J. Dairy Sci. TBC
https://doi.org/10.3168/jds.2024-24988

Potential of 2 Northern European brown seaweeds (*Fucus serratus* and *Fucus vesiculosus)* as enteric methane inhibitors in dairy cows

M. Thorsteinsson,[1]†* É. Chassé,[1]† M. V. Curtasu,[1] M. Battelli,[2] A. Bruhn,[3,4] A. L. F. Hellwing,[1] M. R. Weisbjerg,[1] and M. O. Nielsen[1]
[1]Department of Animal and Veterinary Sciences, AU-Viborg, Research Centre Foulum, Aarhus University, DK-8830 Tjele, Denmark
[2]Department of Agricultural and Environmental Sciences, University of Milan, Milan, Italy
[3]Department of Ecoscience, Aarhus University, DK-8000 Aarhus C, Denmark
[4]Centre for Circular Bioeconomy (CBIO), Aarhus University, Denmark

ABSTRACT

The 2 brown seaweeds, *Fucus serratus* and *Fucus vesiculosus*, have demonstrated anti-methanogenic properties in vitro with reductions in CH_4 production ranging from 53 to 63%. This study aimed to investigate the effects of the 2 *Fucus* seaweeds on enteric CH_4 emissions, DMI, ECM, and nutrient digestibility when fed to dairy cows. The experiment was conducted using 4 multi-cannulated lactating Danish Holstein dairy cows, which over 3 experimental periods received either: 1) basal diet (**CON**; diet without any seaweed), 2) basal diet diluted with 4% (DM basis) *Fucus serratus* (**SER**), or 3) basal diet diluted with 4% (DM basis) *Fucus vesiculosus* (**VES**); resulting in one complete 3 × 3 Latin square and one incomplete 3 × 3 Latin square. Each period lasted 21 d and consisted of 14 d of adaptation, followed by 3 d of digesta sampling, and 4 d of gas exchange measurements using respiration chambers. Milk yield and feed intake were recorded daily. Blood samples were collected on d 15 and 17. All parameters were statistically analyzed using a mixed procedure of R. Opposite to what we had expected, neither of the 2 *Fucus* seaweeds reduced CH_4 emissions from the dairy cows as daily CH_4 production was significantly higher for both *Fucus* treatments compared with CON. Additionally, CH_4 yield (g CH_4/kg DMI) and intensity (g CH_4/kg ECM) were significantly higher for SER compared with CON. Milk yield, DMI, and total-tract digestibility were unaffected by the treatments; however, SER resulted in lower milk protein yield (kg/d) and lower milk and blood plasma urea concentrations compared with CON. In conclusion, neither *Fucus serratus* (SER) nor *Fucus vesiculosus* (VES) showed potential as methane-mitigating feed additives when fed to dairy cows at an inclusion level of 4% of DM. The inclusion of the 2 brown seaweeds had no effects on DMI, milk yield, or total-tract digestibility.
Keywords: Anti-methanogenic feed additives, macroalgae, alternative feedstuffs, cattle

INTRODUCTION

During the last decade, seaweeds, including brown, red, and green species, have been intensely evaluated for their anti-methanogenic properties in both in vitro and in vivo studies (Stefenoni et al., 2021; Muizelaar et al., 2023; Thorsteinsson et al., 2023a). The most potent seaweed in terms of reducing enteric methane (CH_4) from ruminants belongs to the red *Asparagopsis* genus (Machado et al., 2014; Kinley et al., 2016). These seaweeds accumulate high concentrations of bromoform and other halomethanes in their tissue, which are effective inhibitors of ruminal methanogenesis (Wood et al., 1968; Machado et al., 2016; Nørskov et al., 2021). In vitro studies have reported up to 99% reduction in CH_4 production (Kinley et al., 2016; Thorsteinsson et al., 2023a), while reductions in enteric CH_4 emission from *Asparagopsis* spp. fed cattle ranges from 26 to 98%, depending on diet composition and dosage (Roque et al., 2019; Kinley et al., 2020; Roque et al., 2021; Stefenoni et al., 2021). However, concerns could be raised regarding the use of this seaweed as an anti-methanogenic feed additive due to reported decreases in milk production and feed intake in dairy cattle (Roque et al., 2019; Stefenoni et al., 2021), transfers of bromine into milk (Stefenoni et al., 2021), and abnormalities or damage to the rumen wall of sheep and dairy cattle when fed *Asparagopsis* spp. (Li et al., 2018; Muizelaar et al., 2021).

The enzyme bromoperoxidase has been found in green, red, and brown marine seaweeds, enabling them to synthesize halogenated carbon compounds including bromo-

Received March 31, 2024.
Accepted July 9, 2024.
†M. Thorsteinsson and É. Chassé contributed equally as first authors.
*Corresponding author: Mirka Thorsteinsson. Tel: 004528973432; Email: mitho@anivet.au.dk; Department of Animal and Veterinary Sciences, AU-Viborg, Research Centre Foulum, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark

The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

form (Wever et al., 1985; Sheffield et al., 1992; Ohshiro et al., 1999). Nevertheless, seaweeds procurable in the Northern Hemisphere in general have non-detectable or substantially lower tissue concentrations of halomethanes as compared with *Asparagopsis* spp. (Carpenter and Liss, 2000; Nørskov et al., 2021). Despite this, the 2 common brown seaweeds *Fucus serratus* and *Fucus vesiculosus* reduced CH_4 production by 53 and 63% per g OM, respectively, when incubated in vitro with corn silage at an inclusion level of 17% (Pandey et al., 2022; Thorsteinsson et al., 2023a). These anti-methanogenic effects can most likely be ascribed to phlorotannins and other polyphenols in brown seaweeds (Vissers et al., 2018; Pandey et al., 2022). Few in vivo studies have been conducted investigating the effects of brown seaweeds as anti-methanogenic feed additives focusing on *Ascophyllum nodosum*, *Sargassum muticum*, *Saccharina latissima*, and a 50:50 mixture of *Saccharina latissima* and *Fucus serratus* (Antaya et al., 2019; Muizelaar et al., 2023; Thorsteinsson et al., 2023b), but none of these studies demonstrated any CH_4-inhibiting effects of the seaweeds. Thus, the objective of this study was to investigate the CH_4-mitigating potential of *F. serratus* and *F. vesiculosus* when fed to dairy cows, since both species have yielded promising results in vitro. Moreover, effects on feed intake, milk production, and nutrient digestibility were also evaluated. To fulfill the objective, multi-cannulated cows were used to obtain data on nutrient digestion as a previous study found significant differences in nutrient digestibility in the different segments of the gastrointestinal tract of dairy fed 4% brown seaweed on a DM basis (Thorsteinsson et al., 2023b). Due to an expected high content of heavy metals, lower nutritional value than traditional feedstuffs, and palatability, the seaweed inclusion rate was reduced to 4% (DM basis) instead of the 17% inclusion rate supplied in vitro (Pandey et al., 2022; Thorsteinsson et al., 2023a). It was hypothesized that the 2 seaweeds would decrease enteric CH_4 emissions from dairy cows as both species, as previously mentioned, have demonstrated anti-methanogenic potential in vitro.

MATERIALS AND METHODS

The experiment was conducted at Aarhus University, AU Viborg – Research Centre Foulum, Denmark, under a license from the Danish Animal Experiments Inspectorate. The experiment complied with the guidelines set out by the Danish Ministry of Environment and Food (act 474 of 15th of May 2014 and executive order 2028 of 14th of December 2020) concerning animal experimentation and care of animals under experiments. Moreover, the experiment was planned under consideration of the ARRIVE Guidelines (Percie du Sert et al., 2020).

Seaweeds

Fucus serratus and *F. vesiculosus* were harvested from wild populations along the shores of the Isefjord, Denmark by Dansk Tang Aps (Nykøbing Sjælland, Denmark) and thereafter airdried at room temperature by using dehumidifiers. After drying, the biomasses were ground to a particle size of 2- to 4-mm. Both species were harvested from October to December 2022.

Experimental design

The experiment was conducted using 4 multi-cannulated lactating Danish Holstein dairy cows, which over 3 experimental periods received either: 1) basal diet (CON; diet without any seaweed), 2) basal diet diluted with 4% (DM basis) *Fucus serratus* (SER), or 3) basal diet diluted with 4% (DM basis) *Fucus vesiculosus* (VES); resulting in one complete 3 × 3 Latin square and one incomplete 3 × 3 Latin square (Figure S1). Seaweed biomass partially replaced all feedstuffs in the CON diet (Table 1). Each of the 3 experimental periods lasted 21 d, where the first 14 d were assigned to adaptation to the diet, followed by 3 d of digesta sampling, and finally 4 d of gas exchange measurements.

Animals and housing

The cows (2 5th parity and 2 1st parity) were fitted with rumen, duodenal, and ileal cannulas, allowing the collection of digesta in different segments of the digestive tract for determination of diet digestibility. All cows were housed in individual pens (400 × 450 cm) with slatted floors and cubicle beds with mattress and sawdust. The cows were milked twice daily at 0515 and 1630 h. At the beginning of the experiment, milk yield was 30.9 ± 6.04 kg/d (average ± SD), DIM was 52.8 ± 14.0 d, BCS was 2.9 ± 0.24, and BW was 640 ± 86.0 kg.

Diet and feeding

Feed was offered to the cows as TMR, which were prepared daily and fed to the cows once a day (1600 h) on *ad libitum* basis. Corn silage, spring growth and 1st regrowth grass/clover silage (perennial ryegrass, hybrid ryegrass, red clover, and white clover) were included in the rations as roughages, while barley, sugar beet pulp, sugar beet molasses, rapeseed meals, mineral supplements, and seaweeds were included as concentrates (CON diet without seaweed). Seaweed biomass partially replaced all feedstuffs in the CON diet (Table 1). The CON ration was formulated according to the Nordic Feed Evaluation System (Norfor; Volden, 2011) using the "NorFor Feed Ration Optimizer" software with an expected milk yield

Table 1. *Dietary and chemical composition of a control (CON) diet without any seaweed, the same diet with either 4% Fucus serratus* or *4% Fucus vesiculosus, and composition of the seaweed biomass.*

	Treatments		
Item	CON	SER	VES
Dietary composition[1]			
Grass/clover silage, spring growth	8.92	8.56	8.56
Grass/clover silage, 1st regrowth	21.2	20.4	20.4
Corn silage	29.7	28.5	28.5
Rapeseed meal	18.3	17.5	17.5
Barley	9.77	9.37	9.37
Sugar beet pulp	7.64	7.34	7.34
Sugar beet molasses	4.25	4.08	4.08
Mineral premix[2]	0.21	0.20	0.20
Seaweed	0.00	4.00	4.00
Chemical composition[1]			
DM, % of fresh feed	39.1 ± 0.85	39.4 ± 0.78	39.7 ± 0.84
Ash	6.46 ± 0.08	7.00 ± 0.19	7.09 ± 0.09
CP	16.0 ± 0.14	15.6 ± 0.20	15.6 ± 0.27
Crude fat	3.03 ± 0.12	3.07 ± 0.06	3.03 ± 0.15
aNDFom	32.2 ± 1.66	32.4 ± 1.25	32.3 ± 0.92
Lignin	17.7 ± 0.78	17.1 ± 0.10	17.2 ± 0.28
Starch	16.7 ± 0.21	17.0 ± 0.38	17.1 ± 0.67
NE_{L20}, MJ/kg of DM[3]	6.53	—	—
Content in seaweed biomass[1]			
Ash, g/kg DM	—	235.1 ± 31.7	284.7 ± 53.8
CP g/kg DM	—	62.5 ± 2.93	62.6 ± 3.55
aNDFom, g/kg DM	—	290.0 ± 23.0	350.0 ± 33.7
Mannitol, g/kg DM	—	27.4 ± 4.69	25.0 ± 1.13
Cadmium, mg/kg DM	—	1.16 ± 0.255	0.685 ± 0.288
Total arsenic, mg/kg DM	—	44.9 ± 6.08	26.1 ± 11.3
Total polyphenol, mg gallic acid/g DM	—	34.3 ± 4.48	24.9 ± 1.30

[1]Average ± SD; % of DM unless otherwise stated, n = 3.

[2]Vilofoss Komix Type 3, declared macro mineral composition (g per kg DM): Ca = 147, Mg = 141, Na = 116, S = 1. Added vitamins and micro minerals (per kg DM): vitamin A = 600,000.10 IU, vitamin D3 = 190,000.10 IU, vitamin E = 4,000 IU, Mn = 4,000 mg, Cu = 1,500 mg, Zn = 4,500 mg, Co = 25 mg, Se = 50 mg in combination with Vilofoss Suplex ADE, analyzed/declared macro mineral composition (g/kg DM): Ca = 139, Mg = 91, Na = 95. Added vitamins and micro minerals (per kg DM): vitamin A = 900,000 IU, vitamin D3 = 200,000 IU, vitamin E = 2,000 IU, Se = 50 mg.

[3]Net energy for lactation at 20 kg DMI/d. AAT_N (amino acids absorbed in the small intestine) NEL was 14.6 g/MJ and AAT_N milk was 14.6 g/MJ for CON, calculated according to NorFor (Volden, 2011).,.

of 10,700 kg ECM per year corresponding to 36.7 kg ECM per d. Roughage:concentrate ratio was 59.8:40.2 for CON and 57.4:42.6 for SER and VES as the seaweeds were considered as concentrate feeds. Titanium dioxide (TiO_2; 2 × 10 g per d) and chromic oxide (Cr_2O_3; 2 × 13 g per d) were used as external markers to determine nutrient flow in the digestive tract. The markers were weighed into degradable bags and dosed directly into the rumen twice daily coinciding with milkings throughout the experiment. The cows had free access to water.

Sampling and recordings

Milk yield, amount of allocated feed and orts were weighed daily throughout the experiment. The composition of the milk was determined on d 18 to 21, while DMI was determined on d 14 to 21. Drinking water was measured by a water-meter (Brødrene Dahl, Brøndby, Denmark) during the sampling and chamber periods. In each period, rumen liquid, duodenal content, urine, and feces were collected at 8 sampling time points over a 3 d period every 9th h to represent every 3rd h of a 24 h period. At each sampling time, duodenal (0.50 L) samples were collected in plastic bags attached to the cannulas, while fecal samples (0.35 L) were collected during voluntary defecation or by grab sampling from the rectum. Digesta samples were pooled across all sampling times and stored at −20°C until analyzed. Similarly, urine was collected at all sampling times during voluntary urinations or upon manual stimulation of the pelvic region. Immediately after collection, pH was measured using a digital pH-meter (Meterlab PHM 220, Radiometer, Brønshøj, Denmark). Rumen fluid (30 mL) was sampled from the ventral ruminal sac using a syringe attached to a rumen sampler device (Bar Diamond Inc., Parma Idaho, USA). The pH in rumen fluid was measured immediately after sampling using the digital pH-meter. Samples were

stored at −20°C for later analysis of VFA, L-lactate, glucose, and NH_3 concentration.

Plasma was sampled by venipuncture of the tail vein or artery on d 15 at 1530 h and d 17 at 0800 h using lithium-heparin stabilized vacutainers (Greiner Bio-One GmbH, Kremsmünster, Austria). The tubes were centrifuged at 3,000 g at 4°C for 20 min and following transferred to cryotubes and thereafter stored at −20°C pending analyses for urea, glucose, BHB, and nonesterified fatty acids.

Gas exchange was measured on d 18–21, using 4 individual transparent polycarbonate respiration chambers based on open-circuit indirect calorimetry. The chambers were a modified version of Hellwing et al. (2012). To counteract any unforeseen differences between chambers, the cows were assigned to one specific chamber for the first 48 h of gas measurement, whereafter the cows were moved to another specific chamber for the remaining 48 h of gas measurement. The cows were assigned to the same 2 chambers throughout the experiment. Flow and concentrations of outlet gases (CH_4, CO_2, O_2, H_2, and H_2S) were measured continuously over the day (Columbus Instruments, Columbus, Ohio, USA). Likewise, temperature, humidity, and pressure were measured continuously (Veng Systems, Roslev, Denmark). Temperature and humidity in the chambers were 13.8 ± 1.83°C and 78.2 ± 3.02%, respectively. To ensure data quality, recovery tests (n = 18 per chamber for CO_2 and CH_4) were performed before, during, and after the experiment by infusing a known amount of pure CO_2 (4250 mL per minute for 5 h) or CH_4 (400 mL per minute for 22 h) into the chambers and comparing it with the amount of gas measured by the system. Across chambers, average recovery values ± SD were 100.2 ± 1.32% for CO_2 and 100.2 ± 1.39% for CH_4. Recovery tests were used to correct the measured gas concentrations. The average of CH_4 and CO_2 recoveries was used to correct O_2 and H_2.

Chemical analyses

During sampling and chamber periods, DM content in fresh feed and orts was determined daily by drying at 60°C for 48 h (AOAC International, 2000). Total mixed rations and digesta samples were freeze-dried and ground on a 1-mm screen (Ultra Centrifugal Mill ZM 200, Verder Scientific, Hann, Germany) before chemical analyses, except that a 0.5-mm screen was used for starch analysis. Total N in digesta, seaweed biomass, and TMR were analyzed using the Dumas principle (Hansen, 1989) in a Vario Max CN (Elementar Analysensysteme GmbH, Langenselbold, Germany). Conversion of CP was calculated as total nitrogen × 6.25. Crude fat in TMR was determined by Soxhlet extraction with petroleum ether (Soxtec 2050, Foss Analytical, Hillerød, Denmark) after hydrolysis with HCl (Stoldt, 1952). Starch in digesta and TMR was digested with heat-stable α-amylase and amyloglucosidase. The reaction was subsequently assayed for glucose (Kristensen et al., 2007) by using a YSI model 2900 analyzer (YSI Inc., Yellow Springs, Ohio, USA). Neutral detergent fiber was determined as aNDFom by adding heat-stable amylase and sodium sulfite (Mertens, 2002) in the ANKOM^{2000} Fiber Analyzer and following corrected for ash (ANKOM Technology; 2017). Chromic oxide content in digesta was analyzed by oxidation to chromate and subsequent determination spectrophotometrically using a Lamba 900 equipment (PerkinElmer Inc., Waltham, Massachusetts, USA; Schürch et al., 1950), while TiO_2 was analyzed according to Myers et al. (2004) with a minor modification (15 mL of 30% hydrogen peroxide was used instead of 10 mL and 5 extra drops before measuring absorbance). Total polyphenolic content in seaweed biomass was determined using LC-MS-MS as ascribed by Curtasu and Nørskov (2024). Cadmium and total arsenic were determined in seaweed biomass using inductively coupled plasma mass spectrometry (iCAPq ICP-MS, Thermo Fischer, Bremen, Germany). Briefly, 0.3 g dry samples were digested in closed quartz vessels in a microwave oven (Multiwave 3000, Anton Paar, Graz, Austria) using 5 mL of nitric acid. The digest was subsequently diluted with Milli-Q water and quantified using ICP-MS. An internal seaweed standard (IAEA-413-Algea, International Atomic Energy Agency, Vienna, Austria) was used for standardization. Ash content in the samples was determined by combustion at 525°C for 6 h.

Rumen liquid (4 mL) for VFA analysis was stabilized with 1 mL of 25% metaphosphoric acid (MPA) solution to reach 5% MPA in the stabilized sample. The concentrations of VFA were determined in stabilized rumen fluid after methanol-chloroform extraction with 2-ethylbutyrate as internal standard, using a gas chromatograph (GC; Trace 1310, Thermo Scientific, Germany) with split/splitless injector at 225°C and a flame ionization detector at 250°C. A 30 m × 0.53 mm × 1 µm HP-FFAP column (Agilent Technologies Inc., Wilmington, DE) was used with helium as carrier gas at 0.3405 atm. The GC oven was programmed to increase from 100 to 200°C at 10°C/min. L-lactate and glucose in rumen fluid were analyzed using the immobilized glucose oxidase electrode technique (Mason, 1983; YSI 2900D, YSI Inc., Yellow Springs, USA). The NH_3 concentration was determined using a Randox AM 1015 kit (Randox Laboratories, Crumlin, United Kingdom) and an ADVIA 1800® Chemistry System (Siemens Medical Solutions, Tarrytown, New York, USA) autoanalyzer.

Beta-hydroxybutyrate was determined in plasma using a method involving a media with oxamic acid to inhibit lactate dehydrogenase followed by measuring the absorbance at 340 nm due to the production of NADH (Harano

et al., 1985), while NEFA was determined using the Wako, NEFA C ACS-ACOD assay method (FUJIFILM Wako Chemicals, Osaka, Japan). The concentrations of glucose, L-lactate, and urea in plasma were measured using a spectrophotometric assay, following the manufacturer's guidelines (Siemens Medical Solutions, Tarrytown, New York, USA). All analyses were performed using an auto-analyzer, ADVIA 1800® Chemistry System (Siemens Medical Solutions, Tarrytown, New York, USA). Milk samples were analyzed for contents of fat, protein, lactose monohydrate, and urea by mid-infrared reflection (MilkoScan™ 7 RM; Eurofins Steins Laboratorium A/S, Vejen, Denmark).

Calculations

Daily DMI was calculated as the amount of DM offered subtracted DM content in orts. The intakes of OM, CP, aNDFom, starch, and ash were calculated by multiplying DMI with the respective nutrient content in the TMR for each period. Net energy contents in TMR were calculated according to NorFor (Volden, 2011). Duodenal and fecal DM flows were calculated separately for each digestive marker, followed by averaging across markers under the assumption that the concentrations in pooled digesta samples were representative of the average daily flow of digesta. From the DM flow and the respective nutrient concentrations in each section in the digestive tract, flows of OM, ash, aNDFom, CP, and starch were estimated. Apparent nutrient digestibility in different sections of the digestive tract was calculated from the nutrient intake in the sampling period and respective flow.

Milk yield was converted to ECM (3.140 MJ/kg), according to Sjaunja et al. (1991):

$$\text{ECM (kg)} = \text{milk yield} \times [(38.3 \times \text{fat} + 24.2 \times \text{protein} + 15.71 \times \text{lactose} + 20.7)/3{,}140],$$

with milk yield in kg; fat, protein, and lactose monohydrate in grams per kg.

Gas exchange was measured as flows (L/d) at standard temperature and pressure (STP; 0°C/ 273.15 K and 101.325 kPa) and subsequently converted from L/d to g/d using the density of each gas at STP (0.716, 1.963, 0.0899, and 1.428 L/g for CH_4, CO_2, H_2, and O_2, respectively). Data were omitted from the short periods each day when chambers were open in connection with milking and feeding. The cows were assumed to have a similar gas production for deleted minutes as the average for other minutes for each measuring period. Gas yield and intensity were calculated based on the DMI and ECM yield during each chamber period, while the respiratory coefficient was calculated as the ratio between CO_2 produced and O_2 consumed (L/L). Gas yield per kg total-tract digested OM was calculated assuming a constant digestibility throughout the sampling and chamber period.

Statistical analyses

All statistical analyses were conducted in R 4.3.2 (R Core Team, 2023). Before the statistical analysis, all observations of the variables were averaged within cow and period. The effect of diet on the various animal responses was analyzed with the following linear mixed model fitted with REML and the "lmer" function from the "lme4" package (Bates et al., 2015):

$$Y_{dpc} = \mu + \alpha_d + \gamma_p + A_c + E_{dpc},$$

where Y_{dpc} is the dependent response variable, μ is the overall mean, α is the fixed effect of diet (d = CON, VES, or SER), γ is the fixed effect of experimental period (P = 1 to 3), A is the random effect of cow (c = 1 to 4), and E_{dpc} is the random residual error assumed to be independent with constant variance and normally distributed.

For hourly CH_4 and H_2 emissions, observations were averaged within time point, cow and period, resulting in 24 observations per cow per period. The data were analyzed with the following model:

$$Y_{dhpc} = \mu + \alpha_d + \tau_h + \alpha_t \times \tau_h + \gamma_p + A_c + E_{dhpc},$$

where Y_{dhpc} is the dependent response variable, μ is the overall mean, α is effect of treatment (d = CON, VES, or SER), τ is the fixed effect of hour (h = 0 to 23), $\alpha_t \times \tau_h$ is the interaction, γ is the effect of period (P = 1 to 3), A is the effect of cow (c = 1 to 4), and E_{dhpc} is the random residual error assumed to be independent with constant variance and normally distributed. Data was analyzed using a first-order autoregressive covariance structure with heterogeneous variance (AR1). Rumen fermentation patterns were analyzed using the same model as for hourly gas emissions, except data only consisted of one observation per sampling point per cow per period. The analysis was performed assuming that the sampling order was more correlated than the sampling time point.

During the statistical analysis, data were examined for homogeneity of the variance by evaluating plots of residuals and by using Bartlett's test, and the residuals were evaluated for normality by using the Shapiro-Wilk test and by evaluating the QQ-plots constructed in R. All presented data were normally distributed. Data is presented in tables as estimated marginal means (EMS) and SEM. Differences between EMS were evaluated using Tukey's method for comparison. Statistical significance

was declared when $P \leq 0.05$ and statistical tendency was declared when $0.05 < P \leq 0.10$.

RESULTS

Feed intake, nutrient digestibility, and milk production

The seaweed biomass used for SER had a total arsenic content of 44.9 ± 6.1 mg/kg DM and a cadmium content of 1.16 ± 0.26 mg/kg DM, while the seaweed biomass used for VES had contents of 26.1 ± 11.3 and 1.16 ± 0.26 mg/kg DM for total arsenic and cadmium contents, respectively (Table 1). Total polyphenol contents in seaweed biomasses were 34.3 ± 4.5 and 24.9 ± 1.3 mg gallic acid/g DM for SER and VES, respectively. Total-tract digestibility of all nutrients was unaffected by treatments, while only minor effects were observed on nutrient intake. Hence, due to the numerically higher DMI and higher ash content in VES, the cows had a significantly higher ash intake compared with CON ($P = 0.04$; Table 2). Water intake was similar between all treatments.

Milk yield (kg/d) and ECM yield (kg/d) were unaffected by the dietary inclusion of either of the 2 brown seaweeds (Table 3), but due to a slightly lower milk production and protein percentage, daily protein yield (kg/d) was significantly lower for SER compared with CON ($P = 0.01$). Moreover, both SER and VES had significantly lower urea concentrations in milk compared with CON ($P < 0.001$).

Gas exchange

SER had significantly higher hourly CH_4 production the majority of the time points (Figure 1a) compared with CON, resulting in higher daily CH_4 production (g/d), yield (g CH_4/kg DMI), and intensity (g CH_4/kg ECM; Table 4). Daily CH_4 production for VES was also significantly higher compared with CON ($P = 0.01$), but hourly CH_4 production only differed between 05.00 and 06.00 h. Hence, no difference was observed between VES and CON in CH_4 yield and intensity. In contrast, CON had a significantly higher daily H_2 production compared with SER ($P = 0.03$), which was caused in particular by a large peak between 17.00 and 18.00 h right after feeding (Figure 1b). Additionally, CON resulted in significantly higher CO_2 yield (g/kg DMI) compared with both *Fucus* diets, while VES also consumed less O_2 per kg DMI compared with CON. If gas yield instead is expressed per kg total-tract digested OM, no effect was observed on CO_2 and O_2, but SER still resulted in higher CH_4 yield per kg total-tract digested OM compared with CON ($P = 0.01$), and both *Fucus* diets yielded significantly less H_2 ($P = 0.02$).

Rumen fermentation pattern and metabolic indicators

Across all sampling time points ruminal fermentation parameters were unaffected, except SER and VES had significantly lower proportions of valerate in total VFA compared with CON ($P = 0.03$; Table 5). However, diurnal differences were observed between the treatments. Figure 2a shows that SER had significantly higher proportions of acetate at the 2 first sampling points after both daily milkings (6, 9, 18, and 21 h) and particularly after the daily feeding (18 and 21 h) compared with CON, while no differences were detected between SER and VES. Moreover, SER had a lower glucose concentration in rumen fluid overall ($P = 0.04$) and at 21 and 0 h (Table 5; Figure 2b). No differences were observed in the diurnal patterns for concentrations of total VFA, pH, ammonia, lactate, and the respective VFAs (data not shown). Urinary pH and blood plasma indicators for metabolic status were unaffected by the treatments, except SER had a lower concentration of urea in blood plasma compared with CON while the concentration for VES was intermediate ($P = 0.02$; Table 6).

DISCUSSION

Nutrient digestibility and rumen fermentation

Fucus serratus and *F. vesiculosus* have been reported to have low in vitro degradability of both DM (13.3–20.3%) and OM (3.2–12.2%; (Thorsteinsson et al., 2023a). Moreover, both species significantly reduced degradability of DM (10.5–19.7%) and OM (9.2–19.7%) when co-incubated with either corn or grass/clover silage (17% seaweed: 83% roughage; Thorsteinsson et al., 2023a). Low degradability has also been reported for other seaweeds, particularly among brown species, as compared with commonly used roughages such as corn and grass/clover silages (Molina-Alcaide et al., 2017; Thorsteinsson et al., 2023a). However, neither of the 2 seaweed species had negative effects on ruminal nor total-tract digestibility of nutrients in the current study. Similar findings were reported in another study feeding up to 4% on DM basis of the brown seaweeds *Saccharina latissima*, *Ascophyllum nodosum*, and *Sargassum muticum*, though, *A. nodosum* lowered total-tract CP digestibility (Thorsteinsson et al. (2023b).

Ruminal OM digestibility did not differ significantly between the treatments, however both seaweed diets, in particular SER, had numerically higher digestibility compared with CON. If not due to random variation, the high contents (>50% in DM) of polysaccharides such as laminarin and cellulose in the brown seaweeds may have supplied the ruminal microbes with easily fermentable

Table 2. *Intake and apparent digestibility in dairy cows fed either a control diet or the same diet with one of 2 brown seaweeds.*

	Treatments[1]				P-value
Item	CON	SER	VES	SEM	Treatment
Water intake, kg/d	75.2	85.3	80.6	10.3	0.43
Intake during sampling period, kg/d					
DM	22.0	22.3	23.5	2.11	0.35
OM	20.6	20.7	21.9	1.96	0.40
aNDFom	7.16	7.19	7.59	0.704	0.35
CP	3.52	3.48	3.68	0.337	0.45
Starch	3.63	3.77	4.02	0.329	0.15
Ash	1.43[b]	1.56[ab]	1.67[a]	0.143	0.04
Apparent ruminal digestibility, g/kg					
DM	345	398	375	42.4	0.61
OM	436	484	462	36.2	0.56
CP	−217	−146	−179	52.2	0.65
Starch	907	918	918	12.4	0.77
Ash	−964	−762	−765	144	0.41
Apparent intestinal digestibility, g/kg					
DM	525	471	478	32.5	0.38
OM	475	407	421	35.0	0.31
CP	696	649	654	14.6	0.11
Starch	903	856	853	22.2	0.24
Ash	729	719	703	23.6	0.63
Apparent total-tract digestibility, g/kg					
DM	693	684	677	7.64	0.42
OM	707	696	692	7.17	0.35
aNDFom	560	542	534	13.4	0.27
CP	632	599	592	15.1	0.19
Starch	992	989	987	2.46	0.50
Ash	479	518	480	17.6	0.27

[a–b]Values within the same line with different superscripts differ ($P < 0.05$).

[1]CON = control diet; SER = 4% *F. serratus* (DM basis); VES = 4% *F. vesiculosus* (DM basis).

carbohydrates by diluting the CON diet (Jönsson et al., 2020; Li et al., 2021). Increased microbial activity could also explain the significantly lower ruminal glucose concentration for SER as starch intake was similar between the treatments (Hua et al., 2022).

Table 3. *Milk production from dairy cows fed either a control diet (CON) or the same diet with one of 2 brown seaweeds.*

	Treatments[1]				P-value
Item	CON	SER	VES	SEM	Treatment
Milk, kg	33.4	31.7	32.2	4.48	0.13
ECM, kg/d	32.6	31.9	31.9	4.89	0.60
Fat, kg/d	1.30	1.31	1.27	0.216	0.79
Protein, kg/d	1.11[a]	1.04[b]	1.08[ab]	0.160	0.01
Lactose, kg/d	1.65	1.55	1.58	0.212	0.14
Fat, %	3.85	4.03	3.95	0.169	0.63
Protein, %	3.36	3.28	3.34	0.110	0.20
Lactose, %	4.93	4.90	4.91	0.0714	0.38
Urea, mM	3.55[a]	3.19[b]	3.25[b]	0.0816	<0.001

[a–b]Values within the same line with different superscripts differ ($P < 0.05$).

[1]CON = control diet; SER = 4% *F. serratus* (DM basis); VES = 4% *F. vesiculosus* (DM basis).

Methane and hydrogen emissions

Previous in vitro rumen fermentation studies have demonstrated that *F. vesiculosus* and *F. serratus* reduced CH_4 production per g OM by 63 and 53%, respectively, when co-incubated with corn silage at a seaweed inclusion rate of 17% of DM (Pandey et al., 2022; Thorsteinsson et al., 2023a). However, we did not observe any CH_4-mitigating effects in the present study. In fact, opposite to what we had hypothesized, daily CH_4 emission (g/d) was significantly higher on both seaweed-containing diets compared with CON, and the inclusion of *F. serratus* in the diet also resulted in higher CH_4 yield and intensity compared with CON. In vivo studies evaluating the 2 brown seaweeds as anti-methanogenic feed additives are limited to the present study and a study by Muizelaar et al. (2023) in which 150 g of a 50:50 mixture of *F. serratus* and *S. latissima*, corresponding to 0.56–0.60% of total DMI, was fed per d to dairy cows. In the study by Muizelaar et al. (2023), CH_4 emissions were unaffected by the inclusion of the seaweed mix. The nature or presence of any anti-methanogenic bioactive components in the 2 *Fucus* species remains to be identified. It is thus not known whether the conflicting results between in vitro and in vivo studies are related solely to different

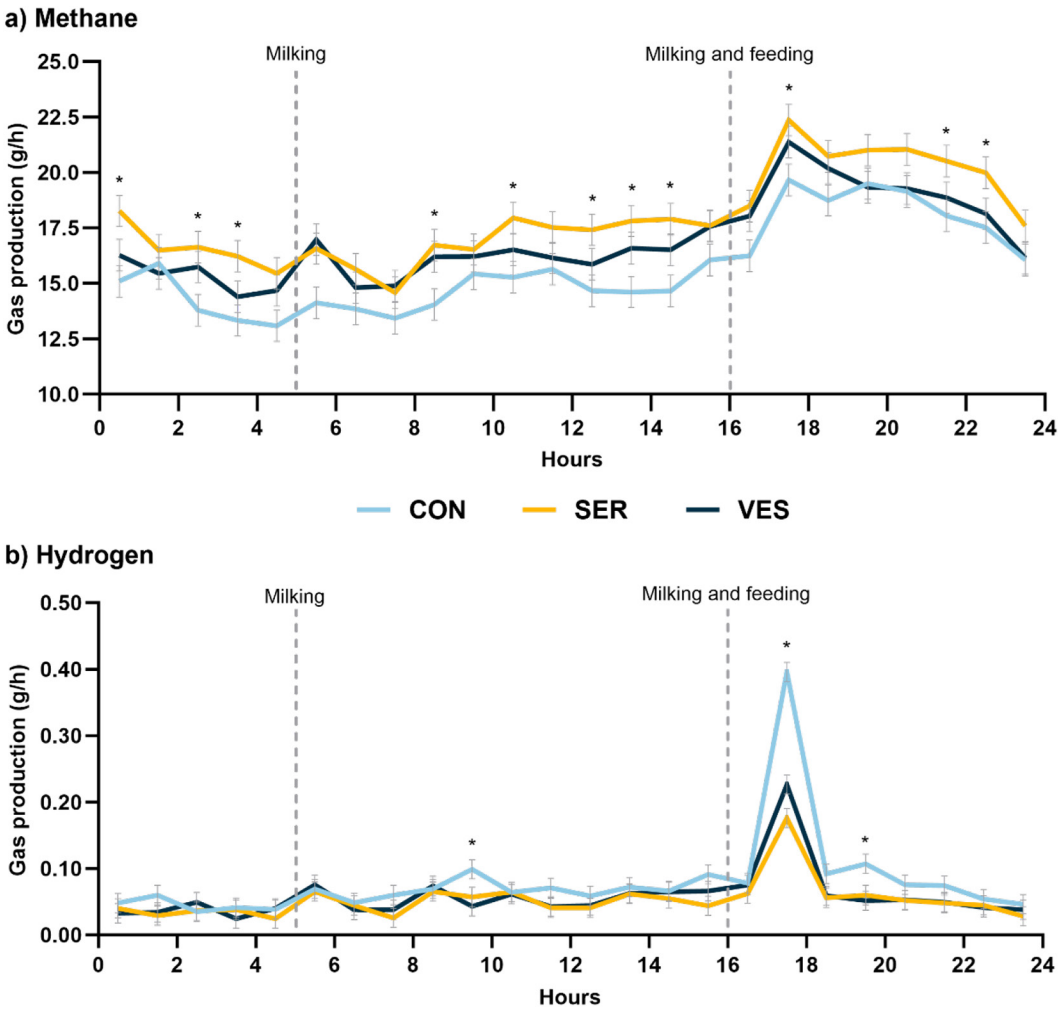

Figure 1. a) Estimated marginal means of hourly methane emission from dairy cows fed a control diet (CON) or the same diet with either 4% Fucus serratus (SER; DM basis) or Fucus vesiculosus (VES; DM basis). Each time point represents the average methane production for 1 h before and after that time (i.e., time point 00.30 h represents the average gas production from 00.00 to 01.00 h). Significant differences between the Fucus treatments and control diet at the individual time points are marked with *. b) Estimated marginal means of hourly hydrogen emission from dairy cows fed a control diet (CON) or the same diet diluted with either 4% Fucus serratus (SER; DM basis) or Fucus vesiculosus (VES; DM basis).

"dietary" inclusion rates (17 vs. 4% on a DM basis, respectively) or variations in contents of bioactive compounds between different collections of the same species depending on e.g., site, environmental factors during growth, and season of harvest (Pavia and Toth, 2000; Pandey et al., 2022). Moreover, it should be mentioned that the seaweed biomass used in the aforementioned in vitro studies by Pandey et al. (2023) and Thorsteinsson et al. (2023a) was freeze-dried, whereas the biomass in the present study was airdried. Hence, differences in post-harvest processing might also contribute to the observed contradictory effects of the seaweeds as CH_4 inhibitors. This is supported by a study Yen et al. (2022) in which prewilting of brown seaweeds before ensiling caused degradation of phlorotannins in the biomass, and thereby potentially affecting the CH_4-mitigating potential. It is also important to note that the harvest of seaweed biomass was spread out over a 3-mo period as the seaweeds were harvest from wild populations, which could have affected the tissue contents of nutrients, metals, and potential bioactive compounds during the experiment. However, both seaweed biomasses had similar contents of total polyphenols across periods.

In contrast to daily CH_4 emissions, H_2 production (g/d) was higher for CON compared with SER, which in particular was caused by a large peak in H_2 emission for CON during the first 2 h after feeding. In the rumen, the hydrogenotrophic pathway is the predominant methanogenic pathway in which methanogens use CO_2 as the carbon source and H_2 as the main electron donor. Therefore, the higher H_2 emission for CON could simply be caused by a lower incorporation of H_2 into CH_4. However,

Table 4. *Daily gas exchange in dairy cows fed either a control diet or the same diet with one of 2 brown seaweeds.*

	Treatments[1]				P-value
Item	CON	SER	VES	SEM	Treatment
DMI in chambers, kg/d	22.3	22.4	23.4	2.24	0.14
Gas exchange, g/d					
CH_4	377[c]	430[a]	404[b]	32.0	<0.01
CO_2	14379	14000	14476	1231	0.21
O_2	9549	9215	9558	739	0.07
H_2	1.78[a]	1.20[b]	1.30[ab]	0.189	0.03
H_2S	0.220	0.218	0.224	0.0143	0.67
Respiration coefficient[2]	1.09	1.10	1.10	0.0106	0.42
Gas yield (g/kg DMI)					
CH_4	17.1[b]	19.2[a]	17.4[b]	0.564	<0.01
CO_2	649[a]	628[b]	621[b]	9.19	0.02
O_2	431[a]	414[ab]	412[b]	9.85	0.04
H_2	0.0817[a]	0.0536[b]	0.0548[b]	0.00674	0.02
H_2S	0.01022	0.00984	0.00946	0.000964	0.44
Gas yield (g/kg dOM)[3]					
CH_4	25.8[b]	29.7[a]	27.1[ab]	1.01	0.01
CO_2	981	969	967	18.9	0.73
O_2	652	639	643	17.8	0.70
H_2	0.124[a]	0.0828[b]	0.0856[b]	0.0107	0.02
H_2S	0.0154	0.0152	0.0147	0.00105	0.74
Gas intensity (g/kg ECM)					
CH_4	12.1[b]	14.0[a]	12.9[ab]	1.11	0.03
CO_2	457	458	459	35.3	0.99
O_2	304	301	304	23.2	0.94
H_2	0.0583	0.0402	0.0416	0.00795	0.06
H_2S	0.00732	0.00741	0.00699	0.000826	0.30

[a–c]Values within the same line with different superscripts differ ($P < 0.05$).

[1]CON = control diet; SER = 4% *F. serratus* (DM basis); VES = 4% *F. vesiculosus* (DM basis).

[2]Respiration coefficient calculated as CO_2:O_2 ratio (L/L).

[3]Gas yield expressed per kg total-tract digested organic matter, assuming similar digestibility in sampling and chamber period.

brown seaweeds also contain several compounds such as phloroglucinol and the sulfate-containing carbohydrate fucoidan that might have acted as alternative [H]-sinks (Nishino et al., 1994; Park et al., 2012; van Zijderveld et al., 2010; Martinez-Fernandez et al., 2017). Therefore, although the *Fucus* species might show limited potential as anti-methanogenic feed additives, they may be of future interest to use, either as whole or biorefined biomass, in combination with other potent CH_4-mitigating feed components such as the red seaweed *Asparagopsis* spp. to scavenge the substantial excess H_2 upon inhibition of methanogenesis (Roque et al., 2019; Stefenoni et al., 2021).

Table 5. *Ruminal fermentation characteristics in dairy cows fed either a control diet or the same diet diluted with one of 2 brown seaweeds.*

	Treatment[1]				P-value
Item	CON	SER	VES	SEM	Treatment
pH	6.13	6.17	6.13	0.0663	0.90
Ammonia, mM	3.13	3.25	3.33	0.720	0.91
Glucose, mM	1.07[a]	0.574[b]	0.812[ab]	0.104	0.04
L-Lactate, mM	1.12	0.918	1.11	0.452	0.91
Total VFA, mM	143	143	148	2.43	0.32
% of total VFA					
Acetate	56.4	59.6	58.5	1.20	0.14
Propionate	23.0	20.7	22.4	0.886	0.21
Butyrate	15.3	15.0	14.5	0.728	0.37
Isobutyrate	0.604	0.597	0.598	0.0223	0.95
Valerate	2.61[a]	2.03[b]	2.05[b]	0.271	0.03
Isovalerate	1.29	1.17	1.27	0.179	0.87
Caproate	0.824	0.936	0.726	0.083	0.28
A:P ratio[2]	2.46	2.90	2.63	0.147	0.18

[a–b]Values within the same line with different superscripts differ ($P < 0.05$).

[1]CON = control diet; SER = 4% *F. serratus* (DM basis); VES = 4% *F. vesiculosus* (DM basis).

[2]Ratio between acetate and propionate.

Nitrogen metabolism

In a meta-analysis by Huhtanen and Hristov (2009), the CP concentration in feed was found to be the most important dietary factor influencing milk protein synthesis. The inclusion of the 2 seaweeds decreased the CP concentration in the SER and VES diets on a DM basis. Moreover, phlorotannins are suggested to bind to both the feed protein and endogenous proteolytic enzymes, forming molecular complexes that are poorly degraded,

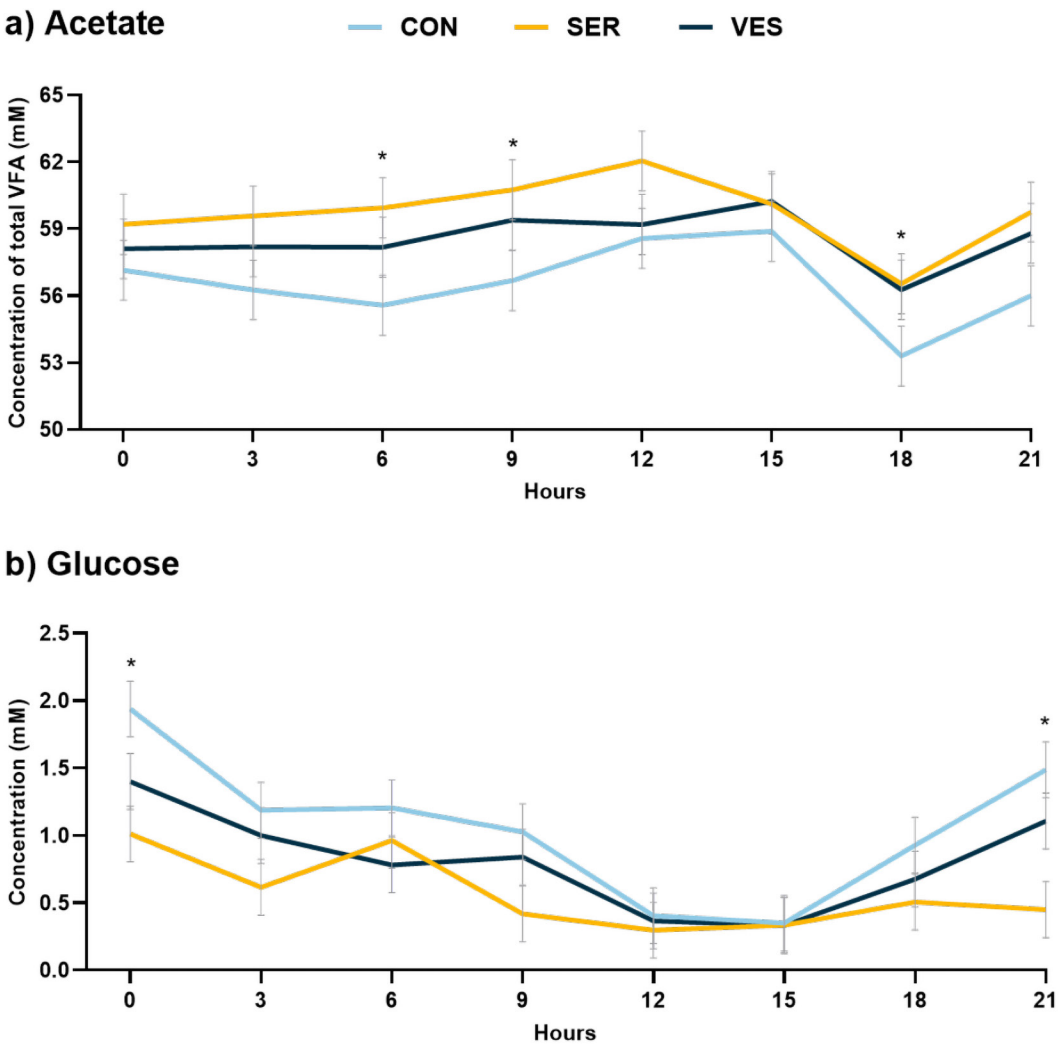

Figure 2. a) Estimated marginal means of acetate at the individual sampling points in rumen fluid from dairy cows fed a control diet (CON) or the same diet with either 4% Fucus serratus (SER; DM basis) or Fucus vesiculosus (VES; DM basis). Significant differences between the Fucus treatments and control diet at the individual time points are marked with *. b) Estimated marginal means of glucose at the individual sampling points in rumen fluid from dairy cows fed a control diet (CON) or the same diet diluted with either 4% Fucus serratus (SER; DM basis) or Fucus vesiculosus (VES; DM basis).

Table 6. *Metabolic and health status indicators of dairy cows fed either a control diet (CON) or the same diet with one of 2 brown seaweeds.*

	Treatments[1]				P-value
Item	CON	SER	VES	SEM	Treatment
Urinal pH	8.06	8.04	8.06	0.0248	0.85
Blood indicators of metabolic status					
Glucose, mM	4.00	3.90	3.91	0.0695	0.55
Urea, mM	2.50[a]	2.23[b]	2.30[ab]	0.163	0.02
BHB, mM	0.743	0.968	1.044	0.122	0.19
NEFA[2], µEq/L	226	231	156	39.8	0.08

[a–b]Values within the same line with different superscripts differ ($P < 0.05$).

[1]CON = control diet; SER = 4% *F. serratus* (DM basis); VES = 4% *F. vesiculosus* (DM basis).

[2]NEFA = nonesterified fatty acids.

and thus reducing CP digestibility (Waghorn, 2008, Belanche et al., 2016; Vissers et al., 2018). In the present study, SER resulted in significantly lower milk and blood urea levels, and daily milk protein yield compared with CON, while VES resulted in a significantly lower milk urea level. Apparent intestinal and total-tract digestibility of CP digestibility was similar between treatments but numerically lower for both seaweed treatments. If more animals had been enrolled in the study, significant differences might have been detected on CP digestibility. Similar results have been reported by Thorsteinsson et al. (2023b) when *A. nodosum* was fed to dairy cows at the same dietary inclusion rate as in this experiment; though, in contrast to our study, CP digestibility was also affected by the inclusion of the seaweed.

It is important to stress that the seaweed biomass in the SER diet exceeded the maximum levels allowed in individual feedstuffs, as defined by the European Commission, for arsenic (max. 40 mg/kg DM) and cadmium (max. One mg/kg DM) (European Commission, 2013; 2019), and hence they would not be allowed as feeds for dairy cows in commercial herds, irrespective of the dietary inclusion level. Similar high levels of As and Cd were measured by Thorsteinsson et al. (2023b) in other brown seaweed species evaluated as CH_4 inhibitors. This illustrates that the high contents of certain minerals will be a major constraint for the use of certain seaweed species as feeds in commercial herds.

CONCLUSION

Neither *Fucus serratus* (SER) nor *Fucus vesiculosus* (VES) reduced CH_4 emission from dairy cows when the 2 brown seaweeds were included in diets at an inclusion rate of 4% of dietary DM. In contrast, daily CH_4 emission (g CH_4/d), yield (g CH_4/kg DMI), and intensity (g CH_4/kg ECM) were increased on the SER compared with control diet (CON). Interestingly, SER and VES yielded less H_2 per kg total-tract digested OM. Further research is needed to elucidate whether this implies a shift in ruminal H_2 metabolism. Dietary inclusion of the 2 brown seaweeds had no effect on ECM yield, DMI, or total-tract nutrient digestibility except for lower daily milk protein yield in SER compared with CON cows.

ACKNOWLEDGMENTS

The authors express their thanks to the barn staff at AU Research Centre - Foulum, especially Tanja Skovbo, Nina Engelbreth, Michael Christensen, and Jørgen Nielsen, for the caretaking of the animals during the experiment and to Tina Press Christensen Bitsch for skillfully overseeing the mixing of the feed. Also sincere thanks to lab technicians for performing the laboratory analyses (AU Viborg – Research Centre Foulum, Tjele, Denmark) and to Torkild Jakobsen and Theresa Otten for assisting in experimental planning and samplings. The authors would also like to give their thanks to Dansk Tang Aps for delivering the *Fucus* biomass.

Funding The study was funded by Innovation Fund Denmark (Grant-ID: 9067-00024B). The authors declare no conflicts of interest.

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ORCIDS

M. Thorsteinsson https://orcid.org/0000-0003-4620-1767
É. Chassé https://orcid.org/0000-0002-8651-4971
M. V. Curtasu https://orcid.org/0000-0001-9823-6692
M. Battelli https://orcid.org/0000-0003-2689-4199
A. Bruhn https://orcid.org/0000-0002-7940-1338
A. L. F. Hellwing https://orcid.org/0000-0002-2881-399X
M. R. Weisbjerg https://orcid.org/0000-0002-6514-9186
M. O. Nielsen https://orcid.org/0000-0002-8364-835X