibility of uCP in the intestine, so we could not fully evaluate the protein value. When the S2 fraction was used to replace grass silage in the diet, it resulted in a relatively higher increase in uCP with increased N concentration in the diet than the other seaweed fractions. Basically, uCP is an estimate of the sum of microbial crude protein (MCP) and undegraded feed protein in the rumen (RUP) that enters the duodenum of the animal. While it is not possible to differentiate between these two sources, it is likely that the A2 fraction of *Alaria* species had a relatively high proportion of RUP, since OMD (digested organic matter) decreased with increasing amount of the A2 fraction in the diet.

Using the proportion of product as the independent variable and the undigested residues at each level of inclusion as the dependent variable, the regression model obtained for the A2 fraction was: $Y = 0.122x + 0.217$, where Y is indigestible organic matter. Solving this equation by assuming $x = 1$, the Y value for the A2 fraction was 0.339. The corresponding value for the *Saccharina* S2 fraction was 0.151. This clearly demonstrates that when the A2 fraction was included in the diet (at increasing levels), the amount of undigested organic matter was greater (lower OMD) than when the S2 fraction was included. Similarly, the *Palmaria* P5 fraction had less indigestible organic matter (0.121) than the A2 fraction, resulting in increased OMD at higher levels of this fraction in the diet. Based on these observations, and provided that organic matter is available for microbes to produce MCP, the increases in uCP determined in this study seem to be realistic. Molina-Alcaide et al. (2017) studied the effect of raw seaweeds on total polyphenol content, gas kinetics and rumen fermentation parameters in a batch culture system of rumen microorganisms *in vitro*. They found that total polyphenol values varied widely between seaweed species and between seasons (range 1.46–50.3 mg/g DM). The red seaweeds *Mastocarpus stellatus* and *Porphyra* sp. showed the highest DM effective degradability (range 424–652 g/kg), while the brown *Pelvetia*

Table 9
Effect of increasing levels of different seaweed fractions in a grass silage-based diet (g/g dry matter) on proportion of butyrate in total volatile fatty acids (mmol/mol). The fractions are defined in Table 1.

Fraction	Level												P-value[1]				
	0		0.1		0.2		0.3		0.4		0.5						
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE[2]	F	L	F × L	Q	F × Q
A2	105	4.8	105	4.7	105	4.7	106^{abc}	4.7	107^{ab}	4.7	109^{abc}	4.8	0.02	0.19	< 0.01	< 0.01	ns
A5	105	4.8	106	4.7	107	4.7	109^{a}	4.7	111^{a}	4.7	114^{a}	4.8					
A6	105	4.8	105	4.7	106	4.7	107^{abc}	4.7	108^{ab}	4.7	110^{abc}	4.8					
P2	105	4.8	106	4.7	108	4.7	–		–		–						
P5	105	4.8	105	4.7	106	4.7	108^{ab}	4.7	110^{a}	4.7	112^{ab}	4.8					
P6	105	4.8	106	4.7	107	4.8	–		–		–						
S1	105	4.8	103	4.7	103	4.7	103^{bc}	4.7	103^{b}	4.7	104^{b}	4.7					
S2	106	4.8	104	4.7	103	4.7	102^{c}	4.7	102^{b}	4.7	102^{b}	4.8					
S5	105	4.8	105	4.7	105	4.7	106^{abc}	4.7	107^{ab}	4.7	109^{abc}	4.7					
S6	105	4.8	105	4.7	104	4.7	104^{abc}	4.7	105^{ab}	4.7	106^{abc}	4.8					

^{abcd} Different superscript letters within columns indicate significant differences between estimates ($P < 0.05$) determined by Tukey's multiple comparison.
[1] F: Fraction; L: Linear; Q: Quadratic.
[2] SE: standard error.

Table 10
Effect of increasing levels of different seaweed fractions in a grass silage-based diet (g/g dry matter) on pH. The fractions are defined in Table 1.

Fraction	Level												P-value[1]				
	0		0.1		0.2		0.3		0.4		0.5						
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE[2]	F	L	F × L	Q	F × Q
A2	6.56	0.052	6.58	0.052	6.59	0.052	6.60	0.052	6.62	0.052	6.63	0.052	< 0.01	< 0.01	ns	ns	ns
A5	6.56	0.052	6.57	0.052	6.58	0.052	6.60	0.052	6.61	0.052	6.62	0.052					
A6	6.56	0.052	6.57	0.052	6.58	0.052	6.60	0.052	6.61	0.052	6.62	0.052					
P2	6.57	0.052	6.59	0.052	6.60	0.052	–		–		–						
P5	6.54	0.052	6.55	0.052	6.57	0.052	6.58	0.052	6.59	0.052	6.61	0.052					
P6	6.57	0.052	6.58	0.052	6.60	0.052	–		–		–						
S1	6.54	0.052	6.55	0.052	6.56	0.052	6.58	0.052	6.59	0.052	6.60	0.052					
S2	6.56	0.052	6.57	0.052	6.58	0.052	6.60	0.052	6.61	0.052	6.62	0.052					
S5	6.55	0.052	6.56	0.052	6.57	0.052	6.58	0.052	6.60	0.052	6.61	0.052					
S6	6.56	0.052	6.58	0.052	6.59	0.052	6.60	0.052	6.61	0.052	6.63	0.052					

[1] F: Fraction; L: Linear; Q: Quadratic.
[2] SE: standard error.

canaliculata and the green *Cladophora rupestris* (misidentified and previously reported as *Acrosiphonia* sp.) showed the lowest values (Molina-Alcaide et al., 2017). However, these results are not directly comparable to those obtained for extracted seaweed protein fractions in the present study.

Branched chain fatty acids (isoacids) are usually generated from degradation of branched chain amino acids, and their presence can be an indication of protein degradation or a balance between degradation and synthesis (Gaillard et al., 2018). In this study, there was a reduction in iso-valerate concentration with increasing level of only the *Alaria esculenta* species (data not shown). This is in line with findings by Molina-Alcaide et al. (2017) indicating that this species generates the lowest proportion of isoacids.

Overall, some of the seaweed protein fractions examined in this study seem to have great potential to increase uCP *in vitro*. The processing procedure, which increased the protein content and reduced the salt content of the seaweed biomass, was positive for all three species. However, future *in vivo* studies are needed to clarify the actual benefits of the extracted protein fractions in ruminants. Recently, Lamminen et al. (2018) demonstrated positive effects (compared with soya bean) of adding microalgae (*Spirulina platensis*, *Chlorella vulgaris* and *Nannochloropsis gaditana*) to the diet of dairy cows, as indicated by lower urinary N and urinary urea N excretion. These findings are supported by the quadratic rise in uCP with increased level of different seaweed protein fractions in the diet in the present study. This was related to the N concentration in the diet (Fig. 2) and could be attributable to better efficiency of N use by microbes, resulting in uCP, which could be beneficial for productivity in dairy cows. Lamminen et al. (2018) also found that, in line with milk production responses, one of the microalgae species tested (*Spirulina platensis*) resulted in the highest efficiency of N utilisation for milk production and the lowest milk urea N concentration. This suggests more efficient nutrient utilisation with this specific species than with the other macroalgae species tested in their study. A recent study by our research group demonstrated that all three seaweed species used in the present study pose a very low health risk from heavy metals when consumed by humans (Roleda

et al., 2019). Thus the risk when fed to animals is probably also low (Monagail et al., 2018). In *in vivo* studies, measurements of the iodine content are critical, due to its toxicity to the animals, whereas in *in vitro* studies it is not necessary to take iodine content into account. However, the three species investigated here are rich in iodine and the concentration is species-specific (*Saccharina* > *Alaria* > *Palmaria*), so the absolute content is important in determining the species-specific daily allowable consumption without adverse health effects (Roleda et al., 2018). Moreover, post-harvesting processes such as chemical treatment, drying, storage and transport are some challenges with the use of seaweed fractions in commercial diets that still need to be resolved.

4.1. Effects on CH_4 and VFA production

Reduced production of CH_4 by inclusion of seaweed in the diet has been reported by Kinley et al. (2016) for the red *Asparagopsis taxiformis* as a result of its bromoform content and by Wang et al. (2007) for the brown *Ascophyllum nodosum* for an unknown compound. We found that increased levels of *Palmaria* fractions did not have any inhibitory effect on CH_4 production or on VFA production. This is likely due to the absence of compounds, *e.g.* bromoform among others, that might contribute to inhibition of CH_4 production. Moreover, different seaweed species also differ markedly in their *in vitro* rumen degradation, which also varies seasonally as reported by Molina-Alcaide et al. (2017).

We observed a reduction in CH_4 production with increasing level of *Alaria* fractions. The main reason is the lower OMD, as shown in Fig. 3. The lower OMD of *Alaria* could also be due to their higher content of tannins (polyphenols), which are known to reduce digestibility and CH_4 production (Gemeda and Hassen, 2015). The polyphenol content was higher in *Alaria* species than in the other two species tested, regardless of collection site and season, as also found by Roleda et al. (2019). The effect of different inclusion levels of the seaweed protein fractions was determined by evaluating fermentation parameters such as VFA, pH and OMD. However, the interaction between treatment (protein fraction) and DM proportion was highly significant for CH_4, which means that the response depended on the seaweed fraction used. One side-effect of using seaweeds could be reduced digestibility. In the present study the *Alaria* fractions tended to decrease OMD, which is comparable to the effect of *Asparagopsis taxiformis* in inhibition of CH_4 production and in reducing digestibility (Kinley et al., 2016). A reduction in OMD with increasing level of *Alaria* protein fraction is also supported by a meta-analysis conducted by Ramin and Huhtanen (2013) showing a positive relationship between digestibility and CH_4 production. High OMD without increased VFA production may reflect increased microbial protein and/or reduced degradability or hydrolysis of some carbohydrates in the solution used for determination of OMD, but not fermented during the *in vitro* incubation. On average, the effect of increased level of protein fractions on OMD was not significant (see Table 3). Similarly, Lamminen et al. (2018) found that OM digestibility was not affected by the algae supplements used in their study. Methane production was closely related to total gas production, with a prediction error in regression analysis of only 2.6% of the mean. The pH measured at the end of the incubation was within the optimum range for microbial growth and nutrient degradation (Wales et al., 2004). The increase in pH with increasing level of *Alaria* fractions was consistent with a decrease in ruminal total VFA concentration, since ruminal pH has been shown to be negatively related to total VFA concentration in a meta-analysis of ruminant studies (Kolver and De Veth, 2002).

5. Conclusions

Positive effects on uCP and other *in vitro* fermentation parameters were obtained by replacing grass silage with different protein fractions isolated from three seaweed species, and these positive effects were species-specific (*Saccharina* > *Palmaria* > *Alaria*). Higher values of uCP were achieved with increasing levels of protein-enriched and salt-reduced fractions of *Saccharina* (S2) and *Palmaria*. The reduction in CH_4 production was more pronounced with inclusion of *Alaria* protein fractions than with protein fractions from the other seaweed species. This indicates that the main reason for increased CH_4 production is the increased amount of OM fermented. The increase in estimated uCP observed when grass silage was partly replaced by seaweed protein fractions remaining after an extraction procedure warrants further study in *in vivo* experiments on ruminants.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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