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Marine macroalgae as an alternative, environment-friendly, and bioactive feeding resource for animals

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Preface

This dissertation is submitted in fulfillment of the requirements for the degree of Doctor of Philosophy (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University, Norway. This PhD dissertation is based on a review and three research articles or manuscripts produced from works conducted at FBA, Nord University, and the collaborating institutions: the University of Oslo, Norway, the University of Copenhagen and Aarhus University, Denmark, and the University of Porto, Portugal from the period of 01.03.2019 to 15.12.2022. This research was supported by the Nord University PhD fellowship (224000-154), Regionale Forskningfond (RFF) Trøndelag, Norway (former: RFF-Midt-Norge) under three projects: Marine Seaweeds; Project no.: 299081, MicroSea; Project no.: 313553 and AutoFeed; Project no: 320745 and the Norwegian Agency for International Cooperation and Quality Enhancement in Higher Education (HK-dir) under the Norwegian Partnership Program for Global Academic Cooperation (NORPART) with support from the Norwegian Ministry of Education and Research (MER), and the Ministry of Foreign Affairs (MFA) (CEER project, Project no: 2021/10345).

The PhD project team was comprised of the following members:

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Deepak Pandey,

Steinkjer, Norway, 15 December 2022

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Paper I **Pandey, D**., Mansouryar, M., Novoa-Garrido, M., Næss, G., Kiron, V., Hansen, H.H., Nielsen, M.O., Khanal, P. (2021). Nutritional and anti-methanogenic potentials of macroalgae for ruminants In Xingen Lei (ed), Seaweed and microalgae as alternative sources of protein: CH14 – Developing seaweed/macroalgae as feed for ruminants. Burleigh Dodds Science Publishing, UK. DOI: 10.19103/AS.2021.0091.14.

Paper II **Pandey. D**., Hansen, H.H., Dhakal, R., Aryal, N., Rai, S.P., Sapkota, R., Nielsen, M.O., Novoa-Garrido, M., Khanal, P. (2022) Interspecies and seasonal variations in macroalgae from the Nordic region: Chemical composition and impacts on rumen fermentation and microbiome assembly. J Cleaner Production. https://doi.org/10.1016/j.jclepro.2022.132456.

Paper III **Pandey, D**., Næss G., Fonseca, A.J.M., Maia, M.R.G., Cabrita, A.R.J., and Khanal, P. (2022). Differential impacts of water blanching on the chemical composition, carbohydrate profiling, and *in vitro* digestibility of two brown macroalgae (Ochrophyta, Fucales): *Ascophyllum nodosum* and *Fucus vesiculosus*. Under review (Algal research, Manuscript number: ALGAL-D-22-00728)

Paper IV **Pandey, D**., Doncheva, A.I., Sapkota, R., Kiron, V., Dalen, K.T., and Khanal, P. (2022) The brown macroalgae *Ascophyllum nodosum* and *Fucus vesiculosus* favorably modulate the cecal microbiome in high fat diet-induced obese mice. Manuscript.

List of abbreviations

Acronym	Abbreviation
AN	*Ascophyllum nodosum*
AOAC	Association of Official Analytical Chemists
As	Arsenic
ASVs	Amplicon sequence variants
BAT	Brown adipose tissue
BOHB	Beta-hydroxybutyrate
Br	Bromine
BUN	Blood urea nitrogen
Ca	Calcium
Cd	Cadmium
CF	Crude fiber
CH_4	Methane
Co	Cobalt
CO_2	Carbon dioxide
CP	Crude protein
Cu	Copper
DM	Dry matter
DPPH	1,1-Diphenyl-2-Picrylhydrazyl
EC	European Commission
EAA	Essential amino acids
EAAI	essential amino acid index
Fe	Iron
FV	*Fucus vesiculosus*
GA	Guluronic acid GA
GC	Gas chromatography
GGT	Gamma-glutamyl transferase

GonFat	Gonadal fat
H_2	Hydrogen
HF	High fat
HNO_3	Nitric acid
HPLC	High-performance liquid chromatography
HTB	High-temperature blanching
I	Iodine
K	Potassium
LS	Least square means
LSU	Livestock units
LTB	Low-temperature blanching
MA	Mannuronic acid
Mg	Magnesium
Mn	Manganese
MS	Maize silage
N	Nitrogen
Na	Sodium
NDF	Neutral detergent fiber
NDFom	Ash corrected neutral detergent fiber
NEFA	Non-esterified fatty acids
NFE	Nitrogen free extracts
Ni	Nickel
NTS	Norwegian transgenic center
–OH	Hydroxyl group
OM	Organic matter
OM	Organic matter
OMD	Organic matter degradability
P	Phosphorus

PAD	Pulsed amperometric detection
Pb	Lead
Pb	Lead
PCR	Polymerase chain reaction
PGE	Phloroglucinol equivalents
PUFAs	Polyunsaturated fatty acids
S	Sulphur
SCFA	Short-chain fatty acid
Se	Selenium
SubFat	Subcutaneous fat
TC	Total cholesterol
TD-NMR	Time Domain-Nuclear Magnetic Resonance
TG	Triglycerides
TGP	total gas production
TMAH	Tetramethylammonium hydroxide
TPC	Total polyphenol content
TSC	Total sugar content
UCPH	The University of Copenhagen
UiO	The University of Oslo
UV	Ultraviolet
VCO_2	Volume of carbon dioxide consumption
VFA	Volatile fatty acid
VO_2	Volume of oxygen consumption
Zn	Zinc

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Abstract

Marine macroalgae, also called seaweeds, possess good nutritional traits and bioactive properties and, thus, may serve as sustainable and alternative feeding components for livestock. However, there are wide species-specific and seasonal variations in macroalgal chemical composition. Such variations can alter nutritive values, digestibility, and bioactivities of macroalgae, such as enteric methane (CH_4) mitigation from ruminants and effects on animal health. This study aimed to test the hypotheses that a) the potential of macroalgae as a sustainable ruminant feeding resource is affected by their species- and season-specific variabilities in chemical compositions, b) macroalgal chemical composition and digestibility can be optimized by specific post-harvest hydrothermal pre-treatments, c) the content of macroalgal bioactive compounds can be associated with health-beneficial outcomes in animals.

Initially, 12 different macroalgae species harvested from the Norwegian coast were characterized for seasonal (autumn vs. spring) and interspecies differences in chemical composition. Then, the impacts of macroalgae on *in vitro* rumen fermentation characteristics were explored using them as feed additives (20% dry matter (DM) inclusion). Afterward, post-harvest hydrothermal processing (water blanching) was applied aiming to optimize the chemical composition and *in vitro* digestibility (for ruminants and monogastric animals) of the polyphenol-rich brown macroalgae species *Ascophyllum nodosum* and *Fucus vesiculosus*. Finally, the effects of the same two brown algae as feed ingredients (5% DM inclusion in a high-fat (HF) diet) on energy metabolism and cecal microbiome were explored in mice.

Macroalgae demonstrated favorable nutritional composition with greater contents of crude protein (CP) and minerals in the spring than in the autumn. The bioactive compound, total polyphenol (TPC), was greater in the autumn in brown species. High levels of macroalgal TPC were negatively associated with ruminal feed fermentability when using macroalgae as feed additives. However, the TPC-rich brown

species, mainly the *A. nodosum* and *F. vesiculosus* from autumn harvest, mitigated ~48–63% of *in vitro* ruminal CH_4 production but also suppressed feed degradation by inhibiting cellulolytic bacteria. The digestibility of both TPC-rich brown algae did not improve or slightly deteriorated with water blanching in both ruminant and monogastric animal models. Nevertheless, high-temperature blanching effectively lowered selective excess minerals (Na, K, and I) and arsenic in both macroalgae biomass, thus improving the safety of macroalgal biomass as animal feed. When included in the HF diet and fed to mice, both *A. nodosum* and *F. vesiculosus* reduced obesity-linked bacteria while increasing short-chain fatty acid-producing bacteria in the cecum compared to the mice fed only the HF diet. This improvement in cecal microbiota was accompanied by a ~40% reduction in body fat mass.

Hence, selective red, green, and brown macroalgae with low TPC and high CP can be promising feed additives for ruminants. The TPC-rich brown algae could be utilized as anti-methanogenic components for ruminants and health-promoting dietary ingredients for monogastric animals. In the future, dose-response studies are required to identify appropriate inclusion levels of macroalgae for effective methane emission reduction from ruminants and assess the impacts on animal health and production parameters. Moreover, efficient macroalgal biorefinery/processing approaches are needed to extract high-value bioactive compounds from macroalgae biomass, which could be utilized for methane-mitigating or health-promoting purposes for animals.

Sammendrag

Marine makroalger, også kjent som tang, har gode ernæringsmessige og bioaktive egenskaper, og har derfor potensiale til å brukes som alternative, bærekraftige komponenter i fôr til husdyr. Det er imidlertid store arts- og sesongavhengige variasjoner i makroalgenes kjemiske sammensetning. Slike variasjoner kan påvirke næringsverdi, fordøyelighet og bioaktive egenskaper til makroalgene, for eksempel reduksjon av enterisk metan (CH_4) fra drøvtyggere og effekter på dyrehelse. Denne studien hadde som mål å teste hypotesene om at a) makroalgenes potensiale som en bærekraftig ressurs som drøvtyggerfôr påvirkes av algenes arts- og sesongspesifikke variasjoner i kjemisk sammensetning, b) makroalgenes kjemiske sammensetning og fordøyelighet kan optimaliseres ved spesifikk hydrotermisk forbehandling etter høsting, og c) innholdet av makroalgenes bioaktive forbindelser er assosiert med forbedret helse hos dyr.

Først ble kjemisk sammensetning for 12 forskjellige makroalger som var høstet fra norskekysten kartlagt med hensyn på sesongvariasjoner (høst vs. vår) og artsforskjeller. Deretter ble virkningen av makroalgenes egenskaper som fôrtilsetningsstoffer (20% tørrstoff-inkludering) på *in vitro* vomfermentering undersøkt. Videre ble hydrotermisk prosessering etter innhøsting (forvelling) utført med sikte på å optimalisere den kjemiske sammensetningen og *in vitro* fordøyelighet (for drøvtyggere og enmagede dyr) av to polyfenolrike brune makroalgearter, grisetang (*Ascophyllum nodosum*) og blæretang (*Fucus vesiculosus*). Til slutt ble effekten av de samme to brunalgene som fôringredienser (5% tørrstoff-inkludering i en fettrik (HF) diett) på energimetabolisme og mikrobiomet i blindtarmen (caecum) utforsket hos mus.

Makroalger hadde en gunstigere næringssammensetning på våren enn på høsten, med større innhold av råprotein og mineraler på våren. Imidlertid var den bioaktive forbindelsen total polyfenol (TPC) større om høsten hos brune algearter. Høyere nivåer av makroalge-TPC var negativt assosiert med gjæring av drøvtyggerfôr når makroalger

ble brukt som fôrtilsetningsstoffer. Videre reduserte TPC-rike brune arter, hovedsakelig grisetang og blæretang høstet på høsten, ~48–63% av *in vitro* CH_4-produksjon, men undertrykte samtidig fôrnedbrytingen ved å hemme de cellulolytiske bakteriene. Fordøyeligheten ble uendret eller noe dårligere med forvelling for begge de TPC-rike makroalgene i dyremodeller, med både drøvtyggere og enmagede dyr. Imidlertid reduserte forvelling ved høy temperatur effektivt overskudd av utvalgte mineraler (Na, K og I) og arsen i biomassen til begge makroalgene, noe som forbedret deres sikkerhet som dyrefôr. Både grisetang og blæretang førte til forbedret mikrobiota i blindtarmen når de ble inkludert i HF-dietten og gitt til mus. Musene viste reduserte nivåer av fedmekoblede bakterier og økte nivåer av kortkjedede fettsyreproduserende bakterier sammenlignet med musene som ble matet med HF-diett uten makroalger. Dette ble ledsaget av en ~40% reduksjon i kroppsfettmasse.

Derfor kan utvalgte røde, grønne og brune makroalger med lav TPC og høyt innhold av råprotein være lovende fôrtilsetningsstoffer for drøvtyggere. De TPC-rike brunalgene kan brukes som metanreduserende komponenter for drøvtyggere og helsefremmende kostholdsingredienser for enmagede dyr. I fremtiden er det nødvendig med «dose-respons-studier» for å identifisere passende inklusjonsnivåer av makroalger for effektiv metanreduksjon fra drøvtyggere og å vurdere de generelle innvirkningene på helse- og produksjonsparametrene til ulike dyr. For å kunne utnytte stoffer som kan brukes til metandempende eller helsefremmende formål for dyr er det dessuten nødvendig med effektive bioraffinerings- eller behandlingsmetoder for å trekke ut bioaktive forbindelser av høy verdi fra makroalgenes biomasse.

1 Introduction

1.1 World population and food demands

With the continuously growing human population, it is projected that the Earth will be inhabited by around 9.7 billion people by 2050, resulting in a 26% increase in the population compared to 2019 (7.7 billion) (United Nations, 2019). This growth in the global population is likely to be predominantly contributed by low and middle-income countries, particularly in sub-Saharan Africa and Central and South Asian regions (United Nations, 2019). By 2050, it is estimated that the overall demand for food will be elevated by a minimum of 35–56% as compared to the baseline of 2010 (van Dijk et al., 2021). There are increasing concerns about how the global food production system will be able to fulfill the elevated food demands of people, also by tackling the unprecedented impacts of climate change and urbanization in the future.

The current global human diet is primarily dominated by cereal (grains) followed by animal products, fruits, and vegetables, but considerable variations exist across the countries in the relative proportions of these products in the diet, particularly cereals and animal products (FAO, 2018a). Animal products contribute to a similar energy supply as cereals to individuals in high-income countries, while the contribution of animal products in low- and middle-income countries is much lower as compared to cereals (FAO, 2018a). As the affordability of food is continuously improving in low and middle-income countries with ongoing economic growth, the share of animal products in the diet is also gradually increasing in those countries, which may lead up to a 78% increase in the global demand for animal products by 2050 (Alexandratos and Bruinsma, 2012). This implies that the future global demand for animal products would potentially be raised by a greater proportion than the demand for other foods where livestock animals would play an important role.

1.2 Role of the livestock sector in food security

The livestock sector has long been playing a critical role in human nutrition by supplying most animal products used for human consumption. This sector alone accounts for ~40–50% of the total agricultural production (Herrero et al., 2016) and contributes to 15% of calories and 31% of global total protein consumption in the form of meat, dairy products, and eggs (Godde et al., 2021). Among the livestock, ruminant species (cattle, buffaloes, sheep, and goats) occupy >70% of total livestock units (LSU), followed by the monogastric species, mainly chickens and pigs, collectively accounting for 25% of LSU (FAO STAT, 2022). The trend in the last 20 years shows that the population of most of those livestock species has been growing steadily over time (FAO STAT, 2022) **(Figure 1)**. This steady growth in the number of livestock is also expected to continue in the upcoming few decades implying that these livestock species will continue to be key players supplying nutritious animal products for humans (Rosegrant et al., 2009). As in the past, the livestock sector may achieve considerable growth in the future by improving feed management and animal productivity with the further intensification of production systems and expanding agricultural land (Herrero et al., 2016). However, this growth will not be straightforward since the livestock sector in the future may face additional challenges, such as environmental issues associated with their own production systems and the ongoing climate change that can adversely affect feed production on the land.

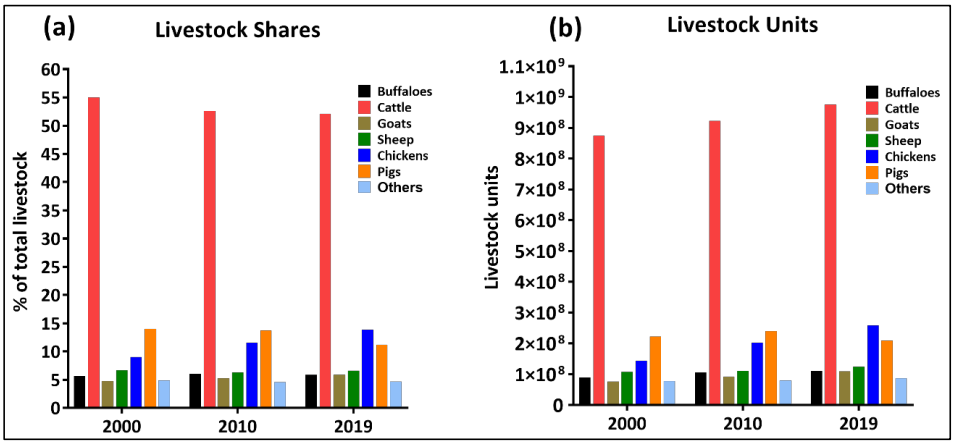

Figure 1: Composition of livestock species in the world in 2000, 2010, and 2019. The livestock units (LSU) in the FAO database were calculated using the reference coefficients for different livestock types relative to the grazing equivalent of one adult dairy producing 3000 kg of milk annually, fed without additional concentrated foodstuffs (=1 LSU) (FAO, 2011). The graphs are produced from the FAO database under the Creative Commons Attribution-Non Commercial-Share Alike 3.0 IGO (CC BY-NC-SA 3.0 IGO) (FAO STAT, 2022).

1.3 Challenges of the livestock production system

Although the livestock feed ingredients comprise several non-human edible by-products such as grass, straw, hay, and plants, they also consume different human-edible products, including cereals and legumes. The livestock production system occupies 30–40% of the world's cultivable land and 32% of the freshwater, thus competing with the human food production systems for resources (Mottet et al., 2017, Herrero et al., 2016). This competition between humans and animals can be counterproductive to human food security (Van Zanten et al., 2019) and should be minimized as much as possible. With the increasing land degradation, depletion of fresh water, and climate change, the feed production system in the future may be severely affected, which can cause shortages of feeding resources for livestock (Makkar et al., 2016). Hence, recognizing new and alternative feeding resources with minimal competition as human foods and the more efficient utilization of the locally available feed resources would play a vital role in managing sufficient and quality

feeding materials for sustainable livestock production in the future (Makkar et al., 2016).

Another major challenge to the livestock production sector is to minimize enteric methane (CH_4) emissions that mostly originate from the ruminant's digestive system via enteric fermentation of feeds. Ruminants are considered responsible for ~18% of total anthropogenic CH_4 releases — a potent greenhouse gas with 28 times higher global warming potential than carbon dioxide (CO_2) (Mizrahi et al., 2021). The process of enteric methanogenesis could also be associated with up to 15% of gross energy loss from the feed lowering the feed efficiency (Van Nevel and Demeyer, 1996). Reducing this ruminal CH_4 production and increasing the feed efficiency and productivity of ruminants has been of great interest to researchers, farmers, and policymakers (González-Recio et al., 2020). Thus, future livestock production should discover efficient measures to minimize this carbon footprint while also enhancing animal feed efficiency and productivity. Identifying and utilizing alternative nutritional approaches could be a sustainable solution in this regard.

1.4 Alternative and environment-friendly feeding components

Nutritional or dietary management has been considered one of the effective strategies for managing CH_4 emissions from ruminants. When compared to other CH_4 mitigating strategies such as genetic selection and selective breeding (González-Recio et al., 2020) and the use of anti-methanogenic chemicals (nitrate, chloroform, and 3-nitroxy propanol) (Patra et al., 2017), dietary management is a technically simple and environment-friendly approach with low or no negative impacts on animal health, performance, and environment (Haque, 2018). Feeding a high-fat-containing creamy diet to lambs for an extended period led to a reduction of up to 87% of CH_4 production compared to the lambs-fed grass hay diet (Haque et al., 2014). Other nutritional manipulations, such as increasing the proportions of concentrate, starch, and legumes while reducing the fibrous components, grass, or improving the forage quality, could

lead to up to 40% reductions in enteric CH_4 production from ruminants (Benchaar et al., 2001, Knapp et al., 2014). The CH_4 reductions achieved with such nutritional strategies are attributed to the enhanced feed efficiency, and their application at the farm could be limited due to practical and economic reasons (Knapp et al., 2014, Haque et al., 2014). Some of these approaches may adversely affect rumen function upon an extended application period. Thus, novel dietary ingredients with promising anti-methanogenic potential are required for better nutritional manipulations while maintaining the proper rumen functions and animal health.

In recent decades, marine macroalgae have gained extensive research interest as a potential alternative and anti-methanogenic feed resources due to their reasonable level of basic nutrients such as protein (Mæhre et al., 2014, Dawczynski et al., 2007) as well as their high contents of minerals, carbohydrates (fiber), and bioactive compounds (Rupérez, 2002, Cabrita et al., 2016, Holdt and Kraan, 2011). In addition, certain macroalgae have demonstrated their great potential to reduce enteric CH_4 production from ruminants both *in vitro* (Machado et al., 2014, Maia et al., 2016) and *in vivo* (Kinley et al., 2020, Li et al., 2016) when included in the ruminant feed. Due to these nutritional attributes and bioactive properties, macroalgae have emerged as an attractive bioresource that could be utilized to sustainably rear livestock in the future and minimize their contribution to greenhouse gas emissions.

1.5 Taxonomy and occurrence of macroalgae

Marine macroalgae, commonly called seaweeds, are plant-like macrophytes naturally growing in seawater, and thousands of macroalgal species exist in the littoral zone across coastal areas worldwide (Makkar et al., 2016). Many species of macroalgae are also being cultivated artificially via aquaculture, which, in fact, covers more than 95% of the total global production of macroalgae (Ferdouse et al., 2018). Macroalgal biomass accounted for ~30% of the total global aquaculture production in 2019 (120 million tonnes) and generated ~36 million tonnes of wet biomass (Cai et al., 2021).

Based on their color or pigmentation, macroalgae are divided into three large taxonomic groups: brown (phylum: Ochrophyta), green (phylum: Chlorophyta), and red (phylum: Rhodophyta). Macroalgae vary not only in their pigmentation but also in size and habitat in the coastal ecosystem. Brown macroalgae are the largest (up to 45 m in length) and mostly occupy the upper intertidal to the subtidal zone, while red species spread from the low intertidal zone to a depth of 100 m, while green species are usually found in the shallow waters and tide pools of the intertidal zone (Makkar et al., 2016). The three macroalgae groups (types) exhibit great variability in their chemical compositions, including the contents of nutrients and bioactive components that may influence their nutritional and bioactive properties, as described in the following sections.

1.6 Chemical composition of macroalgae

The wet biomass of macroalgae contains 70–90% of water, and their biomass needs to be quickly consumed or dried after harvesting to avoid deterioration and microbial degradation (Makkar et al., 2016). In this work, the chemical compositions of macroalgae are described on a dry matter (DM) basis unless stated otherwise.

Protein Contents

The contents of protein in macroalgae vary widely with their types and species. Brown species generally contain the least amount of crude protein (CP) as compared to the green and red species (Dawczynski et al., 2007, Biancarosa et al., 2017, Vieira et al., 2018) and therefore exhibit the lowest value as a protein source among the macroalgae. Although a few brown species, such as *Undaria pinnatifida* and *Fucus serratus,* are reported to contain 17–20% of CP (Fernández-Segovia et al., 2018, Marsham et al., 2007), CP content in brown macroalgae usually remains <15% of DM (Vieira et al., 2018, Biancarosa et al., 2017, Fleurence, 1999). Red macroalgae have shown the highest CP content, which ranges from 3–50% (Marsham et al., 2007, Yanshin et al., 2021). Certain red species, particularly *Porphyra tenera* and *Palmaria*

palmata have exhibited a similar CP level as one of the standard protein feeds, soybean (Fleurence, 1999). Green macroalgae remain in the middle of red and brown species with a CP content of 5–33% of DM, *Acrosiphonia,* and *Ulva* spp., being the two richest species (Mæhre et al., 2014, Tayyab et al., 2016). Based on their protein contents, red and some green species of macroalgae could potentially act as important alternative protein sources for animals.

However, the reported CP levels in macroalgae could have been overestimated because of the indirect analysis method where total nitrogen (N) content is first determined and then multiplied by N to protein conversion factor of 6.25 (Angell et al., 2016b, Biancarosa et al., 2017). This indirect estimation is based on the traditional assumption that food protein contains 16% of N, and all the N is bound to the protein. However, as various non-proteinaceous compounds, including ammonia, nucleic acids, nitrate, urea, chlorophyll, and alkaloids, also contain N, and some amino acids exist in the free form (Mæhre et al., 2018), the indirect method based on N to protein conversion factor of 6.25 often results in overestimation of the protein levels. This issue becomes more important for macroalgae as 22–45% of their total N exits as non-protein N (Biancarosa et al., 2017), and they contain 3.4–24% of free amino acids (Vieira et al., 2018). Thus, recently, a lower or species-specific N to protein conversion factor has been recommended for estimating macroalgae's CP content, which provides more accurate protein levels in macroalgae (Angell et al., 2016b, Biancarosa et al., 2017).

Quality and digestibility of macroalgal protein

While considering an alternative feed as a protein source, not only the concentration of protein but also its quality would be pivotal. Two different measures are traditionally used to evaluate the protein quality: 1) essential amino acid index (EAAI) — a geometric mean of ratios of the essential amino acids (EAA) present in the test protein with the content of the same EAA in the standard protein (e.g., whole egg protein) or reference pattern and 2) amino acid score (chemical score) — a ratio of the

content of an EAA in the test protein with the same amino acid in the requirement pattern (requirement) (FAO, 2018b, Oser, 1959, Dawczynski et al., 2007). An ideal protein should have a chemical score of 1.0 (or 100% if expressed as %) for each EAA and a high EAAI value (1 or greater). Despite the variability among studies, the proportion of EAA in macroalgal proteins ranges from 24 to 90% of the total amino acids, and red macroalgae species have higher EAA proportions than the brown and green species (Gaillard et al., 2018, Dawczynski et al., 2007, Mæhre et al., 2014). The proportions of EAA and chemical scores suggest that proteins from several macroalgae species could be of superior quality than the proteins from cereals and leguminous plants (Mæhre et al., 2014) and may be similar or even higher than the conventional protein sources such as fishmeal, soybean meal (Angell et al., 2016a), casein, and ovalbumin (Vieira et al., 2018). Thus, selective macroalgae could be valuable resources that can at least partly replace traditional protein sources for livestock based on their protein contents and quality.

Although the information on the digestibility of macroalgal proteins and amino acids is limited to a few species, it varies considerably. Both the *in vivo* (sheep) (Gülzari et al., 2019) and *in vitro* (monogastric) total tract digestibility of CP was found to be higher for red and green (64–90%) species than that of brown species (55–85%) (Wong and Chikeung Cheung, 2001, Tibbetts et al., 2016, Kazir et al., 2019). Similar to this trend, *in situ* studies in dairy cows illustrated that selective red: *Porphyra* sp., *P. palmata,* and green: *Cladophora* sp. and *Ulva* sp. have a higher total tract degradability (75–90%) of amino acids as compared to brown species including *L. digitata* (~61%) and red species, *Mastocarpus stellatus* (66%) (Gaillard et al., 2018). A certain portion of the CP, as well as amino acids, was able to escape the rumen degradation and thus was available for intestinal digestion, indicating that macroalgae could be suitable sources for both the rumen-degradable and rumen escape proteins for the ruminants (Tayyab et al., 2016, Gaillard et al., 2018). This property of the macroalgal proteins escaping the rumen degradation can be of great benefit to ruminants as this can be

digested and absorbed in the abomasum or small intestine. However, all these results suggest that both the contents and digestibility of protein (or amino acids) are generally lower for brown macroalgae than for other macroalgae. This heterogeneity in the digestibility of the proteins across macroalgae types and species may be associated with their content of other compounds, such as carbohydrates and polyphenolic compounds, which can interact or bind with protein impeding the degradation of CP (Vissers et al., 2018, Gülzari et al., 2019). Thus, the analysis of the contents of complex polysaccharides and polyphenolic compounds in the macroalgae biomass also seem important in the context of determining the digestibility of the macroalgae protein.

Carbohydrates contents

Carbohydrates constitute the major part of the macroalgae biomass but with wide interspecies variability (4–84% of DM) (Schiener et al., 2015, Holdt and Kraan, 2011). Macroalgal carbohydrates have unique composition and function, making them different from terrestrial plants (Roesijadi et al., 2010). The unique difference in the structure of carbohydrates also exists between the macroalgae with their types and species, but they can be broadly categorized as structural and storage carbohydrates (Rioux and Turgeon, 2015).

Brown macroalgae: The principal structural carbohydrates in brown macroalgae include alginates (alginic acid) and fucoidan, whereas laminarin and mannitol are the major storage carbohydrates. Alginate is the most dominant cell wall polysaccharide accounting for 15–50% of brown algal dry weight and plays an important role in the cellular integrity and flexibility of macroalgal biomass (Charoensiddhi et al., 2016).

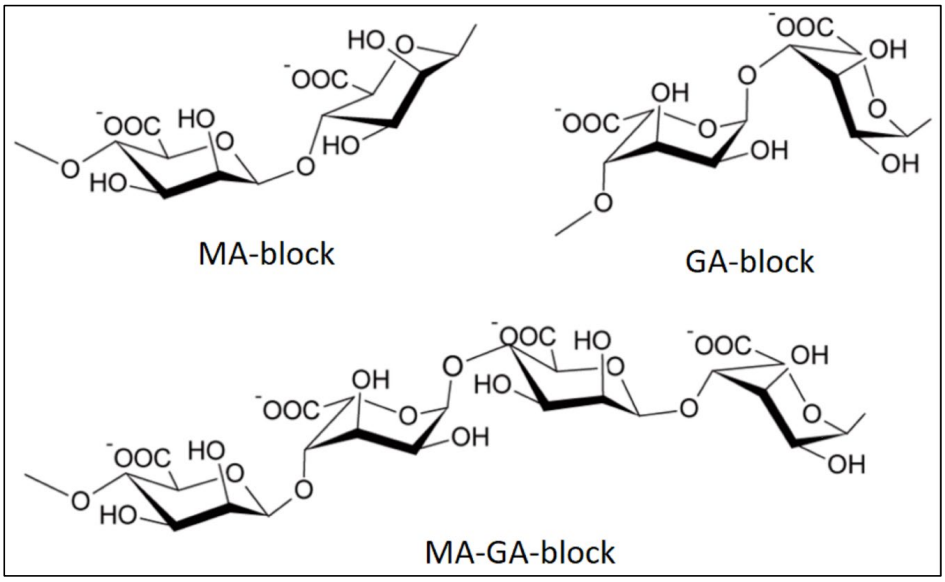

Figure 2: Representative chemical structure of building units of alginate. Adapted with permission from Goñi et al., 2020.

Alginate comprised of two C-5 epimers of uronic acids: β-D-mannuronic acid (MA) and α-L-guluronic acid (GA) polymerized by β-1,4-glycosidic bonds forming either an identical MA–MA or GA–GA or a mixture of MA–GA blocks **(Figure 2)** (Rioux et al., 2007, Manns et al., 2014). The proportion of uronic acids (or MA/GA ratio) and their position in alginate vary with the macroalgal species, which is associated with their physicochemical properties, such as their gel-forming properties (hardness or viscosity) (Manns et al., 2014, Larsen et al., 2003). Alginate with a higher proportion of GA or GA-GA blocks leads to harder and firm gels and vice versa (Khajouei et al., 2021, Draget et al., 1997). Due to its gel-forming property, alginate is widely used as an emulsifier or gelatinizing agent in the food and textile industries (McHugh, 2003). Species with the highest alginate content include *Ascophyllum nodosum* (22–30%), *Laminaria digitata* (25–47%), *Laminaria hyperborea* (17–38%), and *Sargassum* spp. (17–45%) (O'Sullivan et al., 2010).

Fucoidan or fucan, a sulfated polysaccharide, is another major structural polysaccharide in brown macroalgae which comprises 1–32% of the dried macroalgal biomass (García-Ríos et al., 2012). The position of sulfate groups and branching of the fucoidan backbone varies depending upon the macroalgae species (Ale and Meyer, 2013). In general, fucoidan is composed of α-(1,3) and α-(1,4) linked monomeric units of α-L-fucose (fucopyranose) molecules (C-6) sulfated at C-4 or C-2 which can incorporate a branching with a sulfated α-L-fucopyranose or other monosaccharides such as glucuronic acids, galactose, glucose, xylose and acetyl group **(Figure 3)** (Bilan et al., 2008, Rioux et al., 2010, Ale and Meyer, 2013). This carbohydrate also plays an important role in cellular integrity by cross-linking the alginate and cellulose molecules in the cell wall (O'Sullivan et al., 2010). Fucoidan is considered a high-value polysaccharide for both humans and animals due to its diverse bioactive properties: anti-angiogenic, anti-inflammatory, anti-coagulant, anti-tumor (Cumashi et al., 2007), anti-oxidative (Palanisamy et al., 2017), anti-obesity, anti-diabetic, and prebiotic (Shang et al., 2017).

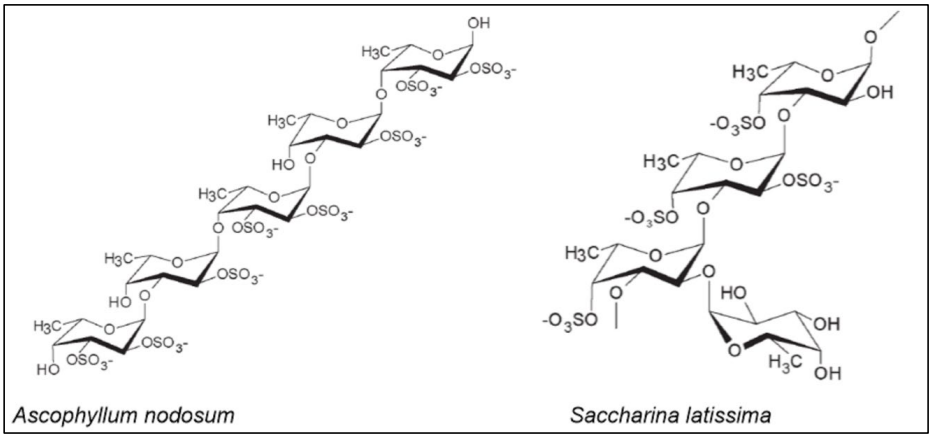

Figure 3: Representative chemical structure of building units of fucoidan from *Ascophyllum nodosum* and *Saccharina latissima*. Adapted with permission from Ale and Meyer, 2013.

Among the two main storage polysaccharides, laminarin accounts for up to 35% of the DM of brown algae (Kadam et al., 2015). A laminarin chain comprises 20–25 units of β-D-glucans (1,3 linked) or glucopyranose molecules, with few 6-O-branching

and β-(1,6)-intrachain linkages (Kadam et al., 2015, Goñi et al., 2020). The reducing end of a laminarin chain either contains glucose or mannitol, resulting in a G or M chain, respectively **(Figure 4)** (Rioux et al., 2007). The degree of branching of the laminarin chain could be related to the water solubility as a highly branched chain has higher water solubility than its less branched counterparts (only soluble in hot water at 60-80 °C) (Rupérez et al., 2002). Laminarin is a bioactive polysaccharide with anti-cancer, anti-inflammatory, antioxidant, antimicrobial, and prebiotic activities (Zargarzadeh et al., 2020, Vigors et al., 2020, McDonnell et al., 2010). Therefore, this polysaccharide draws tremendous interest in applying for animal health benefits. The second storage carbohydrate, mannitol (sugar alcohol), is also present independent of laminarin, accounting for 0-27% of DM depending upon the species of algae (Manns et al., 2014, Schiener et al., 2015). Besides acting as a source of energy for algae, mannitol also contributes to maintaining osmoregulation, protecting algae from reactive oxygen radicals (Rioux and Turgeon, 2015). Due to its chemical inertness, minimal hygroscopicity, and resistance to being metabolized and absorbed in the human intestine (~25%), mannitol is used as a bulking agent or low-calorie sweetener in the food industry, pharmaceutical products, and as an osmotic diuretic drug and in surgeries such as of brain (Rioux and Turgeon, 2015, Soetaert et al., 1999).

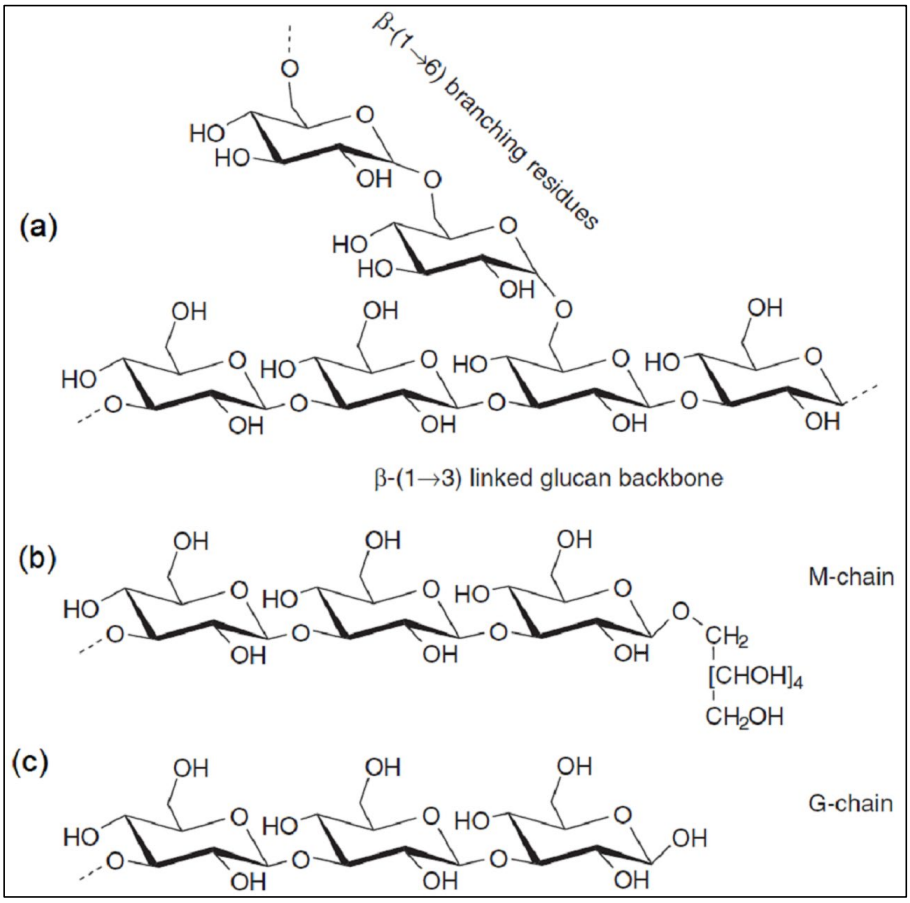

Figure 4: Representative chemical structures of building units of laminarin in three different forms. (a) β-(1, 3) linked backbone of glucan with β-(1, 6) intrachain branching, (b) M-chain with mannitol residue, and (c) G-chain with glucose residue in the reducing end. Adapted with permission from Goñi et al., 2020.

In addition, brown macroalgae contain other carbohydrates, such as cellulose, galactan, xylan, etc., at a variable level depending on the species (Zheng et al., 2022).

Green macroalgae: Like brown species, green macroalgal biomass can constitute a variable level of carbohydrates depending upon their species: *Cladophora* sp. (39%), *Ulva pertusa* (52.3%), *U. lactuca* (35-59%), *Enteromorpha intestinalis* (39%) (Lee et al., 2014, Postma et al., 2018, Rohani-Ghadikolaei et al., 2012). The most prevalent

structural polysaccharides in green species are ulvan (water-soluble) and cellulose (water-insoluble), but they also contain a low amount of starch (1-4% of DM) (Zheng et al., 2022).

Ulvan is a sulfated polysaccharide mostly comprised of repeating units of disaccharides in different combinations: 1) sulfated rhamnose and glucuronic acid (A_{3S}), 2) sulfated rhamnose and iduronic acid (B_{3S}), 3) sulfated rhamnose and xylose (U_{3S}) and 4) sulfated rhamnose and sulfated xylose ($U_{2'S3S}$) **(Figure 5)** (Robic et al., 2009, Yanagisawa et al., 2013, Ray, 2006). The A_{3S} and B_{3S} are the two most prevalent repeating disaccharide forms present in ulvan (Robic et al., 2009).

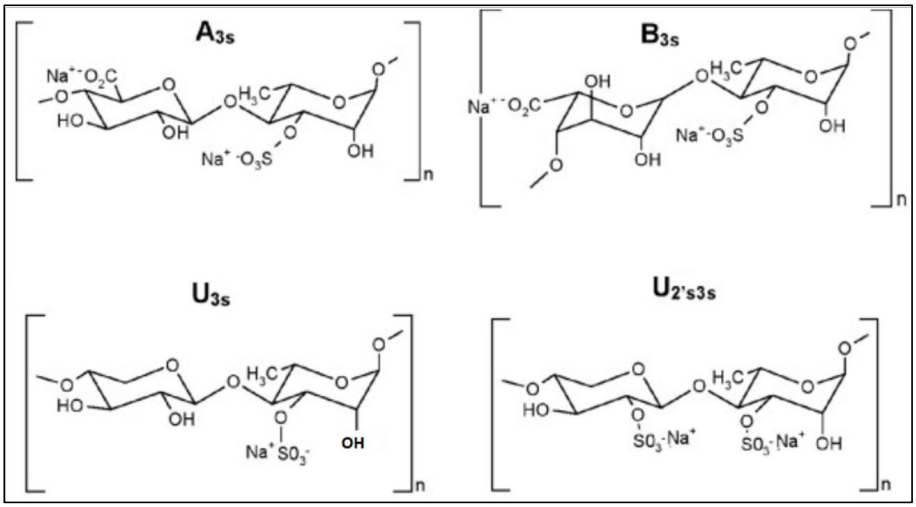

Figure 5: Representative chemical structures of major repeating units in ulvan. Adapted with permission from Robic et al., 2009.

Some studies have indicated that as brown macroalgal polysaccharides, ulvan can be fermented by selective gut microbiota such as *Bifidobacteria* and *Lactobacillus* and thus confer health benefits to the host (Seong et al., 2019, Pratap et al., 2022).

Red macroalgae: Red macroalgae are well known for their abundance of carbohydrates, including carrageenan, agar, agarose, and agaropectin. Certain species

predominantly contain agar (agarose and agaropectin) and are called agarophytes, while others comprise a high level of carrageenan, referred to as carrageenophytes (Zheng et al., 2022). *Gelidium* spp. and *Gracilaria* spp. are two examples of agarophytes (15–31% agar in the cell wall), while *Chondrus crispus*, *Kappaphycus alvarezii*, and *Eucheuma denticulatum* are the most widely used carrageenophytes (30–80% of the cell wall) (Rioux and Turgeon, 2015).

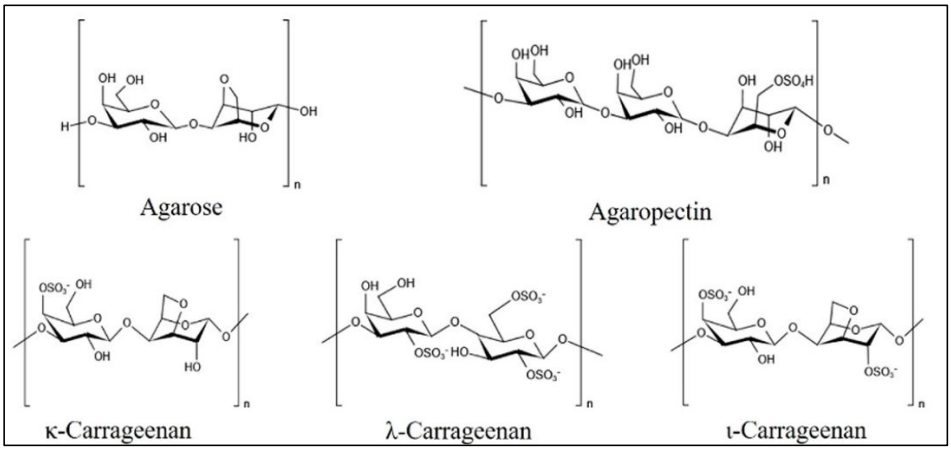

Figure 6: Basic structures of agarose, agaropectin, and three major carrageenan isomers. Adapted with permission from Zheng et al., 2022.

Agar and carrageenan are chemically related complex polysaccharides, and both are sulfated galactans, although the degree of sulfation is higher in carrageenan than in the agar (Gómez-Ordóñez and Rupérez, 2011) **(Figure 6)**. Carrageenan is composed of a backbone of α-(1,3) or β-(1,4) linked D-galactose and (3,6)-anhydro-D-galactose units which may contain elements such as ammonium, calcium, magnesium, potassium, and one or two sulfate groups (De Ruiter and Rudolph, 1997, Rioux and Turgeon, 2015). On the other hand, agar is made up of alternating units of α-(1,3) linked -D-galactose and β-(1,4) linked L-galactose with a (3,6)-anhydrous-L- galactose with a sulfate group in C-4 or C-6 position of galactose ring and as carrageenan, it also contains elements such as sodium, calcium, and magnesium (Rioux and Turgeon, 2015). Among the

various isomeric forms of carrageenan found in red macroalgae, kappa (κ), iota (ι), and lambda (λ) are three main isomers and differ on the number of sulfate groups and their position in the galactose ring (Gómez-Ordóñez and Rupérez, 2011). On the other hand, agar exits in two forms: agarose (neutral and linear) and agaropectin (acidic and branched polysaccharide with sulfate, methyl, or methyl pyruvate groups) (Rioux and Turgeon, 2015).

Carrageenan is readily solubilized in water and forms a highly viscous solution (Stanley, 1987). In contrast, agar is only soluble in hot water (>85 ^{o}C) and forms a gel as it cools down to 32–43 ^{o}C (Rioux and Turgeon, 2015). Due to their gelling properties, agar and carrageenan have long been used as gelatinizing and stabilizing agents in the food industry and other various industrial or medical applications (De Ruiter and Rudolph, 1997, Holdt and Kraan, 2011).

The discrepancies in the relative contents, structure, and biochemical properties of carbohydrates across the macroalgae type and species could have important implications for the digestibility of macroalgal biomass in livestock and hence their feed value.

Digestibility of macroalgal carbohydrates

Limited available information indicates that macroalgal carbohydrates have a very low to medium-high digestibility depending upon the type of carbohydrates and livestock species. Sulfated polysaccharides extracted from brown macroalgae *A. nodosum* (fucoidan) (Chen et al., 2018) and red macroalgae *Gracilaria rubra* (galactose and fucose) were found indigestible with salivary α-amylase and small intestinal enzymes of humans *in vitro* (Di et al., 2018). However, studies with macroalgae-eating Orkney sheep illustrated a certain level of digestibility of complex polysaccharides in ruminant animals due to the benefit of rumen microbial activity. Many of the rumen bacterial isolates collected from the rumen contents of Orkney (North Ronaldsay) sheep efficiently utilized mannitol, mannose, and xylose (Orpin et al., 1985). However,

only limited bacterial isolates belonging to *Clostridium butyricum, Prevotella* spp., *Selenomonas ruminantium*, and *Streptococcus bovis* were able to degrade a significant proportion of laminarin (~58–95%) and alginate (~5.8–80%) and a low extent of fucoidan (1.7–20.3 %), cellulose and agar (Williams et al., 2013, Orpin et al., 1985). Hence, storage polysaccharides mannitol and laminarin seem to be better utilized, while structural carbohydrates may be poorly digestible in ruminants. The polysaccharide utilization was higher in macroalgae-fed than the sheep fed with other pastures, and this was associated with the enrichment of those polysaccharide-degrading bacteria and ciliated protozoa, *Dasytrichia ruminantium* (Orpin et al., 1985). This suggests that rumen microbiota could be modulated by macroalgae feeding, and polysaccharide degradation may be enhanced with the adaptation of microorganisms to macroalgae-based diets.

On the other hand, macroalgal polysaccharides are partially fermented by selective gut microorganisms in the large intestine of animals, including the monogastric species (Di et al., 2018, Chen et al., 2018). Gut bacteria such as *Bacteroides*, *Bifidobacteria*, and *Lactobacilli* can degrade these polysaccharides (particularly, laminarin and fucoidan) to produce short-chain fatty acids (SCFA) (e.g., acetate, butyrate, propionate) which have the inhibitory properties against harmful gut bacteria such as *E. coli* and *Salmonella* leading to *the* improved intestinal health for the host (Seong et al., 2019, Reilly et al., 2008, Charoensiddhi et al., 2017). Therefore, despite being less digestible, macroalgae polysaccharides could be important ingredients to maintain the healthier gut status of the animals.

Lipid contents

Macroalgae generally contain a low lipid level which ranges from 0.3–7% of their dry matter (D'Armas et al., 2019, Lorenzo et al., 2017, Rodrigues et al., 2015). However, the macroalgal fat is enriched with long-chain polyunsaturated fatty acids (PUFAs), such as ω-3 (e.g., eicosapentaenoic acid, C20:5; and docosahexaenoic acid, C22:6) that can improve the cardiovascular health (Mæhre et al., 2014, Cvitković et al., 2021).

Among three types, red and brown macroalgae are considered better sources of such long-chain fatty acids, while the fatty acid composition of the green species resembles that of terrestrial plants (Biancarosa et al., 2018, Mæhre et al., 2014). Despite the low-fat content, mainly brown and red macroalgae could enrich ω-3-PUFA in animal tissues such as meat, mainly for monogastric species (Ribeiro et al., 2013).

Ash and mineral contents

Macroalgae contain a high ash level, ranging from 7–73% of DM of their biomass depending upon the species (Pereira, 2011, Cabrita et al., 2016). This considerable variation in the ash contents originates from the characteristic differences of macroalgae species, their type, and the harvest season (Tayyab et al., 2016). Macroalgae are enriched with macrominerals, such as sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg), and these four elements can constitute ~97% of the total macrominerals (Rodrigues et al., 2015). They also have a good profile of microminerals/trace elements, mainly dominated by iodine (I), iron (Fe), manganese (Mn), and zinc (Zn) (Biancarosa et al., 2018, Rodrigues et al., 2015). The overall mineral content in macroalgae is greater than that of terrestrial plants by a minimum of 10-fold (Rupérez, 2002, Pereira, 2011). Hence, macroalgae have the potential to fulfill most of the mineral requirements of both humans and farm animals (Cabrita et al., 2016). In addition, macroalgal minerals may be more efficiently delivered into animal tissues because of their chelating properties (Evans and Critchley, 2014), suggesting that the dietary inclusion of macroalgae may prevent mineral deficiency or replace mineral supplementation in animal diets.

The mineral abundance in macroalgae is ascribed to their living environment, characterized by a high level of salinity and mineral elements and their unique cellular composition. The macroalgal cell wall contains anionic polysaccharides such as alginate, carrageenan, and ulvan that form ionic bonds with the cationic elements available in the seawater, concentrating the minerals (Mišurcová, 2012, Kidgell et al., 2019). Through similar mechanisms, macroalgae also accumulate heavy metals, including

arsenic, cadmium, lead, and mercury (Mišurcová, 2012). However, this property of concentrating high levels of minerals and heavy metals has been described as one of the limiting factors of using a significant proportion of macroalgae in feed as selective minerals, such as Na and I, can exceed the dietary requirements and in some cases, the maximum tolerable levels of animals and humans (Nielsen et al., 2020, WHO, 2012). Hence, to minimize the risk of mineral toxicity, careful formulation of macroalgae-based diets depending on the content of specific minerals in the macroalgae biomass, the requirements of a particular mineral for a specific animal, and their physiological states are needed (NRC, 2005, Cabrita et al., 2016). Specific post-harvest biomass processing, such as hot water blanching, could be beneficial to optimize the level of minerals, reducing such risk and improving the profile of other compounds in the macroalgae biomass (Nielsen et al., 2020).

Bioactive compounds

In addition to the basic nutrients and mineral elements, macroalgae produce several bioactive compounds that exhibit diverse biochemical properties that potentially improve human and animal health (Gupta and Abu-Ghannam, 2011). The major bioactive compounds include complex polysaccharides, polyphenols, carotenoids, tocopherols, and bioactive peptides (Holdt and Kraan, 2011). Since the role of different complex polysaccharides in animal nutrition and their bioactive properties have already been discussed, only selective other bioactive compounds will be described in the following sections.

Polyphenols: Polyphenols are a heterogeneous group of compounds that contain a minimum of one hydroxyl group (–OH) attached to the aromatic rings (Brglez Mojzer et al., 2016). Together with some terrestrial fruits, vegetables, seeds, and essential oils, macroalgae are the major sources of polyphenols (Gómez-Guzmán et al., 2018). Polyphenols are secondary metabolic products and assist macroalgae in maintaining cellular integrity against waves and desiccation and coping with the stressful living environment of high irradiation, salinity, nutrient fluctuation, biofouling, and herbivory

(Amsler and Fairhead, 2005, Schoenwaelder and Clayton, 1998). The major polyphenolic compounds vary with the macroalgae types: phlorotannins in the brown species while bromophenols, flavonoids, and phenolic acids in the red and green species (Gómez-Guzmán et al., 2018). In general, brown macroalgae contain greater polyphenolic compounds than other algae, which may range from 0.1% in *L. digitata* (Schiener et al., 2015) to 12-14% of dry weight in *A. nodosum* and *Fucus vesiculosus* (Ragan and Jensen, 1978). Therefore, brown species could serve as valuable sources of these phenolic compounds.

Macroalgal polyphenols have been found to be associated with diverse biological activities in *vitro* and *in vivo*. Phlorotannins from diverse brown macroalgae have shown potent antioxidative activities against different oxidative radicals (Farvin and Jacobsen, 2013). They have also exhibited promising antimicrobial properties against intestinal pathogens of pigs (Ford et al., 2020) and reduction of *in vitro* ruminal CH_4 production (Wang et al., 2008, Vissers et al., 2018). Another phenolic compound, bromoform, present in red macroalgae, *Asparagopsis* spp., seems to be an active compound contributing to effectively reducing the enteric CH_4 production *in vitro* (Machado et al., 2014) and *in vivo* in cows (Kinley et al., 2020, Roque et al., 2019b) and sheep (Li et al., 2016) even at a low macroalgal inclusion. Thus, the relative content and type of polyphenolic compounds in the biomass could be vital while determining the bioactive potential of macroalgae and their benefits to animal health and environmental health.

Pigments: Macroalgae comprise various pigments, including chlorophylls, carotenoids, and phycobiliproteins (Aguilera et al., 2002). The chlorophyll *a,* which is present in all macroalgae, gives a greenish color to the algae, and this pigment is masked by fucoxanthin in brown and phycobiliproteins in red macroalgae delivering the characteristic brown and red color, respectively (Pangestuti and Kim, 2011). Chlorophyll *a* is the primary photosynthetic pigment, whereas accessory pigments, such as chlorophyll *b,c,d*, and carotenoids (carotenes and xanthophylls) also contribute

by capturing and passing energy from the sunlight that chlorophyll itself can not absorb (Cikoš et al., 2022). Besides photosynthesis, macroalgal pigments, particularly the carotenoid (xanthophyll), serve as photoprotective compounds for macroalgae (Häder and Figueroa, 1997). Moreover, fucoxanthin from brown macroalgae is involved in antioxidative (Foo et al., 2017), anti-bacterial (Karpiński and Adamczak, 2019), anti-obesity (Maeda et al., 2008), and anti-inflammatory (Su et al., 2019) properties. Therefore, the relative content and type of pigments in the macroalgae seem to be of great value for the bioactive potential of macroalgae.

Other bioactive compounds: Macroalgae also contain several other bioactive compounds, including tocopherols, peptides, vitamins, terpenes, etc., that also exert animal health benefits (Holdt and Kraan, 2011). However, the details of these compounds are not discussed here because that is not a central focus of this study.

1.7 Seasonal variations in the chemical composition of macroalgae

The section above on chemical composition suggested that macroalgal nutritional and bioactive composition varies across their types (or phylum) and among the species within a type. Those nutritional and bioactive attributes of macroalgae can be further influenced by their growing season and geographical location. The contents of protein and ash (minerals) for the temperate/North Atlantic macroalgae are reportedly higher in the winter or spring (Tayyab et al., 2016, de la Moneda et al., 2019, Rødde et al., 2004). In contrast, carbohydrates and polyphenols have generally been found to be higher during the summer or autumn season (Schiener et al., 2015, Molina-Alcaide et al., 2017, Connan et al., 2004). However, for certain species such as *P. palmata* and *L. digitata,* the maximum values of polyphenols have been obtained in spring (Connan et al., 2004, Roleda et al., 2019) and for *A. esculenta and S. latissima* in the winter (Roleda et al., 2019). This indicates the seasonal effects on the macroalgal chemical composition vary not only with the macroalgae species but also depending on the specific nutrients or bioactive compounds. Hence, evaluating the interspecies, type-

specific, and seasonal variabilities in macroalgal chemical composition and its subsequent impacts on the nutritive values and bioactive properties seem extremely important while developing macroalgae as livestock feeds.

1.8 Macroalgae as potential feeds for ruminant livestock

Although macroalgae have a long history of being used as livestock feed, it was limited to a specific period of the year and regions of the world. In Scandinavian countries such as Norway, Iceland, Finland, Sweden, and European countries: France, Germany, and UK (Scotland), and the USA, macroalgae used to constitute the ration of sheep, cattle, as well as of horses, and pigs during the time of feed scarcity until the beginning of 20th century, especially during the winter season (Applegate and Gray, 1995, Evans and Critchley, 2014, Makkar et al., 2016, Chapman, 2012). Later, the conventional feed analysis technologies labeled macroalgae as having poor nutritional values and, thus, less relevant for animal feeding, which scrutinized the feed application of macroalgae (Evans and Critchley, 2014). However, as the search for alternative and sustainable animal feed resources outside the cultivable land has intensified recently, macroalgae have reattracted extensive research interest and reconsidered as relevant feed ingredients for different livestock (Maia et al., 2019, Øverland et al., 2019).

As ruminants have the benefits of an additional stomach, the rumen, characterized by extensive microbial activity capable of degrading complex carbohydrates, macroalgae are considered more suitable for ruminants than other livestock species (Maia et al., 2016). However, the relevance of particular macroalgae species would obviously be based on the degree of degradability in the ruminants. The apparent DM digestibility (in deer) of brown macroalgae, *A. esculenta* (~80%), was found to be much higher as compared to the other two brown species: *A. nodosum* (63.5%) and *F. vesiculous* (64%), however all of these species had better digestibility than the winter forages such as balsam fir, lichen, red maple, rye and white cedar

suggesting their capabilities of replacing of winter forages for ruminants (Applegate and Gray, 1995). Similarly, studies with seven different macroalgae from North Norway indicated that *in vitro* (Molina-Alcaide et al., 2017) and *in situ* (Tayyab et al., 2016) ruminal degradability of macroalgae ranges from low (24–44% for *Pelvetia canaliculata*) to reasonably high (58–86%) for certain other species such as *P. palmata*. Therefore, not all but selective macroalgae species with high rumen or total tract degradability could be relevant for ruminant nutrition purposes. However, the possible dietary inclusion levels and impacts of macroalgae in overall rumen fermentation need to be further assessed.

The rumen degradability of macroalgae will determine their potential dietary inclusion rate and subsequent impacts on rumen fermentation characteristics (e.g., total gas, volatile fatty acids; VFA, organic matter degradability; OMD, CH_4 production) and animal performance. The *in vitro* batch (de la Moneda et al., 2019) and Rusitec fermentation (Maia et al., 2019) studies revealed that selected red (e.g., *Gracilaria vermiculophylla, P. palmata*, *P. umbilicalis*), brown (e.g., *L. digitata*, *S. latissima*) and green species (e.g., *Cladophora rupestris*, *Ulva rigida*) generate no undesirable effects in rumen fermentation at 20-25% DM inclusion in concentrate or mixed ruminant feed, suggesting their potential to constitute a significant part of ruminant's feed. In agreement with this, another *in vitro* and *in situ* study in Barbarine sheep also indicated no evident effect of green macroalgae, *C. linum,* and *Ulva lactuca* at <200 g kg^{-1} inclusion in the concentrate mixed diets but led to a linear depression on feed degradability when the macroalgal inclusion exceeded 200 g kg-1 feed (Rjiba-Ktita et al., 2017). The inclusion level could be even lower for other species with low digestibility (de la Moneda et al., 2019). Interestingly, a study with 20 different tropical macroalgae (at 16.7% of OM inclusion in the flinders grass) demonstrated that selected red (*Asparagopsis taxiformis*), brown (*Dictyota bartayresii*), and green (*Cladophora patentiramea*) macroalgae can lower *in vitro* ruminal CH_4 production (72 h) by 98.9%, 92%, and 66.3%, respectively as compared with decorticated cottonseed (DCS)

(Machado et al., 2014). At the same time, *A. taxiformis*, and *D. bartayresii* led to a 46.8% and 38.7% lower total VFA compared to DCS, demonstrating their adverse effects on feed degradation and potentially animal productivity (Machado et al., 2014). Other *in vitro* (Kinley et al., 2016) and *in vivo* studies in sheep (Li et al., 2016) and cows (Roque et al., 2019b, Kinley et al., 2020) with *Asparagopsis* spp. further demonstrated that they could be high-value anti-methanogenic feed ingredients for ruminants even at a low dietary inclusion (<2% of the DM). However, these species cannot be considered nutritious alternative ruminant feeds at high inclusions because of adverse effects on feed degradability and animal performance. These results imply that macroalgae with high nutritional value might have less anti-methanogenic potential and vice versa. Macroalgae that hold promising nutritional value, as well as CH_4 mitigating potential, could be ideal environment-friendly feeding resources for ruminants. Screening such novel species from the large natural reservoir of macroalgae could be an important research task in the future.

The anti-methanogenic properties of the macroalgae have been ascribed to their secondary polyphenolic metabolites, such as bromoform for red species, *Asparagopsis* (Roque et al., 2019a, Machado et al., 2018) and phlorotannin for brown species (Wang et al., 2008, Vissers et al., 2018). Bromoform and other halogenated analogs of CH_4 have been shown to affect the population and activity of rumen microorganisms, including methanogens and protozoa, and different enzymatic processes of enteric CH_4 production (Machado et al., 2018, Patra et al., 2017, Roque et al., 2019a). The anti-methanogenic mechanisms of other polyphenolic compounds, including phlorotannins, have not been well characterized. Although the species-specific effect of phlorotannins from *A. nodosum* on certain rumen microorganisms has been noted (Wang et al., 2009), the impacts of macroalgae with different levels of polyphenols on the rumen microbiome, particularly the methanogens, and methanogenesis, has not been described. The anti-methanogenic property of macroalgae from the Norwegian coast and their effects on the rumen microbiome has rarely been studied. In the context of

over 100,000 km-long Norwegian coastal line that harbors more than 400 species of macroalgae (Stévant et al., 2017), it would be interesting to characterize the nutritional values of a larger number of species and evaluate whether any macroalgae from this region also encompass the ani-methanogenic potentials. Such characterization of macroalgae would be important to identify the potential species to target for ruminant nutrition or as dietary ingredients to mitigate the enteric CH_4 emissions from the ruminant production sector.

1.9 Macroalgae as potential feeds for monogastric animals

For monogastric livestock, such as pigs and poultry, only a few macroalgae species could be relevant as alternative sources of nutrition but at reasonably low inclusion levels (Makkar et al., 2016, Øverland et al., 2019). Since monogastric animals do not secrete enzymes capable of degrading complex polysaccharides, macroalgae with high carbohydrate contents would have low digestibility in those animals (Holdt and Kraan, 2011). However, small dietary inclusions of macroalgae (<5% DM) or their extracts were found beneficial to improve gut health and productivity of chickens (Kulshreshtha et al., 2014), weaning pigs (Reilly et al., 2008), and rodents (Kim et al., 2018) due to the anti-microbial, antioxidant, and prebiotic effects of polyphenols and polysaccharides. Hence, macroalgal bioactive compounds could be effective nutraceuticals that can potentially replace the use of antibiotics or growth promoters for monogastric livestock species (Ford et al., 2020). Moreover, selective protein-rich macroalgae could be relevant for extracting and valorizing the macroalgal protein for monogastric animals via biorefinery approaches (Bikker et al., 2016).

1.10 Macroalgae as health-promoting dietary ingredients for humans

The biochemical properties of macroalgae or their bioactive compounds can potentially be utilized to improve human health. Studies in murein models revealed that feeding of polyphenolic extracts obtained from brown macroalgae (e.g., *Ecklonia stolonifera*, *Lessonia trabeculate*) could improve blood metabolic profile (e.g., lower

fasting glucose, and insulin, better serum lipid) in diabetic animals as they can lower α-glucosidase and lipase activity (Iwai, 2008, Yuan et al., 2019). Moreover, selective brown macroalgae (e.g., *U. pinnatifida, Laminaria japonica*) or their extracts enriched with fucoxanthin or polysaccharides have been shown to alleviate the body and fat weight gain in diet-induced obese mice by reducing the differentiation of preadipocytes and lipid accumulation and inflammation of adipose tissue (Grasa-López et al., 2016, Yang et al., 2017, Zheng et al., 2021). Due to these properties, brown macroalgae have emerged as potential anti-obesity and anti-diabetic dietary ingredients that may contribute to minimizing the global epidemic of obesity and type 2 diabetes (T2DM) in humans.

One of the typical features of obesity is the dysbiosis of gut microbiota, characterized by an increased proportion of Firmicutes and a reduced proportion of Bacteroidota (Bacteroidetes) (Turnbaugh et al., 2008, Ley et al., 2005). The changes in the composition alter functional attributes of gut microbiota, such as profiles of short-chain fatty acids (SCFA), including acetate, propionate, and butyrate produced from the dietary polysaccharides (Chen et al., 2018, Charoensiddhi et al., 2017). Enhanced SCFA production in the gut is associated with improved intestinal health, and they can regulate the other metabolic and genetic processes related to nutrient catabolism, absorption, and adipogenesis (Turnbaugh et al., 2008, Lu et al., 2016). Limited studies have illustrated that macroalgal compounds, mainly complex polysaccharides and polyphenols, can prevent the unfavorable dysbiosis of the gut microbiome by lowering the proportions of Firmicutes but promoting the Bacteroidota that lead to reduced weight and fat gain and improved gut health (Zheng et al., 2021, Yuan et al., 2019, Jiang et al., 2021). However, as most studies focus on the extracts derived from macroalgae rather than the whole biomass, little is known about how intact macroalgae rich in polyphenols and polysaccharides affects the gut microbiota and obesity development during exposure to an energy-dense high-fat diet. It is also unclear how the intake of brown macroalgae-based diets influences the pattern of energy expenditure in animals.

Anti-obesity drugs aim to reduce energy absorption or increase expenditure, resulting in less fat mass deposition and redistribution of adipose tissue (Wan-Loy and Siew-Moi, 2016). Hence, understanding of effects of macroalgae on these parameters would be important to characterize their effectiveness as potential anti-obesity agents. Since the North Atlantic seacoast is the home for many macroalgae with abundant polyphenols and polysaccharides, such as brown species in the Fucaceae family (e.g., A. *nodosum* and *F. vesiculosus*) (Catarino et al., 2017), some of these species might have the potential to prevent the diet-induced obesity and further metabolic disorders.

1.11 Major bottlenecks of using macroalgae in animal feed

Despite a great reservoir of ~10,000 species in nature and promising nutritional and bioactive attributes, merely more than 10 macroalgae are currently being used for animal feeding purposes (Makkar et al., 2016, Costa et al., 2021). One of the possible reasons could be the insufficient screening of macroalgae species for their potential to support livestock nutrition and health. Therefore, the identification of numerous macroalgae with novel nutritional and bioactive potentials is required to enhance the utilization of macroalgae as an alternative, environmentally friendly, and health-promoting livestock feed. Furthermore, understanding seasonal impacts on macroalgae chemical composition is needed to achieve these potentials at the highest levels. Such characterization needs to be coupled with the in vitro and in vivo feeding trials that could provide a better insight into their digestibility and the impacts of dietary inclusion on animal performance and health.

Besides this, efficient solutions to the bottlenecks hindering the large-scale application of macroalgae biomass in animal feed: excessive contents of certain potentially toxic elements and polyphenols (mainly for brown species), and low digestibility of complex carbohydrates are needed (Cabrita et al., 2017, Bikker et al., 2020, Tabassum et al., 2016). Previous studies suggest that specific post-harvest biomass processing, such as hydrothermal treatments of fresh macroalgae, could be

useful to optimize the level of minerals such as I and toxic metals (Nielsen et al., 2020, Stévant et al., 2018). Hydrothermal biomass treatments can influence the cellular integrity and alter the concentrations and properties of other nutrients, such as sugars and water-soluble nutrients, and bioactive compounds, including polyphenols (Nielsen et al., 2020, Rajauria et al., 2010) and may improve the bioaccessibility of protein or amino acids macroalgae-specific manner (Maehre et al., 2016). These changes in the composition and properties of macroalgal compounds may alter the nutritional value, bioactive potential, and digestibility of the macroalgae biomass in livestock species. However, to our knowledge, studies investigating these three important aspects of hydrothermal pre-treatments: concentrations of nutrients, bioactive compounds, and digestibility of macroalgae in livestock are unavailable. Such studies are therefore needed to shed light on whether any hydrothermal pre-treatments could valorize the feed value for livestock species in practice.

2 Objectives

The overall objective of this PhD project was to establish the role of marine macroalgae as an alternative, environment-friendly, and bioactive feeding resource for livestock. For this, locally based macroalgae species were investigated for their chemical compositions and digestibility in different animal models via *in vitro* simulation studies. Their bioactive potentials and benefits on animal health conferred from the bioactive compounds of macroalgae were studied *in vivo and in vitro.*

Under the major objectives, different sub-objectives were specified as follows:

- To understand the current state of knowledge on the nutritive values and bioactive properties of macroalgae and the status of their utilization as sustainable and environmentally friendly livestock feed **(Paper I).**
- To unravel the interspecies and seasonal variations in the chemical composition of 12 macroalgae species considered relevant for ruminant nutrition and the impacts of macroalgal inclusion on feed degradation parameters, ruminal methane production, and rumen microbiome **(Paper II)**.
- To investigate whether the post-harvest processing of macroalgal biomass, such as hot water blanching, could improve the nutritional profile and enhance the digestibility of the macroalgae in livestock **(Paper III)**
- To understand the role of brown macroalgae on body composition, blood metabolic profile, gut microbial composition, and whole-body energy expenditure in high fat (HF) diet-induced obese mice **(Paper IV)**

3 Hypotheses

- Marine macroalgae harvested in the spring season are more suitable as ruminant feed additives due to the favorable chemical compositions leading to improved degradability in ruminants **(Paper II).**
- Brown macroalgae rich in polyphenols are more effective in reducing enteric CH_4 production from ruminants regardless of the harvesting seasons due to the species-specific modulations of the rumen microbiome and associated rumen fermentation characteristics by their polyphenols **(Paper II)**.
- The chemical composition of anti-methanogenic brown macroalgae can be optimized by post-harvest hydrothermal processing of biomass that minimizes the anti-nutritional compounds such as excess salts, toxic metals, and as well as polyphenols **(Paper III)**.
- Post-harvest hydrothermal processing at high temperature enhances the digestibility of macroalgal nutrients such as carbohydrates and proteins, thereby improving the overall digestibility of the macroalgae in animals **(Paper III).**
- Dietary inclusions of polysaccharide and polyphenol-rich brown macroalgae can contribute to better intestinal health and prevent obesity development in mice exposed to the HF diet by favorably modifying the gut microbiota and body composition **(Paper IV)**.
- The favorable alterations in the gut microbiome and body mass composition of mice supplemented with the brown macroalgae would be associated with their higher whole-body energy metabolism **(Paper IV)**.

4 General methodology

This section provides an overview of the materials and methods used in this PhD work. Further detailed information on the methodologies has been presented in the respective papers included in this thesis.

4.1 Literature matrix preparation

At the beginning of this study, a comprehensive literature review related to this PhD project's field was conducted, and a literature matrix was prepared. The literature review and the matrix were used to identify knowledge gaps in the area and select the macroalgae species that could be relevant for livestock nutrition. Later, this literature review and matrix preparation were extended, which generated another review paper (book chapter). The published book chapter has been included as **Paper I** in this thesis. Based on the survey and literature matrix, 12 different macroalgae species that are predominant in the wild populations or cultivated in the Norwegian coastal waters were selected for the initial research work of this PhD **(Figure 7).**

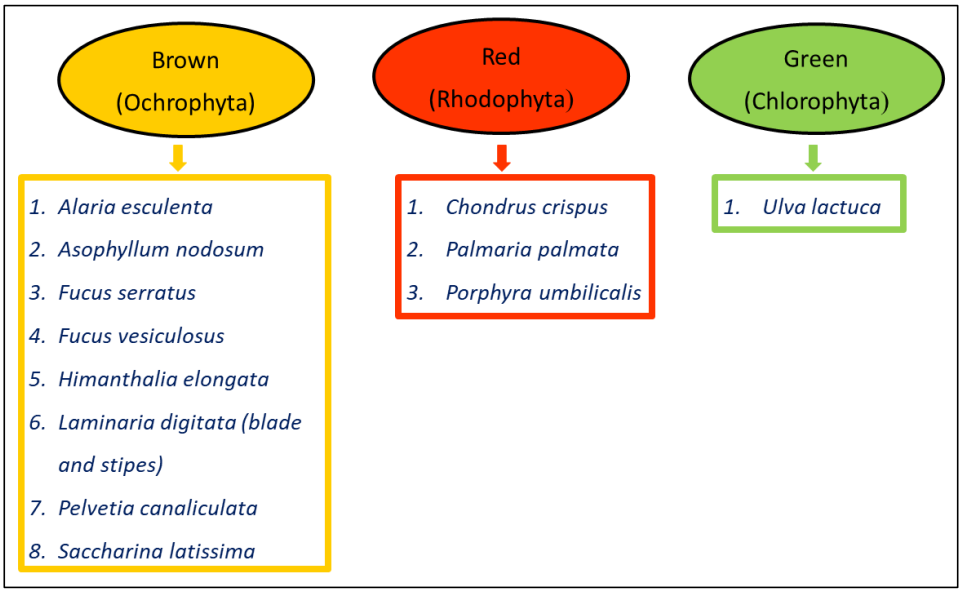

Figure 7: List of studied macroalgae in this study and their phyla (types). Twelve different brown, red, or green macroalgae species were harvested in the autumn and spring during a low tide from Bodø, Norway **(Paper II)**. Two brown macroalgae, *A. nodosum,* and *F. vesiculosus* were harvested in spring for **Paper III** and in autumn for **Paper IV** from Steinkjer, Norway.

4.2 Macroalgae harvesting and sample preparation

In the first experimental study **(Paper II)**, to allow evaluation of both species-specific and seasonal variation among macroalgae, twelve different macroalgae (eight brown, three red, and one green) species were harvested in spring (7–9 May 2019) and autumn (01–03 October 2019) from the natural populations in a coastal area of Bodø, Norway. As macroalgal chemical compositions can be associated with environmental conditions in the habitat, certain environmental parameters: salinity, temperature, dissolved oxygen, and oxygen saturation in the seawater, were monitored before collecting the macroalgae biomass. Macroalgae biomass of each species was manually collected during the low tide from different nearby locations and transported to the laboratory (Mørkvedbukta; Nord University, Bodø) within 2 h of collection. In the

laboratory, macroalgal biomass was cleaned by a three-step washing procedure that involved a stepwise reduction in salinity to minimize the osmotic shock (Tayyab et al., 2016). The cleaned samples were packed in airtight plastic bags and stored at -40 °C until they were lyophilized(freeze-dried) and pulverized to 2 mm size before performing the chemical composition and *in vitro* rumen fermentation digestibility analyses.

Based on the findings of **Paper II**, two brown macroalgae with the highest level of polyphenol and anti-methanogenic properties but lowest digestibility, as well as with reasonably high mineral content: *A. nodosum* and *F. vesiculosus* were selected for the second original research **(Paper III)**. For this study, macroalgal biomass was harvested from the coast of Hoøya, Steinkjer, Norway, and shortly transported to the laboratory of Nord University and cleaned with fresh tap water since there was no availability of seawater in the laboratory. After that, macroalgae samples (n=3) were exposed to the hot water blanching treatments by directly immersing in 10 L of water maintained at 40 °C (Low-temperature blanching; LTB) or 80 °C (High-temperature blanching; HTB) for 5 min in a water oven. Unblanched (raw) samples were only washed three times with fresh water and used as a control (n=3). After the treatments, samples were frozen at -20 °C and later lyophilized before they were ground via a 1 mm sieve. Samples were stored in airtight plastic bags at -20 °C until further analyses **(Paper III).**

The same two brown species: *A. nodosum* and *F. vesiculosus* harvested from Hoøya Steinkjer, Norway, in October-early November 2020 during the low tide and washed with fresh tap water as previously described and lyophilized and prepared as described in paper III. The prepared powdered samples were then used for the diverse chemical composition (Nutrients and bioactive compounds), and mice feed formulations for an *in vivo* feeding trial in mice (**Paper IV)**.

4.3 Chemical composition analyses

In this study, a comprehensive analysis of the chemical composition of macroalgae was performed that covered proximate composition, mineral profile, carbohydrate (sugar) composition, and certain bioactive components: complex polysaccharides, total polyphenol, and brown macroalgal pigment (fucoxanthin) depending upon the research questions addressed in the papers.

Proximate composition

As proximate composition provides a preliminary idea of the nutritive value of feed samples, proximate composition analysis of macroalgae was part of all three experimental studies **(Paper II, III, and IV)**. The proximate compositions were mostly analyzed following the standard protocols of the Association of Official Analytical Chemists (AOAC) (Horwitz, 2010) or the International Organization for Standardization (ISO). While estimating the crude protein (CP) levels based on the Kjeldahl nitrogen, nitrogen to protein conversion factor of 5 was used to minimize the overestimation of CP due to non-protein nitrogen contents in macroalgae (Angell et al., 2016b). For **Papers III and IV**, crude fat content was determined by extraction with 80% petroleum ether and 20% acetone (Commission Regulation (EC) No 152/2009)**.** The level of crude fiber (CF) and neutral detergent fiber (NDF) or ash-corrected NDF (NDFom) were determined via a filter bag technique (Ankom200 Fiber Analyzer, NY, USA).

Mineral composition

Mineral composition analysis was part of all three research papers **(Papers: II, III, and IV)**. However, there were a few variations in the numbers of minerals analyzed across the papers. In **Papers II and IV,** four major macrominerals: sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), and four microminerals: manganese (Mn), iron (Fe), zinc (Zn), and copper (Cu) were determined. For **Paper III**, a more detailed elemental analysis including more macrominerals: sulfur (S) and phosphorus (P) and micromineral: iodine (I), bromine (Br), nickel (Ni), Cobalt (Co), and selenium (Se)

than used in other two papers. For this paper, certain heavy metal elements: arsenic (As), cadmium (Cd), and lead (Pb), were also analyzed. The analysis process of minerals and other elements was preceded by a digestion step where macroalgal powder samples were exposed to nitric acid (HNO3) and hydrogen peroxide or with tetramethylammonium hydroxide (TMAH) (only for I) in a D Microwave digestion system. Then the contents of elements in the digested samples were quantified spectrophotometrically either using a Microwave Plasma Atomic Emission Spectrometer, Inductively-Coupled Plasma Optical Emission Spectroscopy, or an inductively coupled plasma-mass spectrometry depending on concentration and detection limits of the spectrophotometers (European Commission, 2009)**.**

Macroalgal carbohydrates (sugar)

To investigate the impacts of hot water blanching on the composition of macroalgal carbohydrates of *A. nodosum* and *F. vesiclosus*, an extensive sugar composition analysis was performed as reported previously (Hayes, 2012) with some modifications in a commercial laboratory of Celingnis, Ireland **(Paper III)**. For this analysis, finely ground (<850 microns) and homogenized samples were exposed to a two-step acid hydrolysis to degrade the complex macroalgal carbohydrates into sugar monomers: firstly, with 72% H_2SO_4 in a water bath of 30 ^oC (1 h) and secondly, with 4% acid concentration, and autoclaving at 121 ^oC (1 h). This mixture was called acid hydrolysate.

Different sugar components in the diluted and filtered hydrolysates: glucose, fucose, galactose, mannose, arabinose, rhamnose, xylose, and sugar alcohol mannitol, were determined using DIONEX ICS-3000 ion chromatography system equipped with an AS50 autosampler, an electrochemical detector attached with pulsed amperometric detection (PAD), a gradient pump, and a temperature-controlled column and UV-Vis Diode Array Spectrophotometer (Hayes, 2012). After injection, sugars were separated in the Carbo-Pac PA1 guard and analytical columns within 16 minutes and detected using a standard Dionex ‘‘Carbohydrates’’ waveform. In the same acid hydrolysates,

sugar acids: mannuronic, guluronic, glucuronic, and galacturonic, were also quantified by ion chromatography using a custom gradient program that incorporated the sodium hydroxide and sodium acetate. Melibiose was used as the internal standard for all sugar analyses.

Phytochemical (bioactive) compounds

The concentrations of selected phytochemical/bioactive compounds of macroalgae were also determined in this study, as described below.

Total polyphenol extraction and quantification

Total polyphenol contents (TPC) of macroalgae were determined in all three original studies **(Papers: II, III, IV)** to understand variabilities across species and season and their role in bioactive properties and feed degradability. The TPC from the macroalgal powder was extracted by a two-step procedure under darkness: first with methanol (50%) followed by acetone (70%) as described previously (Zhang et al., 2006) with some modifications. The sample-to-solvent ratio was maintained at 1:20 (w/v) for each step, and an acidic medium was created (pH~2) to facilitate the extraction and maintain the stability of the extracted polyphenols. The supernatants collected from those two extractions (after centrifugation at 12000 × *g* for 10 min) were pooled together and used as crude polyphenol extract **(Papers: II and IV)**. Specifically in **Paper III,** crude polyphenolic extracts were filtered, the organic solvent was evaporated by a rotary evaporator, freeze-dried (24 h), and redissolved in deionized water. The TPC in crude polyphenol or freeze-dried extracts was quantified in triplicates by the Folin-Ciocalteu method as described previously using a spectrophotometric microplate reader (absorbance at λ750 nm) (Zhang et al., 2006) and a seven or eight-point standard curve of phloroglucinol dihydrate standard (0–1000 µg mL^{-1}) and hence expressed as phloroglucinol equivalents.

Fucoxanthin contents

For analysis of fucoxanthin content **(Paper III)**, pulverized samples were twice extracted with acetone: first with 80% and second with 100% acetone and then quantified by high-performance liquid chromatography (HPLC) by using acetonitrile/methanol and methanol/ethyl-acetate as eluent gradient and UV detection (445 nm) (Schweiger et al., 2018).

4.4 Antioxidant activities of polyphenol extracts

To measure the effects of water blanching on the antioxidant activity of the extracted polyphenol, a widely used colorimetric method – 1,1-Diphenyl-2-Picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$) radical scavenging assay was performed following the previously published protocols (Cox et al., 2010) with minor modifications **(Paper III)**. Before this, the rotary evaporated and freeze-dried polyphenol extracts were redissolved in deionized water (1000 µg mL^{-1}), and 1 mL of the extract solution (n=3) was mixed with an equal volume of 0.16 mM DPPH solution (in methanol), incubated for 30 min at room temperature under the darkness and read against a methanol blank at 517 nm using a UV-Vis spectrophotometer. Appropriate sample blanks and control samples were also included in the assay. The radical scavenging capacity was calculated by the changes in the absorbance of the solution relative to the control after a correction for the blank samples.

4.5 Digestibility analyses

In vitro *ruminal fermentation characteristics of macroalgae added ruminant feed*

To evaluate the effects of species-specific and seasonal variabilities of macroalgal chemical composition on the digestibility or rumen degradability of macroalgae-added feeds, we performed *in vitro* ruminal fermentation studies simulating the rumen conditions (**Paper II)**. Different parameters that can describe the ruminal feed degradability and function, such as total gas production (TGP), organic matter

degradability (OMD), volatile fatty acids (VFA), CH_4 production, and effects on the rumen microbiome, were evaluated. For this, an automated ANKOM^{RF} gas production system version 11.4 was used, and the experimental setup of the in *vitro* fermentation study is presented in **Figure 8**. Considering the relatively lower fermentability of pure macroalgae as compared to the standard ruminant feed, maize silage (MS) as observed in our pilot study and also based on the previous studies (de la Moneda et al., 2019, Maia et al., 2019), *in vitro* rumen fermentations were performed including macroalgae at 20% DM in the MS (macroalgae: MS,1:4, w/w).

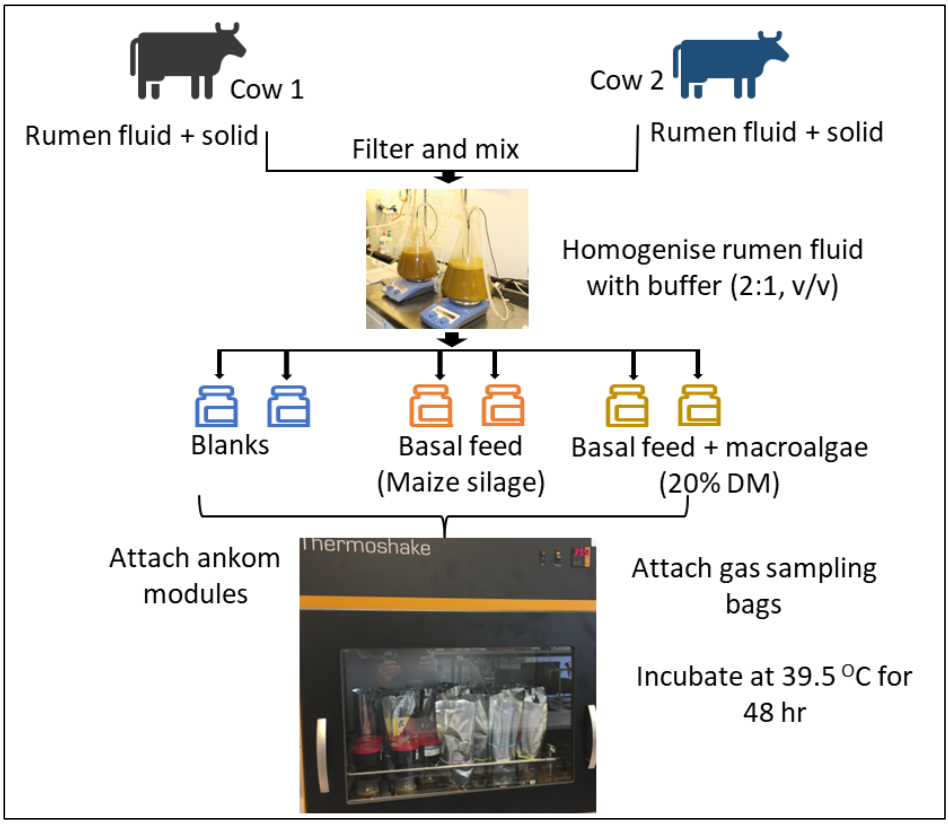

Figure 8: Set up of *in vitro* rumen fermentation studies. The *in vitro* rumen fermentation was performed by simulating the rumen conditions, and rumen microorganisms were acquired as rumen fluid and solids from two rumen-cannulated heifers before the morning feeding.

The rumen fluids, which served as rumen microbial sources for fermentation, were acquired before the morning feeding from the two rumen-cannulated Danish Jersey heifers fed to a maintenance diet of grass silage. The collected rumen fluids were filtered and pooled together before adding the double volume of buffer (1:2, rumen fluid: buffer) containing micro and macrominerals and redox agent (Menke et al., 1979). It was continuously maintained at 39.5 °C and anaerobic condition by flushing with CO_2 gas. This buffered rumen fluid (90 mL) was added to the feed mixture of macroalgae and MS or only MS or blanks (no feed) in a 100 mL glass bottle, flushed with N_2 gas to remove any residual $C0_2$, and fitted with an automatic wireless ANKOM module at the top. To collect the gas samples for measuring the CH_4 production, air-tight gas sampling bags were attached to the selected fermentation bottles from each sample type. The bottles were incubated at 39.5°C in a thermoshaker with an oscillation of 40 rpm for 48 h for the fermentation. Such fermentation was performed twice (in different weeks) with duplicates of each sample type producing a total of four replicates per sample and two gas samples for CH_4.

The pressure generated by the produced gas in the headspace of each fermentation bottle was continuously recorded in a computer wirelessly connected to the ANKOM^{RF} Gas Production System, and the cumulative pressure reading from the whole 48 h of the fermentation was converted to TGP using the ideal gas law. After 48 h, post-fermentation rumen fluid samples were for VFA and rumen microbiome analyses after filtering the fluid with undegraded feed residue through an Ankom filter bag (pore size: 25 µm). The DM and OM contents of the undegraded feed residues were determined gravimetrically and blank-corrected to estimate the DMD and OMD of the feed. The CH_4 percentage in the TGP (48 h) was determined by gas chromatography (GC) using hydrogen as a carrier gas, and the volume of CH_4 produced was calculated by multiplying the CH_4 percentage by TGP. The total VFA production and their profile in the post-fermentation fluid samples were also analyzed by GC, as described previously (Aryal et al., 2021).

Impacts of hot water blanching on digestibility of brown macroalgae

To investigate whether the hot water blanching can optimize the nutritional composition and enhance the digestibility of the TPC-rich and low-digestible brown macroalgae, *A. nodosum* (AN) and *F. vesiculosus* (FV), two other *in vitro* digestibility studies were performed simulating the conditions of the rumen of ruminants and the total tract of monogastric livestock (pigs), respectively **(Paper III)**.

Digestibility in ruminants

The ruminal digestibility of DM, OM, and CP of brown macroalgae that underwent post-harvest hot water blanching was determined *in vitro* according to Tilley and Terry method (Tilley and Terry, 1963) modified by Goering and Van Soest (Goering and Van Soest, 1970). In this study, a different combination (ratio) of ruminal fluid and buffer (1 rumen fluid:4 Kansas State Buffer) (Marten and Barnes, 1979) than used in the previous experiment was used. Macroalgae powder samples, laboratory references (corn silage and meadow hay), and blanks (no feed or macroalgae) prepared in quadruplicates were added with buffered ruminal fluid maintained in anaerobic condition and incubated at 39 °C for 48 h in a water bath. When the fermentation was completed, the undigested feed material was transferred to crucibles (porosity 40-100 µm, P2) and extracted in boiling neutral detergent solution for 1 h (Robertson, 1981). Thereafter, in *vitro* DM, OM, and CP digestibility were calculated and corrected for blanks.

Digestibility in monogastric animals

The *in vitro* total tract digestibility of DM, OM, and CP of macroalgae in monogastric animals was analyzed using a three-step enzymatic method simulating the conditions of the stomach, small intestine, and large intestine of the pig, respectively (Boisen and Fernández, 1997) **(Paper III)**. The digestibility analysis was conducted for 24 h with the following three steps:

1) A 2 h incubation of samples with phosphate buffer (0.1 M, pH 6.0), 0.2 M HCl (pH adjustment to 2.0), pepsin, and chloramphenicol in a water bath at 39 °C with agitation.
2) A 4 h second incubation of the mixture from step 1 after the addition of phosphate buffer (0.2 M, pH 6.8) and 0.6 M NaOH solution (pH adjustment to 6.8), pancreatin solution (containing 100 mg/mL) in a water bath at 39 °C under agitation.
3) A 18 h incubation of the mixture from step 2 with the addition of 0.2 M EDTA solution, acetic acid (pH adjustment to 4.8), and a mixed multi-enzymatic complex containing arabinase, β-glucanase, cellulase, hemicellulase, pectinase and xylanase in a water bath at 39 °C under agitation.

All macroalgae samples, two batches of soyhulls and oat, and blanks were incubated in quadruplicates. After the completion of final incubation, samples with undigested residue were transferred to crucibles, filtered, and rinsed with ethanol and acetone. The DM, OM, and CP content of the residue was determined and corrected for the blanks and used to estimate the *in vitro* total tract digestibility.

4.6 *In vivo* feeding trial with macroalgae-added diet in mice

Design of mice experiment (Paper IV)

Aiming to evaluate whether a dietary inclusion of polyphenol-rich (or fiber) brown macroalgae: *A. nodosum* (AN) and *F. vesiculosus* (FV) could alleviate the development of obesity upon exposure to the energy-dense fatty diet, an *in vivo* dietary intervention study was performed in mice in the final stage of this PhD. The experimental procedures on research animals were performed as per the ethical guidelines of the European Parliament on the protection of animals used for scientific purposes (DIRECTIVE 2010/63/EU) and were further approved by the Norwegian Food Safety Authority (Mattilsynet) (FOTS ID: 23425). After a period of acclimatization, a total of 32 seven-week-old C57BL/6JRj female mice were allocated into four dietary groups,

ensuring a uniform distribution of body weights: Low-fat (LF: 10% energy from fat, N=8), high-fat (HF: 60% energy from fat, N=8), HF supplemented with 5% of AN (HF+AN, N=8), and HF supplemented with 5% of FV (HF+FV, N=8). Mice were provided with their respective diets for 12 weeks, and different phenotypic and physiological parameters were evaluated in the mice at different time points of the experiment.

The body weight of mice was measured every week while body composition (fat mass weight, lean weight, and free fluid content) was measured at four different time points (weeks: one, five, ten, and twelve), using a non-invasive and non-destructive Time Domain-Nuclear Magnetic Resonance (TD-NMR) method. Feed intake was closely monitored throughout the experiment but recorded at two-time points.

Metabolic phenotyping of mice

The whole-body energy expenditure of mice was measured in week 10 of the experiment via open-circuit indirect calorimetry at the Phenotyping Core Facility of the Norwegian transgenic center (NTS), University of Oslo (UiO) as reported previously (Hjorth et al., 2022) with some modifications **(Paper IV)**. The mice were individually caged with enough diet, drinking water in Petri dishes and the bedding material, and subjected to metabolic phenotyping in climatic chambers for measurement of gas exchange (O2 consumption and CO_2 production) and physical activity. The volume of exchanged gas (VO2 and VCO2), physical activity, and total heat production or energy expenditure (EE) by mice were recorded every 20 min for about 60 h in a computer wirelessly attached to the climatic chambers. The measurements from the first 12 h of metabolic caging were discarded (adaptation time), and that from the last 48 h (7 am–7 am) were only included in the statistical analysis.

Blood and tissue sampling and analyses after euthanasia

To investigate the effect of dietary intervention on the blood parameters, fasting blood glucose (6 h) levels, mice fasted for 6 h in week 11 of the experiment, and whole blood glucose was measured by a glucose meter. After week 12, mice were euthanized

by cervical dislocation, and terminal blood samples were collected from the thoracic cavity, processed, and serum was stored at -80 °C. Different blood metabolites: beta-hydroxybutyrate (BOHB), blood urea nitrogen (BUN), enzymatic creatinine 2, glucose hexokinase 3, gamma-glutamyl transferase (GGT), non-esterified fatty acids (NEFA), total cholesterol (TC), and triglycerides (TG) were analyzed using the commercial kits and reagents using Atellica® CH 930 Analyzer.

After euthanasia, various organs: heart, liver, kidneys, and tissues: brown adipose tissue (BAT), gonadal fat (GonFat), subcutaneous fat (SubFat), mesenteric fat, jejunum, and cecum were immediately excised and weighed. From each mouse, ~200 g of cecal contents were collected and snap-frozen into the liquid nitrogen. All samples were later stored at -80 °C until further analyses.

4.7 Effects of dietary inclusion of macroalgae in the rumen and mice cecal microbiome

Microbial DNA extraction and 16S rRNA gene sequencing

To investigate the effects of macroalgae in the rumen microbiome (**Paper II**) and mice cecal microbiome (**Paper IV),** 16S rRNA gene sequencing was performed. For **paper II,** the microbial DNA was extracted from cell-rich pellets obtained after centrifuging post-fermentation rumen fluids (Machado et al., 2018). Five macroalgae species, based on their impacts on TGP production when supplemented to MS (none to high effects on TGP: *P. palmata, S. latissima, P. palmata, U. lactuca, A. nodosum,* and *F. vesiculosus*) were selected. In the case of the mice study, DNA was extracted from the cecal fecal matter **(Paper IV).** The extraction of DNA for both studies was carried out using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, California, USA) and further purified by using Monarch® PCR & DNA Cleanup Kit (New England Biolabs Inc., Ipswich, MA, USA) when required.

For both microbiome studies, the V4 region of the bacterial 16S rRNA gene was amplified by using the universal primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R

(GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011). A sequencing library was prepared by using the Illumina Miseq or Hiseq library preparation kits following the instruction from the manufacturer. This process included one or two-step PCR amplifications with a specific amount of DNA to generate the amplicon libraries, which were cleaned, and quality checked before performing sequencing in Illumina Miseq **(Paper II)** and Hiseq 2500 **(Paper IV)** platforms (Illumina Inc., California, USA). The sequenced files from **Paper II** have been deposited in the NCBI Sequence Read Archive (SRA) under the accession number: PRJNA780171.

Bioinformatics of 16s rRNA sequencing data

The 16s rRNA sequence reads from the Illumina MiSeq, or Hiseq 2500 platforms, mainly were processed using the dada2 plugin (Callahan et al., 2016) in QIIME2 (Bolyen et al., 2019). When the clean reads (Phred score: 33) were generated, they were assigned to the amplicon sequence variants (ASVs) with 97% sequence similarity cutoff via 'feature-classifier classify-consensus-vsearch' (Quast et al., 2012) in SILVA 132 database **(Paper II)** or with 99% sequence similarity cutoff via 'feature-classifier classify-sklearn' (Bokulich et al., 2018) using the SILVA 138.1 database **(Paper IV)**. Afterward, the ASV and taxonomy files were exported to perform different diversity-based analyses.

4.8. Statistical analyses

Statistical analyses of the data were performed mostly using the R Foundation for Statistical Computing Platform, version 4.1.1 (R Core Team, 2021). The chemical composition and all other data measured at a single time point of the experiments were analyzed by one or two-way ANOVA based on their relevance. The models were validated by using different measures — homogeneity of variance by residual plots, normality of residuals by the histogram, or Shapiro test. The potential outliers in the models were tested via OutliersTest, while Cook's distance was used to identify influential observations.

The repeated measurements data collected at different time points (e.g., TGP from each hour of fermentation during *in vitro* fermentation: **Paper II,** mouse body weight and body composition data from different weeks, and metabolic phenotyping data from indirect calorimetry experiment: **Paper IV**) were analyzed as repeated measures using a mixed effect model using lme function. The model contained the macroalgae species and their season of harvest, incubation hours of fermentation, and possible interactions as fixed effects, while different fermentation runs and replicate numbers of the treatments were used as random effects **(Paper II)**. For the data from the mice experiment, the fixed effect of diet, weeks (shifts: day or night for metabolic caging), and the individual mice measured at each time point were included as a random effect. During these analyses, different correlation structures between the measurements and heterogeneous variances were evaluated, and the structure that yielded the best fit was chosen as the final model.

In the end, a Pearson or Spearman's correlation matrix was generated to evaluate the correlation between the chemical composition of diet or macroalgae on the different digestibility parameters **(Paper II and III),** microbiome composition (rumen microbiome: **Paper II**, mice caecal microbiome: **Paper IV**). Whenever the significant effect of main terms or interactions was observed, differences in the least square means (LS means) were compared by Tukey's multiple comparison test. The level of significance was set at $P < 0.05$.

5 Major findings

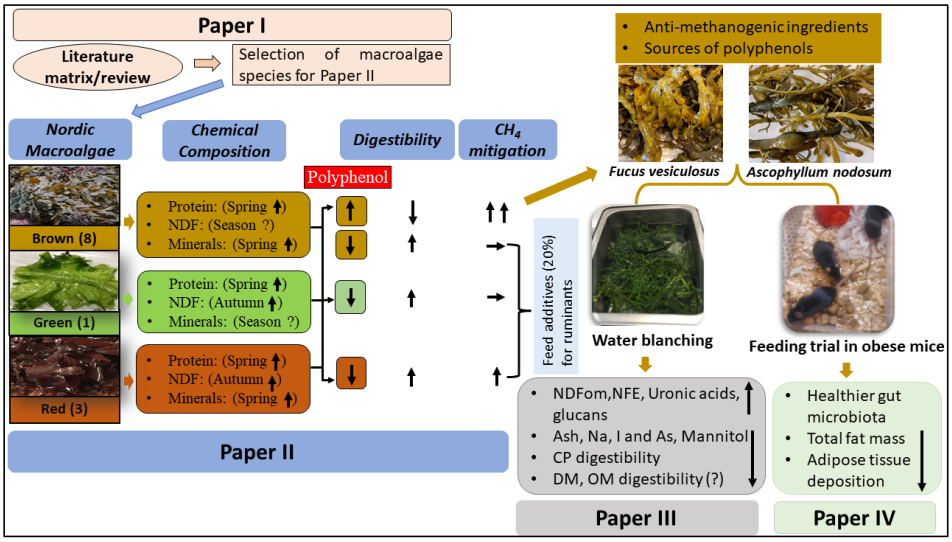

Figure 9: Overview of the papers and main results. This thesis comprised of four papers. Paper I is a review, while Paper II, Paper III, and Paper IV are the original papers. NDF, Neutral detergent fiber; NDFom, Ash corrected neutral detergent fiber; NFE, Nitrogen free extract; CP, Crude protein; DM, Dry matter; OM, Organic matter. Adapted and extended from Pandey et al. 2022.

5.1 Brown macroalgae exhibited greater seasonal variations in chemical composition than other macroalgae types (Paper II)

The chemical composition analyses of 12 macroalgae species harvested in the spring and autumn seasons revealed that red and green macroalgae contained a higher level of CP (~2 fold) than the brown species. In contrast, brown species had a greater level of total polyphenols irrespective of the season of macroalgae harvest. Species in the Fucaceae family, especially the *F. vesiculosus and A. nodosum* illustrated up to 20 times higher TPC compared to the red and green species of macroalgae. The content of NDFom was lower for green species*, U. lactuca,* regardless of the season of harvest than other macroalgae phyla. There was no clear seasonal trend in this parameter, although most red and green species had higher NDFom in the autumn. The contents of CP and ash were generally greater in spring than in autumn, irrespective of

macroalgae types. The higher ash level in spring was mainly contributed by the greater concentrations of Na and K. In general, brown species showed more seasonal variabilities as species in the Fucaceae family, illustrating a 66–86% greater CP, while *S. latissima* and *L. digitata* had 54.5 and ~96% ash in spring than in autumn. On the other hand, brown macroalgae mostly had greater TPC in the autumn than in the spring.

5.2 Impacts of macroalgae addition on *in vitro* feed degradability and CH_4 production mainly depended on their total polyphenol contents (Paper II)

The impacts of macroalgae addition on *in vitro* rumen degradability of the ruminant feed, i.e., maize silage (MS), CH_4 production, and rumen microbiome, were primarily determined by their TPC levels. The feeds supplemented with low or medium TPC-containing macroalgae: *P. palmata, L. digitata, S. latissima, H. elongata,* and *U. lactuca* exhibited similar OMD, TGP, and VFA production to that of MS and were not able to reduce the CH_4 production at significant level regardless of their types and harvesting season. Interestingly, two brown species with the highest TPC content, *F. vesiculosus* and *A. nodosum* led to 62.6 and 48% lower *in vitro* CH_4 production, although both those species impaired the OMD, TGP, and VFA production by up to 37% as compared to the MS fermented alone.

The rumen microbiome analysis of selected macroalgae with high, medium, and low degradability (based on their impacts on TGP), revealed that the two TPC-rich macroalgae caused a significant reduction in the abundance of cellulolytic bacteria (e.g., Lachnospiraceae spp., Ruminococcus spp., Rikenellaceae RC9 gut group) as well as methanogenic archaea *Methanobervibacter* (particularly by *F.vesiculosus*).

5.3 Post-harvest water blanching optimized the chemical composition of brown macroalgae in a species-specific manner (Paper III)

Although the low-temperature blanching (LTB) also reduced some levels of ash and selective minerals, including Na, the high-temperature blanching (HTB) reduced

the 16 and 23% ash, ~25 and ~38% of Na, ~33 and ~40% of K, ~43 and ~59% of P, >73 and 28% of I, ~44 and 40% of Br and 38.2 and 62.7% of As in AN and FV, respectively as compared to their unblanched (UB) biomass. Except for I and Br, all these reductions were higher for FV than for AN.

In contrast, carbohydrate fractions: NDFom (17.6% and 35.6% of in AN and FV, respectively), CF and NFE, and energy contents of both macroalgae biomass were increased by HTB. The HTB also elevated the total sugar content (TSC) of FV by 24.5% but did not affect that of AN. This was associated with a 67.5%, 56.5%, and 33% increase in the concentrations of MA and GA, and glucan, respectively, in FV. On the other hand, mannitol was dramatically reduced in both macroalgae with HTB: ~50% and ~82% in AN and FV, respectively.

5.4 Water blanching did not improve the *in vitro* digestibility of brown macroalgae in animals (Paper III)

In contrast to our hypothesis, none of the water blanching treatments (LTB and HTB) showed any positive effects in the *in vitro* digestibility of studied brown macroalgae in both animal models used in this study. The HTB treatment resulted in a 26% reduction in the CP digestibility of both macroalgae AN and FV in the monogastric animal model and a ~ 42% reduction in the CP digestibility of FV in the ruminant model when compared to their respective UB biomass. In addition, ~8-10% reductions in DM or OM digestibility of both macroalgae were also observed with HTB in monogastric animals and of FV in ruminants.

5.5 Dietary inclusion of brown macroalgae in the HF diet led to healthier cecal microbiota in mice (Paper IV)

The inclusion of polysaccharides and polyphenol-rich brown macroalgae in the HF diet-induced favorable changes in the caecal microbiota of obese mice. The addition of both AN and FV in the HF diet prevented cecal microbial dysbiosis by lowering the proportions of Firmicutes and increasing Bacteroidota in the cecal contents as

compared to the HF diet, which had no macroalgae supplementation. These changes in the microbial composition were associated with the lowered abundances of potentially obesogenic bacterial genera such as *Blautia, Enterorhabdus, Faecalibaculum, Lachnospiraceae, Lactococcus, Lachnoclostridium, Romboutsia,* and *Tuzzerella.* Additionally, both macroalgae-added groups (HF+AN and HF+FV) enriched the potentially beneficial and SCFA-generating bacteria such as *Alistipes, Bacteroides, Muribaculum,* and Rikenellaceae RC9 gut group in the cecal contents. Moreover, the HF+FV group dramatically increased *Akkermansia* compared to the HF diet group.

5.6 The modulations of the mice cecal microbiome by brown macroalgae inclusions were associated with improved body composition (Paper IV)

The Spearman correlation between the bacterial ASVs and mice physiological parameters revealed that the bacterial genera that were inhibited by the HF+AN, HF+FV, or LF diets, such as *Enterorhabdus,* Lachnospiraceae NK4A136group, and Lachnospiraceae UCG006 were positively correlated with body weight, total fat mass, and different adipose tissue weights (GONfat, SUBfat, and BAT). On the other hand, different ASVs accounting for *Alistipes,* and *Muribaculaceae*, *Lachnoclostridium* that were promoted by HF+AN, HF+FV were negatively correlated with body weight, total fat mass, and all types of adipose tissue weights (GONfat, SUBfat, and BAT). In agreement with these correlations, both macroalgae-containing diets had lower total fat mass (41-42%), GONfat (29-31%), and SUBfat (23.5–27.2%) than the HF group, but these reductions did not reach the level of statistical significance. The HF+FV group also showed a slightly improved total SCFA (12.7%) production in the cecal contents than the HF group.

6 General discussions

6.1 Interspecies and seasonal effects in the macroalgal chemical composition

The marine macroalgae sector has been recognized as a sustainable resource of feeding materials for land-based farm animals, mainly ruminant species. However, identifying relevant macroalgae species with high nutritional value and bioactive potential is challenging due to their large variabilities in the contents of nutrients and bioactive compounds across the species and seasons (Tayyab et al., 2016, Molina-Alcaide et al., 2017, Schiener et al., 2015). This could be more critical in regions such as Nordic, where seasonal conditions fluctuate largely throughout the year (Rødde et al., 2004, Lüning, 1993). The current study **(Paper II)** revealed wide interspecies and seasonal variabilities in the contents of protein, ash, minerals, NDFom, and total polyphenols in macroalgae. In general, macroalgae exhibited greater nutritional value in the spring, as evidenced by their higher CP and mineral contents, than in the autumn. However, bioactive properties of macroalgae may be attained greater in the autumn, particularly for brown species, suggested by a higher TPC in the autumn than in the spring. These seasonal trends generally agree with the previous studies from the same region (Gaillard et al., 2018, Tayyab et al., 2016, de la Moneda et al., 2019).

Despite seasonal variabilities, potential nutritional and bioactive values of macroalgae were mainly designated by their phyla or types. Containing higher CP levels, red (*C. crispus, P. palmata,* and *P. umbilicalis*) and green (*U. lactuca*) macroalgae demonstrated their greater relevance as proteinaceous feed resources for livestock than brown macroalgae. These findings are consistent with the previous studies, which also illustrated superior levels of CP in red and green species than in brown species (Rodrigues et al., 2015, Tayyab et al., 2016). When harvested in the spring, the CP contents in the aforementioned three red and green species were comparable to leguminous vegetables such as beans and peas (Rodrigues et al., 2015) and higher than

in cereal crops (Mæhre et al., 2014). Even a few brown species, especially the *A. esculenta* and *S. latissima,* seemed to have an acceptable level of CP (~12–13.6%) if harvested in the spring. As a significant proportion of protein in macroalgae can escape ruminal microbial degradation, these macroalgae could be valuable sources of protein (amino acids) that ruminants can be digested in the small intestine (Tayyab et al., 2016). The macroalgal biomass, however, was dominantly comprised of NDF, as NDFom accounted for up to 62% of macroalgal biomass. Except for the green species, *U. lactuca* (<28% NDFom), it remained within a range of reported NDF levels for forages and silages commonly used as ruminant feeds (Getachew et al., 2004, Castro-Montoya and Dickhoefer, 2020). These observations suggest that red and green species, *C. crispus, P. palmata, P. umbilicalis,* and *U. lactuca*, can be alternative feeding resources to legumes/leguminous plants for ruminants and may also partly replace concentrates. Certain brown species could be potential candidates to replace the low-quality terrestrial forages. The red and green species, with the highest protein contents, may also be relevant for extracting the protein for feeding monogastric animals such as pigs and poultry provided that they have acceptable digestibility.

Macroalgae are abundant sources of bioactive compounds, including polyphenols. Macroalgal polyphenols such as phlorotannin contribute to maintaining cellular integrity and protect them from external stress such as pathogenic attacks, UV-light exposure, desiccation, herbivory, etc. (Connan et al., 2004, Parys et al., 2009, Steevensz et al., 2012). Polyphenols sourced from macroalgae are of particular interest for animal health because of their diverse bioactive properties, which include anti-oxidative (Cox et al., 2010, Farvin and Jacobsen, 2013), anti-microbial properties against gastrointestinal or food pathogens (Ford et al., 2020, Cox et al., 2010) anti-inflammatory properties (Abdelhamid et al., 2018). Brown macroalgae used in this study, notably the species in the Fucaceae family (fucoids), such as *F. vesiculosus, A. nodosum, F. serratus,* and *P.canaliculata* with the most abundant TPC, indicated that they could be the important sources of such polyphenolic compounds **(Paper II)**.

Indeed, polyphenols from fucoid species have shown higher bioactivities, such as DPPH radical scavenging capacity, than those from other macroalgae species (Farvin and Jacobsen, 2013, Jiménez- Escrig et al., 2001). This statement was also supported by a high DPPH radical scavenging of polyphenolic extracts of two fucoid species, *A. nodosum,* and *F. vesiculosus,* in this study **(Paper III)**. Thus, although fucoid species demonstrate lower nutritional value (e.g., low CP), they could be valuable sources of bioactive compounds to improve animal health, such as reducing oxidative stress and intestinal disorders.

Various studies have reported seasonal discrepancies in the contents of nutrients and bioactive compounds in macroalgae (Marinho-Soriano et al., 2006, Rødde et al., 2004, Molina-Alcaide et al., 2017). The seasonal variabilities in the macroalgal chemical composition are attributed to the differences in the intrinsic (e.g., growth stage, thallus structure) as well as external factors such as temperature and nutrient availability in the water (e.g., N, mineral elements), light intensity, and stressors including ultraviolet light exposure, biofouling, pathogenic attacks, herbivory, and desiccation (Rødde et al., 2004, Parys et al., 2009, Steevensz et al., 2012). The better nutritional composition in the spring-harvested biomass of macroalgae might be associated with favorable growing conditions and greater availability of nutrients in the seawater. A lower temperature but a higher oxygen saturation of seawater recorded in the spring than in the autumn also signifies the differential environmental conditions between the two sampling periods of this study. As previously reported, this lower water temperature may also partially be associated with the higher protein contents observed in this study (Marinho-Soriano et al., 2006). This study further revealed that brown species are more prone to seasonal changes than red and green species, as they generally exhibited higher seasonal discrepancies for CP, ash, and polyphenols. This feature was manifested by ~1.5–2-fold greater ash by brown species in the Laminariales: *S. latissima* and *L. digitata,* and 66–86% more CP by fucoid species in spring than in autumn. In addition, most brown species, but more profoundly two fucoids: *F.*

vesiculosus and *P. canaliculata,* remained highly susceptible to seasonal variability with elevated TPC concentrations in the autumn than in the spring. In the North Atlantic or arctic region, the growth of the macroalgal biomass is maximum during the late spring, and it starts to deteriorate during the summer and autumn due to the epiphytic fouling and thallus degradation (Lüning, 1993, Stévant et al., 2017). The greater seasonal variabilities of CP and ash in selective brown species may be associated with their higher susceptibility to epiphytic biofouling that causes biomass deterioration and lowers nutrient uptake and assimilation potential of the thallus (Stévant et al., 2017). For particular brown species, biomass fouling coupled with extended ultraviolet light exposure may be the causal factor inducing further elevation of TPC during autumn as they reside in the upper or middle intertidal regions of the coastal ecosystem (Connan et al., 2004).

Macroalgae species in this study exhibited a rich profile of macro minerals (Na, K, Ca, Mg, P and S) and microminerals (I, Br, Fe, Mn, Zn, Cu), suggesting their capacity to fulfill the mineral requirements of the livestock. However, one or multiple mineral elements, particularly in brown and green macroalgae (mainly in the spring), seem to exceed the recommended dietary levels for different livestock species (NRC, 2005). Certain brown macroalgae encompassing an excessive amount of ash comprised of high contents of K and Na (up to 9% of DM in the spring) **(Paper II)** as well as I, Br, and Fe and certain heavy metals including As **(Paper III)** seemed to pose the risk of mineral toxicity to the animals. Such health risks associated with high mineral contents and heavy metals have also been pointed out in previous studies (Biancarosa et al., 2018, Stévant et al., 2018). Thus, optimization of the mineral contents of macroalgae biomass should be one of the priorities of the macroalgae producers to ensure the safety of using macroalgae as feed products and upgrade their dietary inclusion levels. The outcomes of one of the mineral-optimizing approaches for macroalgae will be discussed later in this thesis.

6.2 Macroalgae as feed additives for ruminants and major factors affecting the ruminal feed degradability and CH_4 mitigation

The contents and variabilities in nutrients and bioactive compounds in the macroalgae will have implications on their digestibility and impacts on the degradability of other animal feeds when macroalgae are supplemented (de la Moneda et al., 2019, Molina-Alcaide et al., 2017). Our pilot *in vitro* study, where pure macroalgae (sole macroalgae as feed) were used as a substrate for the fermentation, revealed a lower degradability (48 h) of macroalgae (>50% lower TGP production) compared to the MS regardless of the macroalgal harvesting season. However, there were different scenarios when macroalgae were used as a feed additive to MS (20% DM inclusion of macroalgae), as only a few macroalgae species impaired the feed degradability of MS **(Paper II)**. These differential effects of macroalgae on the rumen fermentation parameters could be associated with the differences in their secondary metabolites that may affect the rumen microorganisms (Machado et al., 2014). This study illustrates that the relative content of TPC in the macroalgae could be the primary factor determining their effect on ruminal feed degradability as it was inversely associated with crucial rumen fermentation parameters, such as TGP, OMD, and VFA production. Despite the seasonal and interspecies variabilities in chemical composition, macroalgae with low (*L. digitata, S. latissima, P. palmata,* and *U. lactuca*) and medium-high (*A. esculenta* from spring and *H. elongata*) TPC appeared equally relevant as ruminant feed additives with up to 20% inclusion rate regardless of their types. Conversely, brown species with high TPC: *A. nodosum, F. vesiculosus, F. serratus,* and *P. canaliculata* seemed unsuitable as ruminant feed additives, at least to the inclusion level used in this study. These TPC-rich macroalgae, particularly *A. nodosum* and *F. vesiculosus* can impair the ruminal feed degradation by 10-37%, reducing the TGP, OMD, and VFA production, more severely with autumn harvested biomass. The diminished VFA production due to poor feed degradability would lower the energy supply (reduction in total VFA production) to the animals and can affect the animal

performance as VFAs are responsible for 50–75% of the energy supply in ruminants (Faverdin, 1999). Few previous studies have also indicated a negative role of polyphenols in the rumen degradability of macroalgae (Molina-Alcaide et al., 2017, de la Moneda et al., 2019), but such effects were not expressed while using them as feed additives (de la Moneda et al., 2019). This could be due to the far lower concentrations of polyphenols in macroalgae species than the TPC-rich species of the present study and the different combinations of feed mixtures (macroalgae concentrate diets) in the previous study. Therefore, analysis of TPC levels in macroalgae biomass should be one of the most critical parameters while evaluating the potential of macroalgae as a ruminant feed resource.

The adverse effects of brown macroalgal polyphenols (phlorotannin) on feed digestibility are attributed to their ability to impair the degradation of fiber and protein by inhibiting microbial access to the fiber and binding with the protein molecules (Wang et al., 2008, Vissers et al., 2018, Makkar, 2003). Hence, the reduced OMD, TGP, and VFA production of the feed supplemented with TPC-rich macroalgae may be ascribed to the impaired fiber (e.g., NDF) and protein digestibility due to reduced microbial activity in the rumen. The 16S rRNA microbial gene sequencing of the rumen microbiome revealed that the rumen microbial community becomes differentially modulated by the inclusion of low and high-TPC-containing macroalgae in the feed.

The rumen microbiomes of *F. vesiculosus* (more pronouncedly) and *A. nodosum* supplemented fermentation media were characterized by a lowered abundance of rumen bacteria belonging to the phyla Bacteroidetes: Rikenelaceae RC9 gut group and Firmicutes: Lachnospiraceae family, *Ruminococcus* spp. and Ruminococcaceae UCG-010, which are crucial cellulose-degrading bacteria (Mizrahi et al., 2021, Pitta et al., 2010). Similar to these observations with TPC-rich macroalgae, when rumen microorganisms were exposed *in vitro* to phlorotannin extracted from *A. nodosum,* cellulolytic bacteria were significantly suppressed (Wang et al., 2009). Hence, repression of OMD, TGP, and VFA with the TPC-rich macroalgae might be a reflection

of impaired cellulose degradation due to reduced cellulolytic bacteria by their high phlorotannin contents.

In addition to TPC-rich brown macroalgae, feed degradability was also suppressed by a red macroalga, *C. crispus,* which contained high NDF and is known to comprise a large proportion of carrageenan in its cell wall (Rioux and Turgeon, 2015). Contrary to terrestrial plants, macroalgae contain a low level of cellulose, hemicellulose, and lignin (almost absent) in the biomass, but they hold a high proportion of unique complex polysaccharides that vary with the macroalgae types (Holdt and Kraan, 2011, Cabrita et al., 2017). Hence, macroalgal NDF may be mostly comprised of complex polysaccharides (Rjiba-Ktita et al., 2017). The carbohydrate composition analyses of *A. nodosum* and *F. vesiculosus* (**Paper III**) indicated that they are enriched with alginate (~14-24% of DM) and contain a reasonably high level of uronic acids and fucose. Previously, it has been illustrated that complex macroalgal polysaccharides (or sugar components), particularly alginate, fucoidan, and agar, are poorly digestible in ruminants (Orpin et al., 1985, Williams et al., 2013). Since NDFom was the most dominant part of macroalgal biomass, differences in the rumen degradability of macroalgal NDF might also be responsible for the differences in the degradability of the feed mixture. Therefore, brown species rich in TPC and low digestible complex polysaccharides may have limited application for ruminant nutrition purposes.

Besides the nutritive purposes, macroalgae are also considered as potential anti-methanogenic feed ingredients for ruminants as some species have shown potent ruminal CH_4-reducing potential (Kinley et al., 2020, Machado et al., 2014, Maia et al., 2016). This study also supports this idea as most macroalgae species studied exhibited a certain degree of CH_4 mitigating properties when they were added to a ruminant diet. However, two brown species, *F. vesiculosus*, and *A. nodosum,* from the autumn harvest, and a red species *C. crispus,* from the spring harvest, appeared to be the most effective anti-methanogenic species with 62.6%, 48.2%, and 56.5%, respectively, as compared to standard ruminant feed. To the best of our knowledge, such anti-methanogenic

action of *F. vesiculosus* and *C. crispus* has never been reported. Hence, selective macroalgae from the Norwegian natural population may be valuable resources to minimize the CH_4 emissions from the ruminant production sector. As concerns about the environmental impacts of ruminant production are increasing worldwide and different strategies, such as taxation of the livestock sector for their CH_4 emissions or animal products, are being discussed (Wirsenius et al., 2011, Cline, 2020), the dietary inclusion of these macroalgae in the ruminant feed could be a sustainable solution for the economic viability of this sector in future.

Different direct and indirect mechanisms of methane inhibition are reported for anti-methanogenic agents, and they have been reviewed in **Paper I**. The anti-methanogenic property of red macroalgae *A. taxiformis* has been found to be associated with a direct inhibitory effect of halogenated polyphenolic compounds such as bromoform on rumen methanogens (Roque et al., 2019a, Machado et al., 2018). Although brown algal polyphenols or phlorotannin have shown ruminal CH_4 mitigating properties *in vitro* (Vissers et al., 2018, Wang et al., 2008), their effects on rumen methanogenic archaea are still unclear. As expected, the inhibition of major methanogenic archaea, *Methanobrevibacter* spp. (McAllister et al., 1996) was most evident with *F. vesiculosus,* followed by *A. nodosum*, the two brown macroalgae with the highest TPC and CH_4 mitigation **(Paper II)**. The current study also indicated the role of macroalgal TPC in reducing ruminal CH_4 production, showing an inverse correlation between those; however, *Methanobrevibacter* abundance remained uncorrelated with TPC. Hence, brown macroalgae may also contain other anti-methanogenic compounds that directly inhibit the rumen methanogens, or TPC might indirectly affect methanogens. Recent studies have shown that phlorotannin-rich macroalgae or their extracts can impair protozoal activity/population (Belanche et al., 2016, Choi et al., 2021), which could be reflected by a lowered ruminal acetate and butyrate and increased propionate production (Belanche et al., 2016, Zhou et al., 2018). Similar trends of VFA profiles were evident mostly with *F. vesiculosus* in this study. Thus, TPC-

rich brown macroalgae possibly lower the activity/population of ciliated rumen protozoa, which are ecto- and endosymbiotically associated with methanogens and crucial producers of H_2 needed by methanogens to reduce CO_2 to CH_4 (Ushida et al., 1997, Newbold et al., 2015). However, further studies evaluating the effect of TPC on rumen protozoa and methanogens are required to confirm this statement.

This study demonstrates that two predominant Nordic brown macroalgae, *F. vesiculosus,* and *A. nodosum* could be an important resource for developing macroalgae-based-methane mitigating feeding strategies for ruminants. However, their adverse impacts on feed degradability that may compromise animal performance are needed to be minimized. Particularly, optimizing the TPC in the macroalgal biomass and enhancing the digestibility of NDF or complex polysaccharides may minimize the negative impacts on feed degradability and allow a larger dietary inclusion.

6.3 An approach to optimize the chemical composition – hot water blanching

Aiming to optimize the chemical composition (including TPC and minerals) and improve the digestibility of two anti-methanogenic brown macroalgae, *A. nodosum* (AN) and *F. vesiculosus* (FV), their fresh biomass was treated with a low (LTB) and medium-high (HTB) temperature water blanching in the subsequent study **(Paper III)**. This study indicated that HTB is highly effective in optimizing the level of mineral elements in macroalgae biomass. Just a 5 min exposure to HTB not only effectively lowered the excess macrominerals (Na, K, and P) and microminerals (I and Br) but also the toxic heavy metal (As) by (25–73%) without reducing other minerals. The differential responses of mineral elements towards water blanching could be linked to their solubility or leachability in the water, and chemical states (bound or unbound, mono, di, or trivalent), and the elements with higher water solubility are quickly leached (Hou and Yan, 1998, Hou et al., 1997). Hence, the HTB seems beneficial for minimizing the risk of mineral toxicity by removing a significant proportion of selective water-soluble elements from the brown macroalgal biomass. This will improve the

safety status of the macroalgae biomass, possibly allowing a larger macroalgal inclusion in the animal diet. Moreover, this study showed that the impacts of water blanching could be different even for closely related species belonging to the same order and found in the same littoral zone. The removal of ash and mineral elements was generally more evident for FV (except I) than for AN. Previously, it has been reported that macroalgal potential to maintain the integrity of blades during hydrothermal treatments can vary with their species (Stévant et al., 2018). Thus, the ash-storing parts (blades) of the FV thallus might have greater sensitivity to high temperature than that of AN. Since mineral elements in macroalgae mostly remain associated with complex cell wall polysaccharides (Mišurcová, 2012), the differential susceptibility of macroalgae species towards HTB might be related to their variations in the cell wall structure, particularly the proportions of alginate, fucoidan, and cellulose (Rioux et al., 2007).

It is also possible that other valuable nutrients in macroalgae biomass could be lost or altered together with minerals/elements during water blanching affecting their nutritional value. However, the results of **Paper III** did not indicate a loss in valuable nutrients such as protein and carbohydrates in both brown algae, although HTB caused some loss in crude fat in FV biomass. In contrast, the proportion of carbohydrates got enriched by water blanching as CF, NDFom, and NFE levels were linearly raised in both macroalgae species with the increase in blanching temperature (more prominently in FV). These phenomena have also been reported with hot water blanching in other macroalgae species, such as *S. latissima* (Nielsen et al., 2020). Such an increase in carbohydrates improved the energy content of the biomass, potentially adding some nutritive value.

In addition to the content, the composition of carbohydrates or relative proportions of sugars would be important for the nutritive value and utilization of macroalgae biomass as animal feed. Following its higher carbohydrate levels (NDF, NFE), unblanched biomass of AN illustrated greater total sugar content (TSC) than FV,

which is obvious based on the report from the previous study with these two macroalgae (Rioux et al., 2007). During the water blanching, two macroalgae species responded differently as the TSC level was elevated by 24.5% in FV with HTB, but it remained stable in AN with both LTB and HTB. The increase in the TSC in FV biomass could partly be associated with its more significant increase in the carbohydrate fraction and greater loss of ash with HTB. Additionally, this heterogeneity between macroalgae can be related to differential modifications of the cellular components by hydrothermal treatments and the relative degree of hydrolysis that influences the extractability of sugars and phenolic compounds (Rajauria et al., 2010). The composition analysis of both macroalgae indicated that the sugar composition of both AN and FV was primarily dominated by uronic acids (MA, GA, and glucuronic acid), accounting for around half of the TSC (~46–52%). However, there were differences between macroalgae in their relative contents of MA and GA; AN comprising >2-fold greater MA than FV, but both species had a similar level of GA. Being the building blocks of cell wall polysaccharide, alginate, MA, and GA play a vital role in the rheological properties of the alginate (Rioux et al., 2007). A high proportion of MA blocks or MA/GA ratio have been shown to render greater elasticity to the alginate gel, while a lower ratio may lead to less elastic or brittle gel (Draget et al., 2000, Khajouei et al., 2021). Therefore, concentrations of MA and GA in the macroalgae could be closely associated with their potential to maintain cellular integrity in response to external stress such as heat. Thus, the higher content of MA in AN biomass than in FV might have contributed to the greater resistance of its cell wall against HTB.

The increased TSC in FV by HTB was found to be contributed by a 60% rise in the uronic acids (MA: 67.5%, GA:56.5%) and glucans (33%), whereas fucose contents remained stable in both macroalgae. This suggests that the HTB treatment mainly targeted the uronic acids and glucans in FV (also TPC) but was unable to induce significant effects on sugar/carbohydrate components in AN. In contrast, the sugar alcohol, mannitol was sharply reduced by HTB in both macroalgae (~50% and ~82% in

AN and FV, respectively) when compared to their respective UB samples, possibly due to its high solubility in the medium-high hot water as used in the HTB treatment (Ghoreishi and Shahrestani, 2009). This indicates that specific sugar components of high value could be lost during the water blanching, potentially affecting their feed value and bioactive properties.

6.4 Can water blanching improve the digestibility of macroalgae in animals?

With the efficient loss of ash, increases in energy content, and extractability of sugar components (for FV), an improvement in the digestibility of blanched biomass was expected. However, the *in vitro* DM, OM, and CP digestibility analyses with monogastric and ruminant models did not suggest such improvements. Instead, in monogastric animals, both blanching treatments worsened all three digestibility parameters for AN, while only HTB seemed detrimental for FV (all parameters). This impairment was more severe with CP digestibility (~26.5% reduction) for both macroalgae. In the case of the ruminant model, the HTB treatment specifically suppressed the digestibility of FV biomass (all parameters), and CP digestibility remained the most suppressed parameter (42% reduction). This suggests that although water blanching can have a species-specific effect on the digestibility of brown macroalgae depending on the animal models and blanching temperature, HTB is more detrimental to FV biomass and the digestibility of CP. As revealed in correlation analysis, the adverse effects of water blanching on the macroalgal digestibility in both animal models could be associated with increased contents of carbohydrates in biomass (CF, NDFom, NFE, and sugars such as GA) and TPC, but also the reduced mannitol. It has been previously illustrated that macroalgae carbohydrates are mostly resistant to being digested in monogastric animals (Chen et al., 2018, Di et al., 2018) and have restrictive digestibility in ruminant species (Williams et al., 2013, Orpin et al., 1985). The additional adverse effects of HTB in the CP digestibility in FV could be associated with the greater increase in NDFom and TPC levels in the biomass because protein can

exist in the bound states with both NDF (Shayo and Udén, 1999) and polyphenols that can restrict their degradation (Vissers et al., 2018). Besides these, a possible loss of certain readily digestible nutrients, including soluble carbohydrates and free amino acids, during the biomass processing might also have contributed to the impaired digestibility of macroalgae (Maehre et al., 2016, Stokvis et al., 2021). Overall, **Paper III** suggests that water blanching, mainly the HTB, could be an effective tool to minimize the ash, certain excessive water-soluble minerals, and heavy metals, but it seems inappropriate to optimize TPC and enhance the digestibility of brown macroalgae, AN, and FV in livestock.

6.5 Polyphenol-rich brown macroalgae as modulators of gut microbiota and potential anti-obesity ingredients

The findings of **Papers II and III** indicated that polyphenol- (and polysaccharide) rich brown macroalgae, particularly AN and FV, have low digestibility and could have negative consequences on the digestibility of the other animal feeds if included in a high proportion. Instead, they may have other beneficial applications for animals due to the antimicrobial, antioxidative and prebiotic properties of their polyphenols and complex polysaccharides (Gardiner et al., 2008, Ford et al., 2020). Moreover, considering the capabilities of polyphenol or polysaccharide-rich extracts of certain brown macroalgae to favorably modulate the energy metabolism or gut microbiota and obesity (Yuan et al., 2019, Yang et al., 2017), these brown macroalgae could potentially be helpful in suppressing the development of diet-associated obesity and associated disorders in humans. The present study has revealed that these brown algae can significantly shift the cecal microbiota of an obese mice model, which could be linked to favorable changes in body fat mass **(Paper IV)**.

Obesity is often associated with a dysbiosis of the gut microbiome characterized by an elevated population of Firmicutes and suppression of Bacteriodota (Bacteroidetes) (Ley et al., 2005). Due to this shift in composition, the metabolic potential of gut microbiota, such as the utilization of dietary complex polysaccharides

and SCFA production, can be altered, affecting the energy homeostasis of the host that favors obesity development (Ley et al., 2005, Turnbaugh et al., 2006). Hence, reversing this changed proportion of the Firmicutes and Bacteroidota to a normal level could be a primary target of the potential anti-obesity ingredients. In the present study, a short-term (12 weeks) and moderate addition of AN and FV in the HF diet was able to significantly lower the abundance of Firmicutes (≥1.4 folds) while enriching that of Bacteroidota (≥2.2 folds) in the cecal microbiome of obese mice. Consequently, the lowered Firmicutes: Bacteroidota ratios of the HF+AN and HF+FV groups resembled that of the LF diet group. This suggests that a 5% dietary inclusion of AN and FV can contribute to preventing the unfavorable shift of the cecal microbiome towards the unhealthy obese microbiome upon HF exposure.

As expected, the mice in the HF diet group were characterized by the increased abundance of obesity-associated genera (Li et al., 2020, Sun et al., 2020) belonging to Firmicutes: *Blautia, Faecalibaculum, Lachnoclostridium,* different genera in the Lachnospiraceae family, *Peptococcus,* and *Tuzzerella,* as well as some genera in other phyla. These genera also appeared to be correlated positively with mice's body weight and fat weight gain. Interestingly, all those genera promoted by the HF diet were substantially suppressed (1.9–70.7-folds) by both HF+FV and HF+AN diets but more pronouncedly by the HF+FV diet. On the other hand, this bacterial suppression in both macro-algae added groups was accompanied by the enrichment of different genera in Bacteroidota: *Alistipes, Bacteroides*, Rikenellaceae RC9gutgroup, and *Muribaculum,* which often promote leanness and cecal SCFA production (Kim et al., 2018). These changes signal potential obesity-preventing properties of brown macroalgae during HF exposure. Additionally, the HF+FV group of mice demonstrated a sharp enrichment of the *Akkermansia* genus belonging to the phylum Verrucomicrobiota and this genus comprises species (e.g., *A. muciniphila*) that can effectively produce SCFA and contribute to improving gut health, blood glucose-insulin homeostasis and adiposity reduction in human (Dao et al., 2016) and mice (Everard et al., 2013). These statements

were supported by slightly improved total SCFA production (12.5%) (due to increased acetate and propionate) by the HF+FV diet when compared to the HF diet. Furthermore, by almost entirely inhibiting the growth of opportunistic gut pathogens (Wang et al., 2016) such as *Escherichia/Shigella* and *Staphylococcus,* both macroalgae indicated their potential benefits to alleviate the incidence of intestinal disorders in animals. These observations suggest that these macroalgae have some components that adversely affect the potentially obesogenic microbiota but selectively promote beneficial gut microbiota. As previously reported, these prebiotic properties of brown macroalgae are often linked to their polyphenols (Yuan et al., 2019) and polysaccharides (Zheng et al., 2021); differences in the contents of such compounds between macroalgae might have played a role in differential inhibition or promotion of specific cecal microbiota. For example, the polyphenol content in FV was consistently higher than in AN in this study, irrespective of harvesting season and location, whereas carbohydrates (NDF or complex polysaccharides) were higher in AN. The favorable changes in the composition of cecal microbiota and some improvement in SCFA, particularly with FV, indicate that these brown macroalgae indicated their potential anti-obesity property and beneficial effect on gut health.

In agreement with their effects on the microbial compositions, HF+AN and HF+FV diet groups illustrated a reduction of ~40% fat mass and 23–31% of GONfat or SUBfat relative to the HF diet suggesting an improved body composition. Although there were more noteworthy changes in the cecal microbiota and SCFA with the FV, AN also had a similar or slightly better improvement in the body composition parameters. There were some indications of enhanced energy expenditure with the mice in the HF+AN group (insignificantly higher heat production, VO_2, and total activity) compared to HF+FV and HF groups. Hence, the slightly higher energy expenditure also might have contributed to the reduced fat mass in the AN-supplemented group, as increased energy expenditure is associated with reduced fat mass deposition (Jayasooriya et al., 2008). However, the improvements in the total fat mass and other specific fat masses

didn't reach the level of statistical significance, possibly due to a large biological variation among individual mice within a diet group. Another possible reason could be a smaller dose of inclusion (5%) of macroalgae used in this study as compared to other studies with *L. japonica* (16 weeks with 10%) and *U. pinnatifida* (10 weeks with 10%) that recorded a significant reduction in fat and body weight gain (Kim et al., 2018, Li et al., 2020). In agreement with this hypothesis, the same species, *L. japonica* and *U. pinnatifida,* at 5% inclusion in the HF diet, were unable to reduce the body and fat weight in mice during a 16-week feeding trial (Oh et al., 2016). Therefore, with the level of inclusion used in this study, active macroalgal components with anti-obesity properties could have been too diluted in the diet to confer immediate evident effects on body weight and body composition, and potentially a more extended exposure period or larger inclusion dose may be required. Future studies evaluating the impacts of these macroalgae or their purified extracts (polyphenols, polysaccharides, or fucoxanthin) on molecular processes of obesity, such as expression patterns of obesity-related genes and proteins in adipose tissue or liver may provide additional insight into the anti-obesity properties of these brown macroalgae.

7 General conclusions

This study suggested that the potential of macroalgae as livestock feeding resources depends on their contents of protein, NDF, and total polyphenols because these parameters influenced their nutritional value, feed digestibility, and bioactive properties. Additionally, special consideration must be given to intra-species seasonal variability of protein, minerals, and total polyphenols. Selective brown (*A. esculenta*, *H. elongata*, *L. digitata,* and *S. latissima*), red (*P. palmata,* and *P. umbilicalis),* and green (*U. lactuca*), when harvested in the spring, would serve as nutritious and digestible feed additives for ruminants but with none or minimal benefits on mitigation of ruminal CH_4 emission. Brown species, *F. vesiculosus,* and *A. nodosum* can be potential anti-methanogenic and environment-friendly feed additives to mitigate such CH_4 emissions for ruminants. However, they have low feed value and should not be included at a high level in the feed due to the negative effects of their too-high polyphenol on ruminal feed degradability.

To enable the large-scale utilization and safety of brown macroalgae as animal feed, optimization of chemical composition, mainly excessive minerals and polyphenols, as well as enhancing the digestibility of NDF (or complex carbohydrates), are required. A post-harvest water blanching of fresh macroalgal biomass to a medium-high temperature (e.g., 80 °C) seems efficient in minimizing the risk of mineral toxicity associated with their high contents of Na, K, I, and As in brown macroalgae. However, water blanching cannot be considered an appropriate processing method in terms of optimizing the level of polyphenols and digestibility of macroalgae in livestock species as it may further impair the digestibility (mainly of CP), possibly due to the increased proportion of carbohydrates (NDFom, and uronic acids) in the biomass. These effects of water blanching could vary between the macroalgae depending upon their susceptibility toward the hydrothermal treatments.

In addition to the anti-methanogenic ingredients for ruminants, polyphenol, and polysaccharide-rich brown macroalgae, *A*. *nodosum* and *F. vesiculosus* may also be the target species to improve the gut health in animals and as potential anti-obesity dietary ingredients for humans. A moderate and short-term inclusion of these macroalgae was associated with the development of healthier and less obesogenic gut microbiota and improved body composition with lower fat mass in mice exposed to a HF diet.

Overall, this study revealed that several macroalgae from the Norwegian coast with relatively high protein and low polyphenols could be important digestible and nutritious feeding resources for ruminants while polyphenol-rich brown macroalgae could serve as sustainable anti-methanogenic feed additives and potentially the gut health supporting and anti-obesity ingredients for animals and humans in future.

8 Future perspectives

This study generated important knowledge on the nutritive and bioactive potential of the diverse macroalgae for the livestock species, also accounting for their seasonal variabilities. The anti-methanogenic potentials of certain macroalgae were disclosed for the first time. However, as these findings were based on the *in vitro* simulating studies*, in vivo* studies are needed to further unravel the impacts of macroalgae inclusions in animal growth performance and health before using them for livestock feeding purposes. Since the anti-methanogenic properties of selective brown macroalgae were associated with reduced feed degradability and energy supply, establishing the optimal inclusion dose that efficiently minimizes the ruminal CH_4 production without compromising the feed degradation would be important to develop macroalgae-based CH_4 mitigating strategies. Potentially, a macroalgal biorefinery to segregate polyphenols and other nutrients from biomass could improve the digestibility of protein and carbohydrates and the overall digestibility of macroalgae in livestock (Marinho et al., 2016, Bikker et al., 2016). On the other hand, extracted polyphenolic compounds could be utilized for the health benefits of animals and the environment on the required dose.

To the best of our knowledge, this is the first study evaluating the impacts of water blanching, considering both chemical composition and digestibility aspects of macroalgal biomass, focusing on monogastric and ruminant animals. This research has generated important knowledge about the changes that can occur in the composition and digestibility of macroalgae biomass with such hydrothermal processing. However, more efforts are needed to identify ways to improve the digestibility of the complex polysaccharides to enhance the large-scale utilization of macroalgal biomass for animal feeding purposes.

In the context of observed positive effects of polyphenol and polysaccharide-rich brown macroalgae on gut microbiota and body composition in mice, future studies are

required to assess the long-term effects and any potential adverse effects of macroalgal compounds on animal health and identify the optimal dose of macroalgae for the efficient improvement of gut health and prevention of obesity development. This may open the possibility of using these macroalgae as anti-obesity for human health applications or gut health-promoting agents for animals.

9 References

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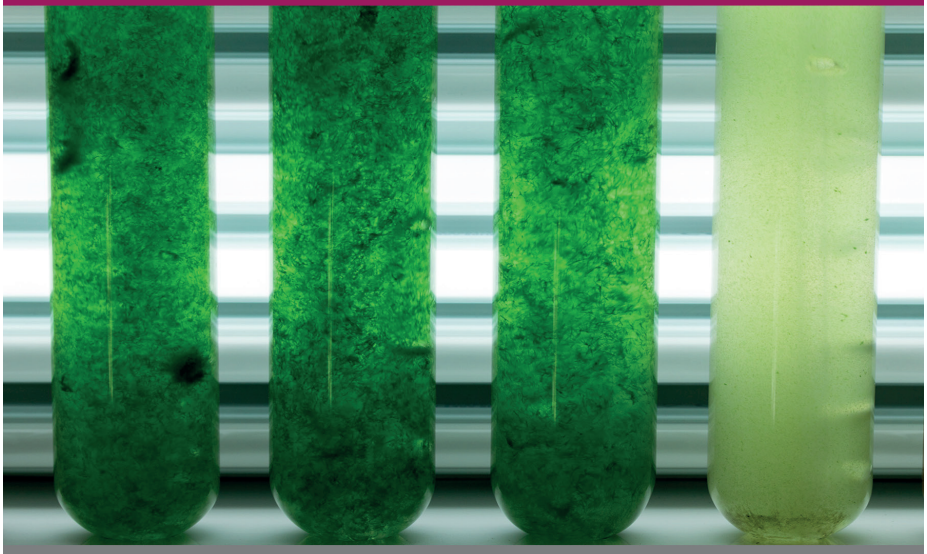
Paper I

BURLEIGH DODDS SERIES IN AGRICULTURAL SCIENCE

Nutritional and anti-methanogenic potentials of macroalgae for ruminants

Deepak Pandey, Nord University, Norway; Morteza Mansouryar, University of Copenhagen, Denmark; Margarita Novoa-Garrido, Geir Næss and Viswanath Kiron, Nord University, Norway; Hanne Helene Hansen, University of Copenhagen, Denmark; Mette Olaf Nielsen, Aarhus University, Denmark; and Prabhat Khanal, Nord University, Norway



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Nutritional and anti-methanogenic potentials of macroalgae for ruminants

Deepak Pandey, Nord University, Norway; Morteza Mansouryar, University of Copenhagen, Denmark; Margarita Novoa-Garrido, Nord University, Norway; Geir Næss, Nord University, Norway; Viswanath Kiron, Nord University, Norway; Hanne Helene Hansen, University of Copenhagen, Denmark; Mette Olaf Nielsen, Aarhus University, Denmark; and Prabhat Khanal*, Nord University, Norway*

1 Introduction

The global human population is rising rapidly and has been projected to be ~10 billion by 2050 (Holt-Giménez, 2019). This implies that by that time, an increase of 60–70% in overall food and ~78% in meat production is required (Estrada et al., 2011; Alexandratos and Bruinsma, 2012; Holt-Giménez, 2019). The livestock sector supplies ~28% of the global protein consumption, but in developed countries this contribution may be as high as 48% (Estrada et al., 2011). Thus, the livestock sector will continue to play a crucial role as a source of high-quality protein for human consumption in the future (Åby et al., 2014). However, ruminant livestock species such as cattle, goats and sheep are responsible for ~17% of the total anthropogenic enteric methane

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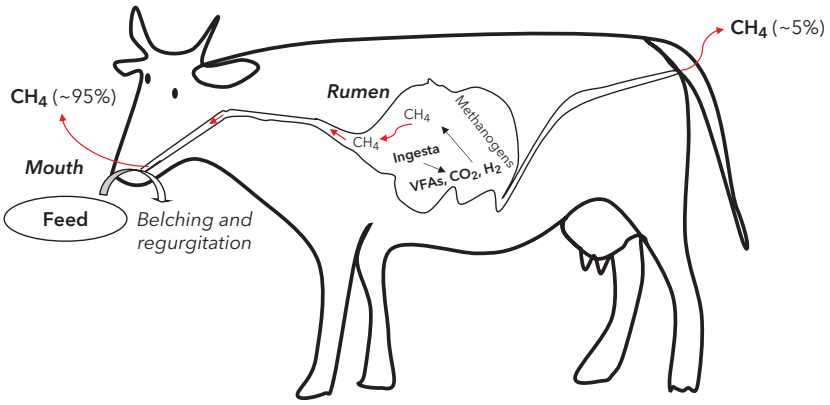

Figure 1 A general outline of methane production and emissions from ruminant animals. Enteric fermentation of ingested feeds occurs in the rumen, where the majority of CH_4 is released from the mouth by eructation. CH_4, methane; VFAs, volatile fatty acids; CO_2, carbon dioxide; H_2, hydrogen.

(CH_4) emissions, via fermentation of feeds in their forestomach (Fig. 1) (Knapp et al., 2014). Ruminants possess a unique digestive system comprised of a four-chambered stomach: rumen, reticulum, omasum and abomasum. The rumen is the residence of a large number of microorganisms, including bacteria, fungi, protozoa and archaea, and these microorganisms play a vital role in feed degradation and energy supply to the host animals (Bergman, 1990; Maia et al., 2016). Feed components, particularly carbohydrates, get partially or completely fermented in the rumen and produce volatile fatty acids (VFAs) such as acetate, propionate, butyrate, and also carbon dioxide (CO_2) and hydrogen (H_2) (Van Nevel and Demeyer, 1996) (Fig. 2). Volatile fatty acids are an important energy source for ruminants, while CO_2 and H_2 may later be reduced to CH_4 by the action of methanogenic archaea before getting eructed from animals into the environment (Bergman, 1990).

Methane is one of the major contributors of global warming and has a 28 times higher global warming potential than another greenhouse gas, CO_2 (Grossi et al., 2019). The CH_4 emission from the rumen represents a loss of up to 15% of gross energy (GE) from the feed, which could otherwise be utilized for animal growth and production (Van Nevel and Demeyer, 1996), and is, therefore, unfavorable for the animal. Enteric methanogenesis is thus both an environmental and nutritional concern, and any interruption in this process could provide nutritional benefits to the animals and result in the release of the less potent greenhouse gases CO_2 and H_2, compared to the highly potent CH_4 (Patra et al., 2017; Grossi et al., 2019). Hence, development of appropriate CH_4 abatement strategies is important to attain sustainable ruminant production systems in the future (Grossi et al., 2019).

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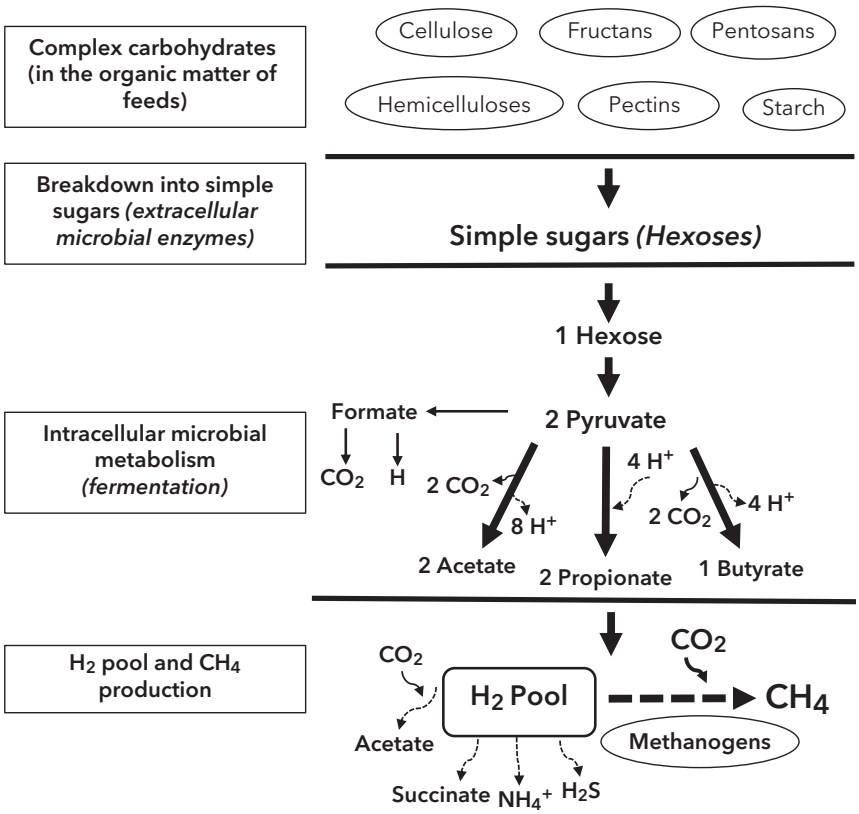

Figure 2 Major pathways involving degradation of carbohydrates, and production of volatile fatty acids and methane by microbial fermentation in the rumen. Adapted with some modifications from (McDonald et al., 2011; Haque et al., 2014; Kohn and Boston, 2000; Ungerfeld, 2013). CH_4, methane; CO_2, carbon dioxide; NH_4, ammonium; H_2S, hydrogen sulfide. Dotted lines represent pathways involving metabolic hydrogen (H) or dihydrogen (H_2).

Several CH_4 mitigation strategies have been suggested to cope with the CH_4 emissions from ruminants. These include (i) mitigation of CH_4 emissions via genetic selection (González-Recio et al., 2020), (ii) use of anti-methanogenic chemical compounds such as nitrate, chloroform and 3-nitroxy propanol (Patra et al., 2017) and (iii) dietary interventions using alternative feed ingredients and nutritional strategies (Knapp et al., 2014). Genetic selection can permanently reduce CH_4 production from an individual animal and can be inherited to the offspring (González-Recio et al., 2020). However, this approach could be technically demanding and time consuming, and convincing outcomes of genetic selection are yet to be obtained (Knapp et al., 2014; González-Recio et al., 2020). The application of anti-methanogenic chemical compounds is an effective strategy in reducing CH_4 emissions; however, their effects can be

transitory and such compounds may have adverse impacts on both animal performance and the environment (Patra et al., 2017). Dietary interventions can be a relatively simple and environmentally friendly approach and can lead to no or lower negative consequences to animal health and performance (Haque, 2018; Benchaar et al., 2001; Haque et al., 2014). Despite some advantages, only a modest reduction (5–40%) in CH_4 emissions by dietary interventions has been reported (Benchaar et al., 2001; Knapp et al., 2014). In this context, identification of alternative and novel feed materials that can substantially decrease enteric CH_4 production without compromising animal health and production would be important to develop novel CH_4 mitigating strategies in the future.

Marine macroalgae (also commonly known as seaweeds) have been identified as an alternative feed resource that can largely decrease enteric CH_4 production from ruminants (Machado et al., 2016; Maia et al., 2016). Macroalgae consist of 6000–10 000 diverse marine species distributed along the coastal regions worldwide, and they can be categorized into three types based on their pigmentation: brown, red and green (Tiwari and Troy, 2015; Makkar et al., 2016; Rajauria, 2015). Within the three categories of macroalgae, there are large species variations with respect to chemical composition (carbohydrates, proteins and minerals), and how digestible the organic components are in ruminant animals (Makkar et al., 2016; Dawczynski et al., 2007). Additionally, macroalgal species produce a wide range of bioactive components, such as halogenated compounds, polyphenols, complex polysaccharides and pigments (O'Sullivan et al., 2010; Charoensiddhi et al., 2017; Machado et al., 2016). Their bioactivities include antioxidative (Kannan et al., 2007; Ling et al., 2015), anti-microbial, immunomodulatory (Turner et al., 2002; Kim et al., 2018), anti-diabetic (Kang et al., 2016), anti-inflammatory, prebiotic (Cañedo-Castro et al., 2019; Reilly et al., 2008; O'Sullivan et al., 2010) and anti-methanogenic properties (Machado et al., 2014; Roque et al., 2019a). This broad range of chemical activities may enhance the future commercial application of macroalgae as multipurpose feed ingredients (Øverland et al., 2019).

Macroalgae have long been utilized as a feed ingredient for ruminant animals in different parts of the world. In many countries, including Iceland, Norway, France, Germany, Sweden, Finland, Scotland and the USA, macroalgae were used as an occasional or regular animal feed, particularly during extreme winter conditions, when the availability of other feed resources was limited (Makkar et al., 2016; Evans and Critchley, 2014; Chapman, 2012; Hansen et al., 2003; Applegate and Gray, 1995). However, there are very few published studies on the application of macroalgae as commercial and regular feed resources for ruminant animals. A brown macroalgae, *Ascophyllum nodosum*, has been reported to be used in small amounts as a feed additive for dairy cows in some organic farms in the USA (Erickson et al., 2012). North Ronaldsay (Orkney) sheep in Northern Scotland are purported to survive by grazing on different macroalgal species: *A. nodosum,*

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Alaria esculenta, Fucus spp., *Laminaria* spp., *Saccharina latissima* and *Palmaria palmata* (Hansen et al., 2003; Makkar et al., 2016). However, their commercial application for farm animals on a large scale is yet to be achieved.

Macroalgal species within all three (red, brown and green) categories have been identified to have CH_4 mitigating properties both *in vitro* and *in vivo* (Machado et al., 2014; Maia et al., 2016; Belanche et al., 2016b). However, when using some macroalgal species as feed, rumen fermentation patterns and total tract digestibility may be negatively affected due to high contents of ash and complex carbohydrates of low rumen degradability (Bikker et al., 2020). This can reduce the overall animal performance, particularly when such macroalgae are fed in large amounts (Bikker et al., 2020). Hence, the implications of the anti-methanogenic properties of macroalgae must be evaluated based on their overall impacts on feed intake, digestibility and animal performance. To be able to exploit macroalgae as potential feed resources, it is essential that species of commercial relevance be extensively characterized from both a biochemical (including anti-methanogenic compounds) and a nutritional point of view as presented in Fig. 3.

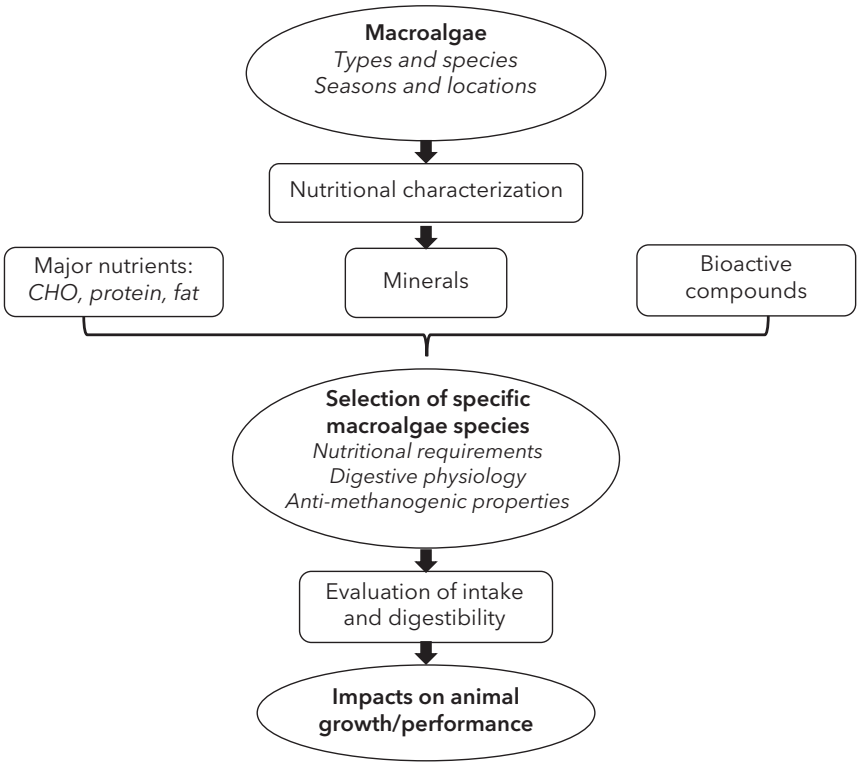

Figure 3 A flowchart for the evaluation of macroalgae as a potential ruminant feed ingredient in future. CHO, carbohydrates.

This chapter aims to evaluate the role of macroalgae as a potential anti-methanogenic ruminant feed resource. Similarly, effects of different intrinsic (macroalgal species, types) and extrinsic (growing season, post-harvest processing) factors on nutritional value as well as concentration of bioactive compounds and anti-methanogenic properties will be discussed. This will enable us to evaluate whether macroalgae can be used as anti-methanogenic dietary additive without compromising overall animal production and performance.

2 Nutritional value of macroalgae

Fresh macroalgae biomass normally contains about 70–90% water and various macro- and micro-nutrient fractions (Kılınç et al., 2013; Biancarosa et al., 2017). In this section, protein, carbohydrate, mineral and lipid contents of various macroalgal species will be described, and their potential as ruminant feeds will be evaluated. Unless otherwise stated, the contents are reported as % of dry matter (DM) to allow comparisons.

Protein: Red macroalgae species generally contain greater levels of crude protein (CP) than brown and green species. Some red species belonging to the genera *Palmaria, Pyropia* and *Porphyra* have been reported to contain 20–50% CP (Tibbetts et al., 2016; Fernández-Segovia et al., 2018; Marsham et al., 2007; Jung et al., 2016). The green macroalgae *Acrosiphonia* spp. and *Ulva* spp. also contain high levels of CP (appr. 31% and 25%, respectively) (Biancarosa et al., 2017; Peña-Rodríguez et al., 2011; Tayyab et al., 2016), whereas CP levels in most of the brown macroalgae are <15% (Dawczynski et al., 2007; Biancarosa et al., 2017). Thus, red and green macroalgae are the most relevant to consider as protein sources for animals.

Macroalgae proteins are reported to have a high quality due to their high proportion of essential amino acids (EAA) (Angell et al., 2016; Mišurcová, 2012). The red species *P. palmata, Porphyra* spp. and *Vertebrata lanosa*, the brown species *A. nodosum* and *Undaria pinnatifida* and the green species *Enteromorpha intestinalis* (*Ulva* sp.) have a higher EAA index and are thus considered to be superior compared to cereals from a nutritional point of view (Mæhre et al., 2014; Dawczynski et al., 2007; Gaillard et al., 2018). The EAA proportion in macroalgae can account for 45.7% of the total amino acids, which is similar to that of the conventional protein feed resource soybean meal (46%) and greater than fishmeal (43.4%) (Angell et al., 2016; Dawczynski et al., 2007; Biancarosa et al., 2017). Although the requirements for EAA would vary based on specific animal parameters (e.g. age, growth stage, production purpose), the EAA content of selected macroalgal species are reported to be able to fulfill the human and animal requirements (Mæhre et al., 2014). Therefore, selected macroalgae, particularly red and green species, could be considered

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as alternative sources of quality feed protein but their biomass yield and technologies for large-scale cultivation must be taken into account. This is, however, beyond the scope of the present chapter.

The significance of alternative proteins in ruminant nutrition depends on their digestibility. Studies regarding *in vivo* digestibility of macroalgae proteins are relatively scarce; however, *in vitro* protein digestibility (IVPD) has been explored for a number of species. The IVPD of the red macroalgae *Chondrus crispus, P. palmata, Sarcodiotheca gaudichaudii* and *Meristotheca papulosa* have been found to be ~85% of the total CP content, whereas IVPD for the brown species: *A. esculenta, A. nodosum, Fucus vesiculosus* and *S. latissima* are reported to be slightly lower (~80%) (Tibbetts et al., 2016). In ruminants, a significant part of the feed CP is degraded via microbial action in the rumen and subsequently utilized in microbial protein synthesis, including synthesis of EAA (Hvelplund and Weisbjerg, 2000). The amount of protein that passes un-degraded by the microbes to the small intestine is called rumen escape protein (REP). The bioavailability and amino acid composition of this fraction becomes particularly important, when feed protein degradability and hence microbial protein supply from the rumen is low (Hvelplund and Weisbjerg, 2000). An in situ study illustrated that 50–70% of the CP from *A. esculenta, L. digitata* and *P. palmata* is degraded in the rumen within 24 h, while for other species including *M. stellatus, Ulva* and *Pelvetia canaliculata* rumen CP degradability was substantially lower (<35%) (Tayyab et al., 2016). Hence, for many of the above mentioned macroalgal species, a large proportion (30–51% of total CP) of protein supply to the small intestine will be REP, and the intestinal digestibility of the REP becomes important for the potential amino acid supply to the animal (Tayyab et al., 2016). In the same study, degradation of CP in the small intestine was negligible for *A. esculenta* and *P. canaliculata*, while this value was similar or greater than the rumen degradability in others (*Porphyra*, *Palmaria*, *Ulva*, *Acrosiphonia*, *Mastocarpus*) (Tayyab et al., 2016). In addition, in situ total tract amino acid degradability of *Porphyra* sp. and *P. palmata*, and green macroalgae *Cladophora rupestris* and *Ulva* sp. has been found to be the highest among macroalgal species (Gaillard et al., 2018). These studies suggest that green and red macroalgae species are interesting new potential sources of rumen degradable and intestinal digestible protein for ruminants.

Carbohydrates: Carbohydrates are generally the most abundant organic compounds in macroalgae and may account for 25–75% of their DM (Jiménez-Escrig and Sánchez-Muniz, 2000; Rioux and Turgeon, 2015). They comprise both soluble and non-soluble carbohydrates and their relative amounts and composition vary depending upon macroalgae type and species (O'Sullivan et al., 2010). The major carbohydrates in macroalgae are unknown in terrestrial plants, and include alginate, fucoidan, mannitol and laminarin in brown; agar, carrageenan and porphyran in red; and ulvan and xylans in green species

(Cherry et al., 2019). Despite being indigestible in monogastric animals, macroalgae polysaccharides, particularly from brown species, have attracted research interest as prebiotics due to their beneficial gut impacts (O'Sullivan et al., 2010). These polysaccharides can partially or completely be fermented in the hindgut by the action of specific gut commensal bacteria producing short-chain fatty acids, and can thereby contribute to inhibit the growth of gut pathogens, such as *Clostridium* spp., *Escherichia coli* and *Salmonella* spp. (Braden et al., 2004; Seong et al., 2019). The prebiotic effect of macroalgae polysaccharides has mostly been studied in non-ruminant animals, including weanling piglets and humans (Reilly et al., 2008; Smith et al., 2011), and information about prebiotic effects for ruminant animals, containing relatively complex digestive systems, is limited. However, Tasco-14, an *A. nodosum*-based commercial additive, has been found to be effective in reducing the fecal shedding of *Escherichia coli* (O157:H7) and *Salmonella* spp. in feedlot cattle and lambs when supplemented in the diet at 2% DM basis (Braden et al., 2004; Bach et al., 2008). Further studies are needed to identify whether such reduced fecal shedding is due to the action of polysaccharides in the hind gut of cattle.

The nutritional value of macroalgae polysaccharides for ruminants depends on whether they can be degraded by the microbial population in the forestomach. Studies on the rumen microbes isolated from macroalgae-fed Ronaldsay sheep have revealed that polysaccharides from brown species, that is, alginate, fucoidan and laminarin, can variably and only partly be degraded by selective rumen microorganisms (Orpin et al., 1985; Williams et al., 2013). Only nine, out of 65, cultured isolates of rumen microorganisms were able to degrade >90% of the laminarin and 70–80% of alginates, but <20% of the fucoidans (Williams et al., 2013). The rumen microorganisms involved in the degradation of macroalgae carbohydrates include *Prevotella* spp., *Clostridium butyricum, Streptococcus bovis, Selenomonas ruminantium, Butyrivibrio fibrisolvens* and *Dasytricha ruminantium* (Orpin et al., 1985; Williams et al., 2013). However, as only a limited number of rumen microbes were included in the studies due to problems associated with microbial cultivation in artificial media, results from these *in vitro* fermentations may not be representative of the whole *in vivo* scenario of rumen degradability of macroalgal polysaccharides. Hence, further *in vivo* studies evaluating the digestibility of these polysaccharides are important to establish their nutritional value.

Minerals: Although mineral contents of macroalgae are affected by both intrinsic (macroalgae types and species) and environmental factors (culture conditions, seasons etc.), they are generally an excellent source of both macro and trace minerals. They are capable of accumulating a large quantity of minerals from seawater, and hence, the levels of various minerals including iodine, sodium, potassium, iron, chlorine and calcium in macroalgae are found to be 10–20 times higher than the levels found in terrestrial plants and fresh

water algae (Pereira, 2011; Gómez-Ordónez et al., 2010; Makkar et al., 2016; Mišurcová et al., 2010). The capacity of macroalgae to concentrate minerals has been linked to their mineral-rich growing environment and the content of unique cell wall polysaccharides such as alginic acid, salts of alginate, agar and carrageenan that can absorb different inorganic ions from the seawater (Mišurcová, 2012). The ash content in macroalgal species can vary between 20% and 72% of DM (Cabrita et al., 2016; Rupérez, 2002; D'Armas et al., 2019). In general, brown and green species contain higher amounts of minerals than red species (Pereira, 2011; Cabrita et al., 2016; Fernández-Segovia et al., 2018). Due to the abundance of minerals in macroalgae, they are considered natural mineral sources for both livestock and humans; for example, they can be used for the prevention of iodine deficiency (Baňoch et al., 2010).

Minerals are important for normal functioning of different hormones and enzymes in the body (Trumbo et al., 2001; Mæhre et al., 2014). However, due to the high mineral contents such as sodium, chlorine, calcium, iron and iodine in many species (*Codium* spp.*, Himanthalia elongata, Laminaria* spp.*, Saccharina* spp., *Bifurcaria bifurcata* and *Ulva* spp.), an excess intake of macroalgae-based diets may result in mineral toxicity, particularly in monogastric species as they are at higher risk due to a generally lower tolerance towards excess mineral uptake than in ruminants (Bikker et al., 2020; Cabrita et al., 2016). Excessive uptake of iodine from macroalgae-based ruminant feeds can be excreted in milk or accumulated in body tissues, leading to undesirably high levels of iodine in animal products that can have adverse consequences for human health (van der Reijden et al., 2017). The maximum recommended level of iodine is 2 mg/kg feed for dairy ruminants in the European Union (EU) (Additives and Feed, 2013), due to concerns of toxic levels in ruminant products destined for human consumption. Therefore, an abundant mineral content limits the inclusion of macroalgae on a larger scale in ruminant diets, unless special precautions are undertaken while formulating diets (Bikker et al., 2020).

Macroalgae are also able to concentrate heavy metals such as arsenic, mercury and cadmium from seawater, which are known to have a range of adverse health impacts, such as cancer and renal dysfunctions (McLaughlin et al., 1999). Particularly the contents of inorganic versus organic arsenic must be considered due to the greater toxicity of the inorganic form, although the predominant form of arsenic in macroalgae is normally organic (~ 90%) (Díaz et al., 2012; Mæhre et al., 2014; Biancarosa et al., 2018). The levels of these heavy elements in 21 macroalgal species from the Norwegian coast were found to be far below the maximum tolerable levels set in the EU region (Biancarosa et al., 2018).

Lipids: Macroalgae generally contain a low level of lipids (<5%) (Makkar et al., 2016; Øverland et al., 2019). Lipids from macroalgae are considered beneficial for human health due to their bioactive properties (Mæhre et al.,

2014). However, it can be insignificant with respect to the supply of (essential) fatty acids in ruminant feeds due to their very low lipid content, and most of it is utilized by rumen microbes (Bikker et al., 2020).

3 Digestibility of macroalgae as a feed or feed ingredients

A broad range (20–97% organic matter, OM) of ruminal and post-ruminal degradability of macroalgae has been reported earlier (Table 1). The rumen degradability of brown macroalgae *A. nodosum, F. serratus,* and *F. vesiculosus* has been observed to be low (<33% of OM) when they were used as a sole ruminant diet (Greenwood et al., 1983). Moderate *in vitro* rumen DM degradability (40–65%) has been recorded for other brown (*M. pyrifera, A. esculenta, L. digitata, P. canaliculata*) as well as red (*M. stellatus, P. palmata, Porphyra* sp.) and green macroalgae (*Ulva lactuca* and *Acrosiphonia* sp.) (Gojon-Báez et al., 1998; Ventura and Castañón, 1998; Molina-Alcaide et al., 2017). However, *in vitro* rumen OM degradability for selected brown (*A. esculenta, L. digitata, L. hyperborea, Sargassum* spp., *S. latissima*) and red (*P. palmata*) species was found to be higher (80–89%) (Hansen et al., 2003; Makkar et al., 2016; Marín et al., 2009). Since the later studies were performed using rumen fluid and microbial inoculum obtained from macroalgae-eating Ronaldsay sheep (Orkney), the greater degradability could be the function of potential adaptation of rumen microbes to those particular macroalgae (Hansen et al., 2003). Hence, a gradual increase in the digestibility of macroalgae may be observed over time and therefore, animals may require an adaptation period to achieve an acceptable digestibility level. However, it is not known whether exposure to macroalgae-based diets at a young age would lead to a better feed digestibility in adults.

Feeding macroalgae at larger doses for a long duration can result in adverse health consequences such as bone and kidney dysfunctions in animals, probably due to mineral overload (Britt and Baker, 1990). Thus, long-term use of macroalgae as sole feed may not be safe, unless excess minerals and potentially toxic heavy metals are removed prior to feeding. Rinsing of macroalgae biomass with fresh cold or hot water (e.g. 40°C) for a short duration (30 min) could be effective in removing excess mineral salts from macroalgae (Magnusson et al., 2016). These types of processing may also enhance palatability of macroalgae and nutrient digestibility, though a loss of soluble nutrients can be expected (Magnusson et al., 2016). Thus, proper post-harvest processing of macroalgae biomass prior to animal feeding may minimize the risks of adverse health impacts on animals and can improve nutrient utilization.

Macroalgae can affect animal feed intake and rumen degradability of the feed, depending upon the inclusion level and the macroalgal species (Choi

Table 1 Effects of macroalgae on rumen fermentation and animal performance

Macroalgae species	Study type	Animal/rumen fluid donor	Dose	Impacts on ruminant nutrition	References
Red macroalgae:					
Asparagopsis armata	*In vivo*	Dairy cow	0.5–1% OM	Reduces CH_4 but also reduces feed intake (−38%), lower milk yield (−11.6%) at 1% OM inclusion	(Roque et al., 2019b)
Asparagopsis taxiformis	*In vitro/ In vivo*	Steers/sheep	0–16.7% OM	Effectively reduces CH_4, enables ADWG at low inclusion, lowers total VFA (≥2% OM inclusion) and OMD at ≥10% inclusion	(Machado et al., 2016; Li et al., 2018; Kinley et al., 2020)
Gracilaria vermiculophyla	*In vitro*	Cow	25% DM	Reduces CH_4, no adverse effects on rumen fermentation	(Maia et al., 2016)
Gigartina sp.	*In vitro*	Cow	25% DM	Reduces CH_4, no adverse effects on rumen fermentation	(Maia et al., 2016)
Mastocarpus stellatus	*In vitro*	Sheep/dairy cow	20% DM	Lowers VFA production and has low in situ DM and crude protein degradability	(Molina-Alcaide et al., 2017)
Palmaria palmata	*In vitro/ in situ*	Sheep/dairy cow	20% DM	High DMD, supplies high level of digestible protein, high amino acid index	(Tayyab et al., 2016; de la Moneda et al., 2019; Gaillard et al., 2018)
Porphyra spp.	*In vitro/ in situ*	Sheep/dairy cow	20% DM	High DMD, provides high digestible and rumen by-pass protein (~50% of total protein content)	(Tayyab et al., 2016; de la Moneda et al., 2019; Gaillard et al., 2018)
Brown macroalgae:					
Alaria esculenta	*In vitro/ In situ*	Dairy cow, goat	20% DM	High apparent DMD and protein digestibility	(de la Moneda et al., 2019; Tayyab et al., 2016)

(*Continued*)

Table 1 Effects of macroalgae on rumen fermentation and animal performance (*Continued*)

Macroalgae species	Study type	Animal/rumen fluid donor	Dose	Impacts on ruminant nutrition	References
Ascophyllum nodosum	*In vitro/ in vivo*	Cattle, lambs, steers	2–5% DM	Alters rumen and gut microbiome, and reduces OMD, Total VFA and CH_4 production	(Wang et al., 2009a; Belanche et al., 2016b)
Fucus serratus	*In vitro*	Sheep	?	Low rumen degradability (15%)	(Greenwood et al., 1983; Makkar et al., 2016)
Fucus vesiculosus	*In vitro*	Sheep	?	Low rumen degradability (26%)	(Makkar et al., 2016; Greenwood et al., 1983)
Laminaria digitata	*In vitro*	Cow/sheep	5–20% DM	~80% *in vitro* OM digestibility, high rumen degradability, enables microbial protein synthesis, provides greater AA supply in intestine	(Belanche et al., 2016a; Hansen et al., 2003; de la Moneda et al., 2019)
Laminaria hyperborea	*In vivo*	Sheep	?	High rumen degradability, high in vitro OM digestibility (~80%)	(Hansen et al., 2003)
Laminaria ochroleuca	*In vitro*	Cow	25% DM	No adverse effects on fermentation parameters (e.g. Total VFA, CH_4)	(Maia et al., 2016)
Macrocystis pyrifera	*In situ*	Bull	?	~ 85% degradability of DM (96 hr) and high by-pass protein	(Gojon-Báez et al., 1998)
Pelvetia canaliculata	*In vitro*	Goat	Sole feed /20% DM	Low DMD, lowers VFA volume	(de la Moneda et al., 2019; Molina-Alcaide et al., 2017)
Saccharina latissima	*In vitro*	Cow/ goat	20–25% DM	Improves DMD and OMD of feed, no effects on VFA production and rumen fermentation	(de la Moneda et al., 2019; Maia et al., 2019)
Sargassum spp.	*In situ/in vivo*	Bull/sheep	10–30% DM	No effect on feed intake and digestibility, ~55–79% DMD and high protein digestibility (>85%)	(Gojon-Báez et al., 1998; Marín et al., 2009)

Undaria pinnatifida	*In vitro/ In situ*	Cow	10% DM	Improves feed digestibility and rumen environment, VFA production, provides quality and digestible protein	(Choi et al., 2019)
Green macroalgae: *Acrosiphonia* sp.	*In situ*	Dairy cow	20% DM	Provides high rumen digestible (~46%) and rumen by-pass protein (~31%)	(Tayyab et al., 2016; Molina-Alcaide et al., 2017)
Chaetomorpha sp.	*In vivo*	Sheep	20–40% DM	No adverse effects on feed digestibility and growth performance up to 30% inclusion	(Rjiba-Ktita et al., 2019)
Cladophora patentiramea	*In vitro*	Steers	16% (OM)	Reduces CH_4, feed degradability and VFA volume	(Machado et al., 2014)
Cladophora rupestris	*In situ*	Dairy cow	-	High protein content, 75% total tract AA degradability	(Gaillard et al., 2018)
Ruppia sp.	*In vivo*	Sheep	20–40% DM	Improves feed intake, no adverse effects on feed digestibility and growth performance up to 30% inclusion	(Rjiba-Ktita et al., 2019)
Ulva rigida	*In vivo/ in vitro*	Sheep/ cow	25% DM	Increases DMD of the feed, no effect on VFA and CH_4 production	(Cabrita et al., 2017; Maia et al., 2019)
Ulva sp.	*In vivo/ in vitro*	Sheep	20–40% DM	High rumen degradable and by-pass protein, no effects in feed intake and degradability for 30% DM inclusion	(Tayyab et al., 2016; Rjiba-Ktita et al., 2019)

DM, dry matter; OM, organic matter; DMD, dry matter degradability; OMD, organic matter degradability; AA, amino acids; ADWG, average daily weight gain; VFA, volatile fatty acids.

et al., 2019; Maia et al., 2019; Rjiba-Ktita et al., 2019). For instance, a low inclusion level of the anti-methanogenic red macroalga *Asparagopsis* taxiformis resulted in an improved average daily weight gain and OM degradability of the feed in cattle when included at ≤5% of OM, but the opposite effects were observed as the dose was increased to 10% OM of the total ration (Machado et al., 2016; Kinley et al., 2020). Hence, an inclusion level of <5% OM appears to be the cut off value for *A. taxiformis* in terms of maintaining the feed digestibility and fermentation parameters, such as VFA production (Machado et al., 2016; Roque et al., 2019a). Another anti-methanogenic red macroalga *Asparagopsis armata*, however, reduced feed intake, weight gain and milk yield at a relatively low inclusion level (≤1% OM) in the feed of dairy cattle (Roque et al., 2019b). This indicates that *Asparagopsis* spp. can be included in the ruminant feed at a low inclusion to achieve beneficial impacts in overall animal performance.

Other, different, red, brown and green species have also shown similar trends as *Asparagopsis* spp.; however, they can possibly be included at higher doses. Increased *in vitro* DM degradability and VFA production were observed with the edible brown macroalgae, *Undaria pinnatifida*, when it was incorporated up to 10% DM in the feed (Choi et al., 2019). Similarly, stable feed digestibility and animal performance were achieved with other brown (*A. esculenta, L. digitata, S. latissima*), red (*G. vermiculophyla, M. stellatus, P. palmata, Porphyra* sp.) and green (*Cladophora* sp. and *Ulva* spp.) macroalgae up to 20–25% of DM inclusion (de la Moneda et al., 2019; Maia et al., 2019). A few other green macroalgae (*Chaetomorpha* sp., *Ruppia* sp., and *Ulva* sp.) produced no significant negative effects on feed intake and digestibility in Barbarine sheep at up to 30% DM inclusion, but feed digestibility was reduced while the inclusion was increased to 40% (Rjiba-Ktita et al., 2019). This suggests that these macroalgal species can be incorporated to 10–30% in the ruminant rations, though more *in vivo* studies are needed to establish a beneficial inclusion level of a broad range of macroalgal species.

Macroalgae are generally low energy containing feeds due to low contents of lipid and starch, a large proportion of complex polysaccharides and relatively large content of ash (Bikker et al., 2020; Angell et al., 2016; Øverland et al., 2019). The GE contents of macroalgae, including *U. lactuca, Ulva rigida, G. vermiculophyla* and *S. latissima*, have been reported to be less (14–15.2 MJ/kg DM) than conventional ruminant feeds such as corn silage, hay silage and commercial concentrates (17.4–18.9 MJ/kg DM) (Maia et al., 2019; Ventura and Castañón, 1998). However, some brown macroalgae including *A. esculenta, A. nodosum* and *F. vesiculosus* have higher GE and digestible energy levels than the terrestrial forages such as winter rye and lichen (Applegate and Gray, 1995). Thus, although the majority of macroalgae lead to a lower energy supply compared to conventional feeds, there is a scope for their future use

as feed additives in the ruminant's rations due to their high mineral contents and promising anti-methanogenic potentials as described in the following sections.

4 Anti-methanogenic properties of macroalgae

In addition to the aforementioned macro- and micro-nutrients, macroalgae are also rich sources of a wide range of bioactive components (such as pigments, tocopherols and various secondary metabolites) (Gupta and Abu-Ghannam, 2011). Macroalgae are gaining interest as anti-methanogenic feed ingredients in ruminants due to their richness of bioactive compounds, particularly halogenated and polyphenolic secondary metabolites that are able to inhibit CH_4 formation during the fermentation of feed in the forestomach (Roque et al., 2019a; Wang et al., 2008). In the following sections, the anti-methanogenic potentials of different macroalgal species will be discussed.

Red macroalgae: The potential of macroalgae to suppress enteric CH_4 formation in ruminants has been evaluated using both *in vitro* and *in vivo* studies (Table 2). The most convincing anti-methanogenic properties have been found among the red macroalgae, particularly *Asparagopsis* spp. (Machado et al., 2016; Roque et al., 2019b; Kinley et al., 2020). It was reported that a 40–98% reduction of CH_4 emission in steers could be achieved by adding as little as 0.1–0.2% (OM basis) *A. taxiformis* to a high grain diet (Kinley et al., 2020). Similarly, a 72-day feeding trial in sheep using the same macroalgae in a mixed ration (3% of the OM of the diet) containing a high proportion of fiber, resulted in an overall 80% reduction of enteric CH_4 production (Li et al., 2018). This is consistent with several *in vitro* fermentation studies, where addition of 0.5–5% OM of this macroalgae along with different substrates resulted in an ~74–99% decline in CH_4 formation over a 72-h incubation period (Roque et al., 2019a; Brooke et al., 2018; Machado et al., 2016). Another red macroalgae species belonging to the same genus, *A. armata* has also been shown to suppress the CH_4 production by ~67%, when fed to dairy cattle at 1% of OM (Roque et al., 2019b). Thus, *Asparagopsis* spp. can be an effective feed additive which can reduce enteric CH_4 production dramatically at a minimal inclusion in the ruminant diet.

The anti-methanogenic property of red macroalgae is not limited to the genus *Asparagopsis*. Two other red species *Gigartina* sp. and *Gracilaria vermiculophyla* have also demonstrated anti-methanogenic attributes in *in vitro* fermentations, but a greater amount of the macroalgae was added (16–18% OM), and the magnitude of reduction was substrate dependent (Maia et al., 2016). For example, a 60% reduction in CH_4 production was observed when *G. vermiculophyla* was supplemented to either meadow hay or corn silage, whereas *Gigartina* sp. reduced CH_4 production by 44%, but only when added

Table 2 Macroalgae species and their anti-methanogenic potential

Macroalgae species	Study type	Inclusion dose (%)	CH_4 ↓ (%)	Main active compound	Reference
Red macroalgae:					
Asparagopsis armata	*In vivo*	1% OM	67.2	Bromoform	(Roque et al., 2019b)
Asparagopsis taxiformis	*In vivo/in vitro*	0.1–16% DM/OM	74–99	Bromoform	(Kinley et al., 2020; Machado et al., 2016; Roque et al., 2019a)
Gracilaria vermiculophyla	*In vitro*	25% DM	63	?	(Maia et al., 2016)
Gigartina sp.	*In vitro*	25% DM	44	?	(Maia et al., 2016)
Brown macroalgae:					
Ascophyllum nodosum	*In vitro*	2% OM	15	Phlorotannin, polysaccharide (?)	(Belanche et al., 2016b)
Cystoseira trinodis	*In vitro*	20% OM	80	Terpenes, phlorotannin (?)	(Dubois et al., 2013)
Dictyota bartayresii	*In vitro*	2% OM	92	Phlorotannin, isoprenoids	(Machado et al., 2014)
Zonaria farlowii	*In vitro*	2% OM	11	Phenolic lipids	(Brooke et al., 2018)
Green macroalgae:					
Cladophora patentiramea	*In vitro*	16% OM	66.3	?	(Machado et al., 2014)
Oedogonium sp.	*In vitro*	75% OM	30.3	?	(Machado et al., 2016)
Ulva sp.	*In vitro*	25% DM	55	?	(Maia et al., 2016)

DM, dry matter; OM; organic matter; ↓, decrease in methane (CH_4) production; ?, unknown.

to meadow hay and not corn silage (Maia et al., 2016). However, no significant anti-methanogenic properties were detected for three other red macroalgae species studied, *M. stellatus, P. palmata* and *Porphyra* sp., when they constituted 8.4–20% fresh matter in the concentrate portion of the goat diet in an *in vitro* fermentation study (de la Moneda et al., 2019). Thus, some, but not all, red macroalgae species have strong anti-methanogenic properties, but their quantitative impact on CH_4 emission may depend on both the composition of the ruminant diet and the type and concentration of bioactive components present in the macroalgae and this requires further investigations in the future.

Brown macroalgae: Certain brown macroalgae species also have CH_4 mitigating potential, but the documentation stems primarily from *in vitro* studies. Two species *Dictyota bartayresii* and *Cystoseira trinodis* were able to reduce *in vitro* CH_4 production by 90% and 80%, respectively, when 16% of OM was added to Rhodes grass (Machado et al., 2014; Dubois et al., 2013). Other brown species, such as *A. nodosum* and *Zonaria farlowii*, have been shown to reduce CH_4 *in vitro* by 11–15% at inclusions of 2% and 5%, respectively (Brooke et al., 2018; Belanche et al., 2016b). However, no anti-methanogenic properties have been detected with 20–25% DM inclusion in feed in other *in vitro* trials, when using *L. digitata*, *L. ochroleuca*, *P. canliculata* and *S. latissima*, (Maia et al., 2016; de la Moneda et al., 2019). This suggests that only specific brown macroalgae species possess anti-methanogenic properties, and these are less powerful than those of the red species. However, further studies are needed to estimate their most effective dietary inclusion rates, and to confirm whether such outcomes are also evident *in vivo*.

Green macroalgae: Methane reduction properties have been observed in a few green macroalgae species. *Cladophora patentiramea* and the fresh water green algae *Odogonium* sp. have been shown to reduce CH_4 production by 66% and 30% *in vitro*, when added at 16% OM to decorticated cottonseed meal *in vitro* (Machado et al., 2014). With a similar inclusion rate in corn silage, another unspecified green macroalga from the genus *Ulva* illustrated a 55% suppression on enteric CH_4 production *in vitro* (Maia et al., 2016). However, the same authors later revealed that 25% DM addition of *Ulva rigida* to a mixed ration *in vitro* did not reduce CH_4 production (Maia et al., 2019). Thus, compared to brown and particularly red species, green macroalgae seem to have limited anti-methanogenic potential, which would require high levels of inclusion in the feed.

The anti-methanogenic macroalgae have also been found to affect other rumen fermentation parameters. With a concomitant reduction in the CH_4 production, they will decrease total VFA amount, feed intake and degradability when included in large amounts in the feed (Machado et al., 2014; Roque et al., 2019b). These effects were clearly observed with various macroalgal species such as *Asparagopsis* spp. (red), *C. trinodis* (brown) and *D. bartayresii*

(brown) either *in vitro* or *in vivo* models, when macroalgae supplementation was gradually increased (Machado et al., 2014; Li et al., 2018; Roque et al., 2019b). Thus, it is important to include macroalgae at an optimum level so that possible negative impacts on rumen fermentation and animal performance are minimized.

4.1 Anti-methanogenic factors in macroalgae and potential mechanisms

Mechanistic insights into the anti-methanogenic properties of macroalgae are needed to identify the most efficient and safe ways of using them as feed additives to reduce enteric methane formation. Enteric CH_4 emissions can be reduced by macroalgae through two different mechanisms: (a) a direct inhibition of the methanogenic archaea themselves or rate-limiting steps in their methane formation or (b) through alteration of the rumen environment by reducing substrate availability or altering the rumen microbiota composition to disfavor the methanogens (Fig. 4).

4.1.1 Direct impacts: inhibition of methanogens and the methanogenic pathways

Macroalgae produce a number of secondary metabolites that protect them from a complex and possibly stressful seawater environment and help them to cope with various microbial infections (Li et al., 2017) and such metabolites may

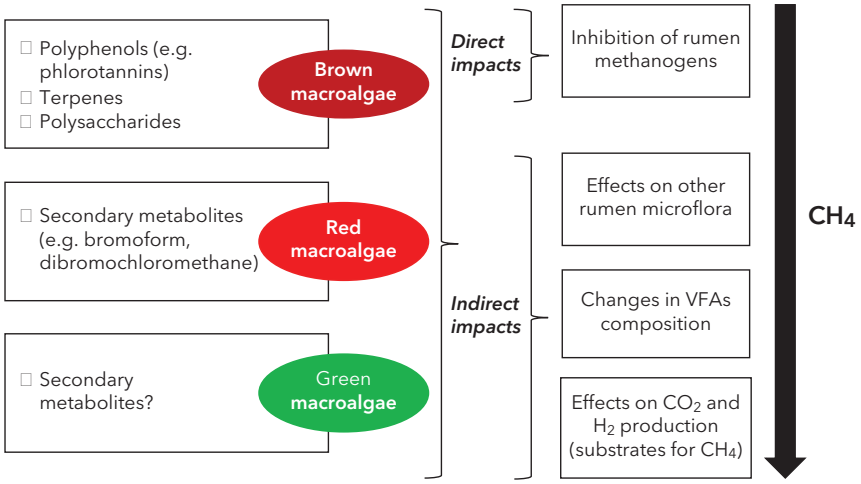

Figure 4 Potential anti-methanogenic factors of macroalgae and their mode of action in minimizing methane production in ruminants. CH_4, methane; VFAs, volatile fatty acids; CO_2, carbon dioxide; H_2, hydrogen.

also to a large extent account for the anti-methanogenic properties of some species. These compounds and their mode of action are described hereunder.

Halogenated compounds: Halogenated compounds are the aliphatic compounds containing one or two carbon atoms that are covalently linked with one or more halogen atoms (fluorine, bromine, chlorine or iodine). These compounds, such as bromoform, chloroform and bromochloromethane, irrespective of their source (synthetic or macroalgae), have shown a strong inhibitory action both *in vitro* and *in vivo* against rumen and other methanogens, significantly lowering their abundance in the rumen even at a low concentration (Paul et al., 2006; Roque et al., 2019a; Machado et al., 2018; Denman et al., 2015). Red macroalgae (e.g. *Asparagopsis* spp.) produce a high level of various brominated and chlorinated halocarbons, including bromoform, dibromochloromethane, chloroform, bromochloroacetic acid and dibromoacetic acid (Machado et al., 2018; Paul et al., 2006). They are structural analogs of CH_4 and other methanogenic intermediates and possess a higher affinity to enzymes, including corrinoid/porphinoid, which catalyzes the cobamide-dependent methyl transfer in methanogenesis (Wood et al., 1968; Yu and Smith, 2000; Roque et al., 2019b). Thus, the halogenated compounds can competitively inhibit the binding of intermediates or methane substrates into the corrinoid/porphinoid enzyme (Yu and Smith, 2000). Moreover, they are also structurally similar to CoM (a cofactor produced specifically by methanogens) which supplies the methyl group to methyl coenzyme-M reductase enzyme during the terminal reductive reaction of methanogenesis (Liu et al., 2011; Roque et al., 2019b; Li et al., 2018). Therefore, anti-methanogenic compounds from red macroalgae seem to exert their effects on CH_4 production directly by either of the two mechanisms: (a) minimizing the abundance of rumen methanogens through their anti-microbial activity or (b) interrupting their functional components such as enzymes, catalyzing the different steps of methane biosynthesis.

The anti-methanogenic property of synthetic halocarbons, such as chloroform, is dependent on the degree of chlorination, and this property can decrease over time due to the sequential reductive dechlorination during methane inhibition (Yu and Smith, 2000). In addition, methanogens have also been shown to develop resistance to synthetic anti-methanogenic compounds such as bromochloromethane when repeatedly exposed, potentially due to the adaptation of methanogens to those compounds (Patra et al., 2017). Although the rate of dechlorination and possibility of developing resistance to anti-methanogenic compounds derived from red macroalgae is unknown, two animal trials in steers and sheep have shown persistent CH_4 mitigating effect of *A. taxiformis* for 3 months (Li et al., 2018; Kinley et al., 2020). This indicates that anti-methanogenic compounds from these macroalgae might have more stable and effective CH_4 mitigation potential than synthetic halocarbons.

The excess intake of bromoform can be hazardous to human and animal health and therefore a maximum uptake level of 0.08 mg/L has been set for drinking water in the USA (EPA, 2012). In addition, synthetic aliphatic halocarbons are reported to cause ozone depletion and thus have environmental concerns (Patra et al., 2017; Roque et al., 2019b). Therefore, the possible toxicity of halogenated compounds from red macroalgae should be investigated to understand their effect on both animal health and environment.

Polyphenols: Polyphenols are a group of phenolic compounds and the concentration of these can account for up to 15% of DM in brown macroalgae (Wang et al., 2009a). The predominant form of polyphenols in brown macroalgae is phlorotannins (PT) and their anti-methanogenic properties have been described in *in vitro* studies (Hierholtzer et al., 2013; Wang et al., 2008). Though the effects of PT specific to rumen methanogenic archaea are not clear, a suppressive effect of condensed tannins (structural analogs of PT) on rumen archaea has been reported. For example, condensed tannins extracted from the terrestrial forage *Leucaena leucocephala* have exhibited a linear reduction of total rumen methanogens belonging to the orders *Methanobacteriales* and *Methanomicrobiales* with increasing doses (Tan et al., 2011). Due to the limited information available on the impacts of PT on rumen methanogens, it is too soon to evaluate whether there is a practical perspective for the use of PT in ruminants. However, because of the chemical and structural resemblances of PT and terrestrial tannins, antimicrobial activity of PT against rumen methanogens can be anticipated (Wang et al., 2008).

The mechanisms of action of PT on rumen methanogens are not known, but are described for other rumen microbes or methanogens isolated from wastewater treatment plant (Hierholtzer et al., 2013; Wang et al., 2009a). It has been revealed that PT can affect the integrity of microbial cell membrane and cell wall, via creating stress and ultimately causing cell lysis (Hierholtzer et al., 2013; Wang et al., 2009b). Other potential mechanisms of PT in relation to anti-microbial effects have been suggested to be via inactivation of extracellular enzymes and proteins necessary for growth and metabolism of microorganisms (Scalbert, 1991).

Species specific and time-dependent impacts of PT against various rumen microorganisms have been observed. For example, 500 μgmL^{-1} PT isolated from *A. nodosum* resulted in the reduction of cellulolytic rumen bacteria *Fibrobacter succinogenes* by 78%, 83% and 65% in 6, 12 and 24 h, respectively in an *in vitro* batch culture (Wang et al., 2009a). The same level of PT caused a 42% decrease in *Ruminococcus albus* without affecting the population of *Ruminococcus flavefacien* during 24 h of cultivation. In contrast, it significantly increased the number of non-cellulolytic bacteria such as *Prevotella bryantii, Ruminobacter amylophilus* and *Selenomonas ruminantium* (Wang et al., 2009a). This suggests that even within an order, various bacterial strains may be

differentially affected by PT and that may also apply to the rumen methanogens. The underlying reason for this selective and differential anti-microbial property of PT is yet unknown. However, this could possibly be linked to the structure of PT, such as degree of polymerization (phloroglucinol units) and the number of reactive hydroxyl groups present (Wang et al., 2008; Hierholtzer et al., 2013). Furthermore, interspecies differences of macroalgae in the methane inhibition potential and the potency of PT from those macroalgae may also play some role in this selective action.

Polysaccharides: Macroalgae contain different kinds of polysaccharides, which are present either as structural components of the complex cell wall or as storage carbohydrates (O'Sullivan et al., 2010). Bactericidal and bacteriostatic effects of these polysaccharides have been documented against various hindgut microorganisms (Smith et al., 2011; Seong et al., 2019); however, specific information about their impacts on the rumen methanogens is yet to be evaluated. Polysaccharides from brown macroalgae (alginates, fucoidan and laminarin) can partially and selectively be fermented by specific bacteria in the rumen (such as *Prevotella* sp., *C. butyricum* and *Selenomonas* sp.) (Williams et al., 2013; Orpin et al., 1985). These polysaccharides have shown selective enrichment of beneficial gut bacteria, including *Bifidobacterium, Clostridium coccoides* and *Lactobacillus*, and a suppression of pathogenic gut microbes, including *E. coli, Salmonella* spp., *Enterococcus* and *Clostridium* spp., in monogastric animals (Charoensiddhi et al., 2017; Seong et al., 2019; Smith et al., 2011). Thus, macroalgae polysaccharides apparently have anti-microbial properties and whether such selective impacts are also evident with rumen microorganisms are not known.

Isoprenoids and terpenes: Macroalgae also produce various types of isoprenoids and terpenes, and over 200 different diterpenes have been identified in the single brown macroalgae of genus *Dictyota* (Chen et al., 2018). Isoprenoids have also been reported in other macroalgae, including *A. taxiformis* and *C. trinodis* (Brooke et al., 2018; Machado et al., 2014; Dubois et al., 2013). Although they have been suggested to contribute to the CH_4 mitigating properties, nothing is yet known about the mechanism underlying the anti-methanogenic effect of such compounds.

4.1.2 Indirect impacts: changes in the rumen environment affecting methanogenesis

In addition to the direct influences, macroalgae macroconstituents and bioactive compounds can affect numerous microorganisms in the rumen leading to changes in fermentation parameters and the overall rumen environment. The factors affected include the relative abundance and activity of non-methanogenic microorganisms, VFA production and availability of substrates

for CH_4 production. The anti-methanogenic macroalgae species such as *A. taxiformis* and *A. nodosum* have been found to reduce the abundance of rumen microbes, including rumen protozoa (Roque et al., 2019a; Belanche et al., 2016b). A group of rumen methanogens (9–25% of total methanogens) live in a mutualistic relationship with protozoa and they generate a large amount of H_2 that is utilized by the methanogens to form CH_4 (Belanche et al., 2014; Newbold et al., 1995). Protozoans also get benefit from the methanogenic H_2 utilization because accumulation of H_2 in the rumen is inhibitory to their growth (Belanche et al., 2014). Thus, changes in the abundance and activity of the protozoa will result in H_2 deprivation in the rumen resulting in reduced methanogenesis (Morgavi et al., 2012).

The incorporation of macroalgae with anti-methanogenic potential in ruminant feed changes VFA production profile. They can change patterns of rumen fermentation from acetate formation towards the formation of more propionate and thus reducing acetate:propionate ratio (Machado et al., 2014; de la Moneda et al., 2019; Belanche et al., 2016b). Acetate formation in the rumen results in the release of metabolic H_2, and this can be used by methanogens to produce CH_4 (Fig. 2). Therefore, reduced acetogenesis and increased propiogenesis are considered as factors indirectly decreasing methanogenesis (Roque et al., 2019a; Wolin and Miller, 1997). It has been noted that anti-methanogenic compounds such as PT and bromochloromethane increase the population of H_2-consuming bacteria, such as *Prevotella* spp., *F. succinogenes* and *Selenomonas* spp., in the rumen (Mitsumori et al., 2012; Denman et al., 2015; Wang et al., 2009a). The available H_2 can be re-channeled towards the formation of propionate, lactate and succinate by the action of H_2-consuming bacteria (Denman et al., 2015; Belanche et al., 2016a) which may also lead to H_2 deprivation. However, when the methanogenesis is highly inhibited (as with *A. taxiformis*), all the metabolic hydrogen produced cannot be captured by this re-channeling towards the formation of aforementioned fatty acids, and some will be eructed by animals (Martinez-Fernandez et al., 2016; Kinley et al., 2020). In fact, the actual causes and effects of macroalgae in changes of VFA, H_2 and populations of bacteria involved in the formation and consumption of these substances are yet to be clearly understood.

5 Processing and seasonal effects on anti-methanogenic properties of macroalgae

Post-harvest processing of macroalgae biomass, such as washing, drying and storage conditions may impact, not only the nutritional contents, but also the bioactive potential of harvested macroalgae (Kadam et al., 2015; Paull and Chen, 2008). The drying of *A. taxiformis* biomass at 45°C for 48 h led to a substantial reduction in the concentration of bromoform and eventual anti-methanogenic

activity (Vucko et al., 2017). Similarly, oven drying at a higher temperature (80°C for 24 h) caused a significant reduction in the level of phytochemicals including polyphenols and flavonoids in *Kappaphycus alvarezzi* as compared to a lower temperature (40°C for 24 h) (Ling et al., 2015). However, the extractability of polyphenols and flavonoids could be greater when macroalgae biomass is semi-dried (e.g. 35–40°C for 2 h) as noted with the semi-dried *H. elongata* biomass (~40% increase) (Gupta et al., 2011). For other macroalgal species such as *F. vesiculosus* and *Porphyra* spp., drying methods (oven, sun or freeze drying) did not alter the amount of bioactive phytochemicals (Jiménez-Escrig et al., 2001). These results indicate that an appropriate drying/processing protocol for macroalgae biomass after harvesting may be beneficial to achieve increased levels of bioactive phytochemicals. Further studies are required to establish such optimal procedures, not in the least, in light of the high cost associated with the commercial production and transportation of macroalgae biomass.

The concentration of nutrients and bioactive components in macroalgae can vary across the seasons and geographical locations (Tayyab et al., 2016; Schiener et al., 2015). In macroalgae harvested in Norway, the level of protein and minerals have been found to be higher in spring than in autumn while polysaccharides (e.g. fucoidan and laminarin) are noted to be higher in summer (Kim, 2012; Rioux et al., 2009; Tayyab et al., 2016; Molina-Alcaide et al., 2017). On the other hand, in the same location, total polyphenol content in brown (*A. esculenta, L. digitata, P. canaliculata* and *S. latissima*), red (*P. palmata, M. stellatus* and *Porphyra* sp.) and green (*Acrosiphonia* sp.*,* and *Cladophora rupestris*) macroalgae have been found to be around two-fold higher in autumn compared to the spring season (Molina-Alcaide et al., 2017; de la Moneda et al., 2019). In agreement with these findings, we have also found that Norwegian brown species (e.g. *A. esculenta*, *F. vesiculosus, P. canaliculata, H. elongata, L. digitata, S. latissima*) harvested in the autumn have higher polyphenol levels compared to those harvested in the spring (Deepak et. al. unpublished data). However, a study from Scotland which included some of the aforementioned macroalgae (*A. nodosum, A. esculenta, L. digitata, L. hypeborea* and *S. latissima*) recorded a higher total polyphenol content during the summer compared to the autumn (Schiener et al., 2015). These variations are associated with the growth stage of the macroalgae and season-specific environmental factors such as temperature, light intensity and nutrient content in the seawater (Mišurcová, 2012; Parys et al., 2009). It has been mentioned that at the beginning of the spring season, vegetative growth of the macroalgae is rapid and the level of polyphenols is low during the rapid growth stage (Parys et al., 2009). Furthermore, due to the geographical differences, variations in these environmental factors may exist within the same season. Therefore, a specific harvesting period needs to be established based on compounds

of interest and the spring season could be appropriate to harvest algae to achieve a maximum level of nutrients.

6 Future perspectives

Bioactive components of certain macroalgal species have the potential to be utilized as anti-methanogenic feed additives for ruminant animals to mitigate enteric CH_4 production. However, only a few species have been evaluated so far in this respect, and the most powerful anti-methanogenic compounds identified (halogenated carbons) are both ozone depleting and having documented adverse health impacts on humans (Roque et al., 2019b; Patra et al., 2017). It is therefore uncertain whether they can be approved (at least within the EU) as CH_4 mitigating instruments. Future studies should be directed towards the screening of a large number of macroalgal species to potentially identify efficient and safe compounds to be employed in climate-friendly feeding of ruminants. In this context, fractionation and/or extraction of targeted bioactive compounds (e.g. polyphenols, polysaccharides) and the characterization of their chemical and functional properties are important.

Utilization of macroalgae biomass as novel ruminant feeds on a larger scale is presently challenged by high costs associated with post-harvest processing as well as limited digestibility of several of the brown algae species that can most easily be cultivated. In addition, there are large variations between and within species in chemical composition and digestibility as well as contents of bioactive compounds depending on season, geographical location and post-harvest processing (Tiwari and Troy, 2015; Paull and Chen, 2008; Tayyab et al., 2016). This should encourage future research to develop cost-efficient techniques to increase the nutritional quality and anti-methanogenic potential of cultivable macroalgae by optimizing cultivation, harvest and post-harvest processing techniques.

7 Conclusions

The relevance of macroalgae as alternative and anti-methanogenic ruminant feeds depends upon their nutritional contents, digestibility, CH_4 mitigating potential, and influences on animal and environmental health. Red macroalgae such as *A. taxiformis and A. armata* seem to be promising anti-methanogenic feed ingredients and do not lead to significant adverse impacts on feed degradability with an inclusion rate of under 5% of OM. However, the impacts on other parameters of animal performances (e.g. feed intake, weight gain and milk yield) and rumen fermentation products, such as total VFA, should be carefully monitored. Moreover, due to a high concentration of halogenated compounds (e.g. bromoform) in those species, their potential adverse effects on human and environmental health must also be assessed. Brown macroalgae, such as

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D. bartayresii, C. trinodis and *A. nodosum*, can be effective anti-methanogenic feed ingredients. Nevertheless, a high phlorotannin and polysaccharide content of these species can negatively impact the rumen degradability at high inclusion levels and thus optimal supplementation levels of these algae need to be carefully maintained. Green algae, including *C. patentiramea*, can also mitigate enteric CH_4 production, but the active anti-methanogenic compounds in green macroalgae are unknown.

Red macroalgae, such as *P. palmata, Porphyra* spp. and *Gracilaria* spp., and the green species *Acrosiphonia*, *C. rupestris*, *Ruppia* and *Ulva* can be used as a nutrient source due to their better nutritional composition and greater degradability compared to other species. Among brown macroalgae, *A. esculenta, Laminaria* spp., *S. latissima* and *U. pinnatifida* could be used as feed additives in up to 10–25% of DM in the ruminant feed provided that the excess mineral content is removed. Overall, macroalgal species could be an important component of future ruminant feed, but further *in vivo* studies are required to identify any potential adverse impacts on animal health and performance.

8 Where to look for further information

Further useful information about macroalgae and their applications can be obtained from the following resources:

- Sustainable use of seaweeds for food and non-food applications: Ed. Tiwari, Brijesh and Troy D. J. 2015. Seaweed sustainability–food and nonfood applications. Elsevier, https://doi.org/10.1016/C2013-0-12836-X.
- Seaweed biology – Novel insights into Ecophyisology, Ecology and Utilization. 2012. Springer. https://doi.org/10.1007/978-3-642-28451-9.
- Application of seaweeds as feed: "Seaweeds for livestock diets" Makkar et al 2016. https://www.sciencedirect.com/science/article/pii/S0377840115300274?via%3Dihub.

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Paper II

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Interspecies and seasonal variations in macroalgae from the Nordic region: Chemical composition and impacts on rumen fermentation and microbiome assembly

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ABSTRACT

Marine macroalgae may serve as sustainable feed resources for ruminant production due to their nutritional attributes, and enteric methane (CH_4) mitigating potential. We aimed to characterize the anti-methanogenic properties of 12 Nordic macroalgae species (eight brown, three red, and one green). Differences in the chemical composition across two harvesting seasons and impacts of addition of macroalgae (20% dry matter basis) on *in vitro* rumen fermentability of maize silage (MS) and associated changes in the rumen microbiome composition were also evaluated. Green and red macroalgae contained twice as much crude protein (CP) as compared to brown macroalgae. The latter had higher mineral and total polyphenol content (TPC: 10 to 20 times). In some brown species, ash and CP contents were up to twice as high in spring than in autumn, but TPC was highest in autumn. The TPC content was inversely correlated with *in vitro* rumen fermentation characteristics: organic matter (OM) degradability ($r = -0.85$; $P < 0.001$), production of total gas ($r = -0.79$; $P < 0.001$), total volatile fatty acids ($r = -0.78$; $P < 0.001$) and CH_4 ($r = -0.53$; $P < 0.03$) per gram of OM. The polyphenol-rich brown species, *Fucus vesiculosus* and *Ascophyllum nodosum*, caused a significant reduction in feed degradability (~25%) due to the suppression of cellulolytic bacteria (*Ruminococcus* spp., *Lacnospiraceae* spp., *Rikenellaceae* RC9 gut group) in the rumen fluid after fermentation. Interestingly, autumn-harvested samples of those two macroalgae decreased the CH_4 production by 62.6% and 48.2%, respectively, and reduced rumen methanogenic archaea (e.g., *Methanobrevibacter* spp.), although the reduction was not directly correlated with TPC. Thus, Nordic macroalgae, depending upon their species-specific unique properties, could be utilized as anti-methanogenic feed additives or feeding resources for ruminants. *In vivo* studies are needed to establish the implications of feeding with these macroalgae on overall animal performance.

1. Introduction

The livestock sector is a critical element of food security worldwide as livestock-based products contribute 13% of calories and 28% of total protein consumed by humans (FAO, 2011). The demand for livestock products is expected to further rise with the growing global population and altered dietary preferences in direction of a higher proportion of animal-derived protein, particularly in developing countries (Alexandratos and Bruinsma, 2012). On the other hand, ruminant livestock such as cattle, sheep, and goats, are major sources of greenhouse gases, and they account for ~18% of the total anthropogenic methane (CH_4) emissions (Mizrahi et al., 2021). Addressing this challenge requires the development of an environmentally friendly, yet productive, livestock sector in the future to fulfill the demands of livestock products without

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increments in the carbon footprint of production. In this regard, changes in feeding strategies can mitigate the greenhouse gas emissions from ruminants (Haque et al., 2014) and various novel feeding materials could play a crucial role in the future to improve the balance between animal productivity and environmental sustainability (Beauchemin et al., 2008).

Marine macroalgae, also called seaweeds, might serve as such alternative feeding materials that can support animal productivity as well as reduce the CH_4 emissions from ruminant livestock (Kinley et al., 2020; Maia et al., 2016). Macroalgae are rich in carbohydrates, dietary fiber, and minerals (Dawczynski et al., 2007) and can contribute to both rumen-degradable as well as bypass protein sources for ruminants (Tayyab et al., 2016). Recent studies have indicated that dietary supplementation of certain macroalgae, such as the tropical red species *Asparagopsis taxiformis*, can reduce CH_4 emissions by targeting the special domain of rumen microbiota responsible for the formation of CH_4, namely the methanogenic archaea (Machado et al., 2018; Roque et al., 2019a). However, the bioactive compounds in *Asparagopsis* spp. responsible for the anti-methanogenic effect are halomethanes, which are ozone degrading, and some are also carcinogenic and may be toxic to animals (Muizelaar et al., 2021). A mild to moderate anti-methanogenic property has also been reported for specific temperate or North Atlantic macroalgae such as *Ascophyllum nodosum* (Belanche et al., 2016b), *Gracilaria vermiculophylla,* and *Ulva* spp. (Maia et al., 2016). However, the responsible anti-methanogenic compounds and their mechanisms of action remain unclear. In a recent study, anti-methanogenic halomethanes, such as bromoform that are found in *Asparagopsis* spp., could not be detected in macroalgae species from the Nordic region (Nørskov et al., 2021). It is therefore highly relevant to clarify if anti-methanogenic properties, potentially associated with safer bioactive compounds, could be detected among Nordic macroalgae species.

Nutritional values, as well as the contents of bioactive compounds in macroalgae, are associated with their phylum (brown, green, or red), genus, and species, and fluctuations can occur across the growing or harvesting seasons (Pandey et al., 2021). Red, as well as green macroalgae, are known to have higher contents of proteins than brown species, but the latter are enriched with higher levels of mineral elements and polyphenols (Molina-Alcaide et al., 2017). In general, protein and minerals are present in the highest concentrations during winter or spring (Rødde et al., 2004; Tayyab et al., 2016), while carbohydrates and polyphenols have been found highest during summer or autumn, but this may vary with the species or type of macroalgae (Connan et al., 2004; Schiener et al., 2015). This seasonal and species-specific variation can affect the digestibility of the algae and the impact on enteric CH_4 production, hence possibilities for utilization as a source of feed or as an anti-methanogenic feed additive for ruminants (de la Moneda et al., 2019; Molina-Alcaide et al., 2017). Information about seasonal and species variation is important to identify the optimal harvest time to achieve maximum nutritional and/or bioactive potential of macroalgae.

Therefore, the current study investigated the interspecies and seasonal variations in the chemical composition of 12 Norwegian macroalgae species, and their impacts on *in vitro* rumen fermentation characteristics, including enteric CH_4 production and composition of the rumen microbiome. Macroalgae selected in this study comprised the dominant wild (e.g., *A. nodosum, Fucus vesiculosus*) and the commercially cultivated genera (kelp species: *Saccharina latissima, Alaria esculenta, Laminaria digitata*) in Norwegian coastal water. These species were chosen as they are considered an important part of the future bioeconomy (Stévant et al., 2017). The activities associated with commercial macroalgae production are continuously increasing in Norway as indicated by the number of companies involved (27 in 2020 vs 10 in 2015), cultivation sites (114 in 2021 vs 83 in 2018), and harvested biomass (336 metric tons in 2020 vs 51 in 2015) (Directorate of Fisheries, 2022). The species included in this research are regarded as highly relevant for different applications, including food, livestock feed, etc., and likely to be in higher demand in the future (Makkar et al., 2016; Skjermo et al., 2014). To the best of our knowledge, this study is the first to evaluate the seasonal impacts on rumen fermentation characteristics of the brown macroalgae species: *F. vesiculosus, Fucus serratus*, and *Himanthalia elongata*. Furthermore, impacts of *F. vesiculosus*, *F. serratus*, *S. latissima,* and *Ulva lactuca* on the rumen microbiome have not been reported previously. Thus, this study aimed to test two hypotheses: A) macroalgae harvested in the spring are more rumen degradable than when harvested in the autumn. This would potentially make them more suitable as feed resources for ruminants due to higher nutritional value, and B) polyphenol-rich brown macroalgae are the most effective in reducing enteric CH_4 production, regardless of the harvesting seasons, due to their species-specific modulations of the rumen microbiome and associated rumen fermentation characteristics.

2. Materials and methods

An outline of the experimental activities and analyses is presented in Fig. 1. Prior to the harvesting of macroalgal biomass, different environmental parameters of seawater were monitored at the sampling locations.

2.1. Monitoring of environmental parameters during harvesting

Environmental parameters in the seawater (temperature, salinity, and dissolved oxygen levels) were recorded during the sampling time at seven different nearby sampling locations, using a sensor equipment STD/CTD SD204 (SAIV A/S Environmental Sensors & Systems, Bergen, Norway). The equipment was dipped about 1 m into the seawater and readings were recorded every second for at least 2 min.

2.2. Macroalgae biomass collection and processing

Twelve different macroalgae (eight brown, three red, and one green) were harvested from wild populations and processed as described previously (Roleda et al., 2019; Tayyab et al., 2016). For *Laminaria digitata*, two different parts of the thallus: blades and stipes were collected, while for other species the whole algal plants excluding the holdfast were included. In brief, about 5 kg fresh biomass for each species was collected manually from different sites within a narrow zone (N 67° 16.466′ E 014° 33.608′ to N 67° 16.550′ E 014° 34.218′) during the spring (07–09 May 2019) and autumn (01–03 October 2019) from the coastal area of Bodø, Norway (Table 1). Within 2 h of collection, the biomass for a given species collected from different sites were pooled based on similarity of environmental conditions at the harvesting sites and were transported to a laboratory (Mørkvedbukta; Nord University, Bodø) for further processing. First, the collected biomasses were washed with seawater followed by a mix of 30% seawater and 70% freshwater, and finally with freshwater to remove any possible contaminants, surface salts, and invertebrates. Then, excess water was drained and the samples were frozen at −40 °C until they were lyophilized for 72 h at −50 °C under a vacuum pressure of <0.1 mbar (Labconco, freeze dryer, Kansas City, MO, USA). Finally, the lyophilized samples were ground to a particle size of 2 mm using a cutter mill (CT Cyclotex TM 193 TM, FOSS, Hillerød, Denmark), and the homogenized material was analyzed for chemical compositions and used for *in vitro* rumen fermentation studies.

2.3. Chemical composition analyses

Dry matter (DM), organic matter (OM), ash, and protein contents of the samples were determined gravimetrically following the principles of the Association of Official Analytical Chemists (AOAC) with some modifications (Horwitz, 2010). Dry matter was estimated by drying the ground macroalgal powder at 105 °C for 24 h. Ash content was estimated by weighing the obtained residue after combustion of samples at 530 °C overnight, and OM was calculated as DM weight minus the

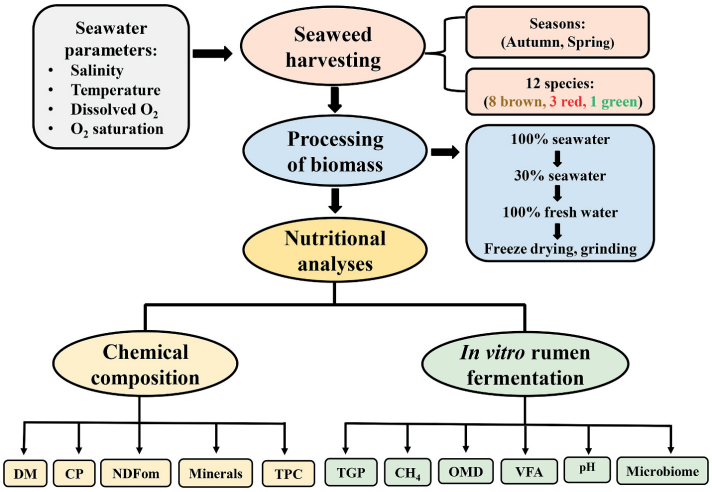

Fig. 1. A flowchart of the macroalgae experiment. Twelve different macroalgae species were harvested from the coastal area of Bodø, Norway, in two different seasons (autumn and spring). Environmental parameters of seawater at harvesting location, chemical compositions, and impacts of macroalgae on *in vitro* rumen fermentation parameters were analyzed. CH_4, Methane; CP, Crude protein; DM, Dry matter; NDFom, Ash corrected neutral detergent fiber; O_2, Oxygen; OMD, Organic matter rumen degradability; TGP, Total gas production; TPC, Total polyphenol content; VFA, Volatile fatty acids.

Table 1
List of harvested macroalgae and their phylum.

Name of macroalgae	Phylum	Harvest time (Autumn)	Harvest time (Spring)	Tidal zone[a, b]
Alaria esculenta	Brown	01 October 2019	07 May 2019	Lower intertidal to subtidal
Ascophyllum nodosum	Brown	03 October 2019	08 May 2019	Mid intertidal
Fucus serratus	Brown	03 October 2019	07 May 2019	Mid intertidal
Fucus vesiculosus	Brown	03 October 2019	08 May 2019	Mid intertidal
Himanthalia elongata	Brown	01 October 2019	09 May 2019	Lower intertidal
Laminaria digitata (blade)	Brown	02 October 2019	09 May 2019	Lower intertidal to subtidal
Laminaria digitata (stipe)	Brown	02 October 2019	09 May 2019	Lower intertidal to subtidal
Pelvetia canaliculata	Brown	03 October 2019	08 May 2019	Upper intertidal
Saccharina latissima	Brown	01 October 2019	09 May 2019	Lower intertidal to subtidal
Chondrus crispus	Red	01 October 2019	08 May 2019	Lower intertidal
Palmaria palmata	Red	01 October 2019	07 May 2019	Lower intertidal
Porphyra umbilicalis	Red	02 October 2019	09 May 2019	Lower intertidal
Ulva lactuca	Green	02 October 2019	08 May 2019	Lower intertidal

For *Laminaria digitata* two structural variants (blade and stipe) were harvested separately.

[a] Connan, S., Goulard, F., Stiger, V., Deslandes, E., Ar Gall, E., 2004. Interspecific and temporal variation in phlorotannin levels in an assemblage of brown algae. DOI: https://doi.org/10.1515/BOT.2004.057

[b] Makkar, Harinder PS, Gilles Tran, Valérie Heuzé, Sylvie Giger-Reverdin, Michel Lessire, François Lebas, and Philippe Ankers. 2016. 'Seaweeds for livestock diets: a review', *Animal Feed Science and Technology*, 212: 1–17.

weight of the ash in the DM. N content was determined by the Kjeldahl method (Kjeltec™ 8400, FOSS Denmark, Hillerød, Denmark) and crude protein (CP) content was calculated using a nitrogen to protein conversion factor of 5, as previously recommended for macroalgae (Angell et al., 2016b). Neutral detergent fiber (NDF) was determined by the filter bag technique (Ankom^{200} Fiber Analyzer, NY, USA) using a neutral detergent solution, with addition of heat-stable alpha-amylase (ANKOM Technology, Macedon, NY, USA), and sodium sulfite (ANKOM Technology, Macedon, NY, USA). The residue obtained was incinerated at 550 °C for 12 h to obtain ash corrected NDF (NDFom).

The contents of individual minerals were determined after a predigestion of 150 mg of samples in a mixture of concentrated nitric acid and hydrogen peroxide (5:1, v/v) using a D Microwave digestion system (Milestone Srl, Sorisole, BG, Italy). Macrominerals (Na, K, Ca, and Mg) and trace minerals (Mn, Fe, Zn, and Cu) were determined by atomic absorption using a Microwave Plasma Atomic Emission Spectrometer (MP-AES 4200, Agilent Technologies, Santa Clara, CA, USA) following the standard protocols of the Official Journal of the European Union 2009 (European Commission, 2009). A calibration curve was created by preparing sets of standard solutions with known concentrations of analytes, and the concentrations of minerals in samples were then determined from the linear regression equation.

2.4. Determination of total polyphenol contents (TPC)

The total polyphenol fraction was extracted in duplicate samples and quantified using the protocol of (Zhang et al., 2006) with some modifications. In brief, 0.5 g of ground macroalgae material was added with 10 mL of methanol-water (1:1 v/v) solution (Merck KGaA, Darmstadt, Germany), pH adjusted to ~2, and shaken (200 rpm) in an orbital shaker at room temperature for 1 h in darkness. The supernatant was recovered after centrifugation at 12000 × g_{av} for 10 min, and the residue thereafter re-extracted with 10 mL of acetone-water (7:3 v/v) solution (Merck KGaA, Darmstadt, Germany) under similar conditions as described for the methanol-water treatment. The supernatants from both extractions were pooled to make the final polyphenol extract, which was diluted 10 times in distilled water for the quantification of TPC.

For quantification of TPC in extracts, a seven-point standard curve was prepared. First, a stock standard solution (500 μg mL^{-1}) of phloroglucinol dihydrate (Acros Organics, Geel, Belgium) was prepared, and the solution was serially diluted to make standards containing 250, 125, 62.5, 31.25, 15.625, and 0 μg mL^{-1} phloroglucinol. Then, TPCs in macroalgae extracts, standard solutions, and blanks were determined in triplicates in a 96-well microplate (Thermo Fischer GmbH, Kandel, Germany) as previously described (Zhang et al., 2006) using a spectrophotometric microplate reader (absorbance at λ750 nm; BIO-RAD,

iMark™ Microplate Reader, California, USA). The mean TPC was calculated as milligram of phloroglucinol equivalents (mg PGE) per g of DM using the formula given in equation (1).

$$\text{TPC(mg PGE/g DM)} = \frac{(Mean\ TPC\ of\ sample(\mu g/mL)xSVxDF)}{DM\ weight\ of\ sample(g)x1000} X100\% \quad (1)$$

where, Mean TPC = average of the total polyphenol concentrations of triplicate samples obtained from the calibration curve, SV= Volume of solvent used for extraction, DF = Dilution factor of the original extract during the quantification assay.

2.5. In vitro ruminal gas fermentation and degradability

2.5.1. Fermentation procedure

The impact of macroalgae on rumen fermentation and gas production was simulated *in vitro* using the ANKOM^{RF} gas production system version 11.4 (Macedon, NY, USA). First, a pilot study was performed to evaluate the *in vitro* fermentation characteristics of pure macroalgae material. Thereafter, further *in vitro* fermentation studies were undertaken using macroalgae as an additive to a standard feed, maize silage (MS), in a ratio of 1:4 (w/w) giving a macroalgal inclusion rate of 20% in DM. The selection of 20% DM inclusion level was also based on previously published literature (de la Moneda et al., 2019; Maia et al., 2019). It should be noted that individual macroalgae were added to MS, and no blend of macroalgae species was used in this study.

Rumen fluid, as a source of rumen microorganisms, was obtained from two rumen-cannulated Danish Jersey heifers maintained at the Large Animal Hospital, University of Copenhagen, Denmark, following the guidelines of the Danish National Committee for the Protection of Animals used for Scientific Purposes (License nr: 2012-15-2934-00648). The donor heifers were fed a basal diet of grass silage containing 612 g/kg NDF, 72 g/kg CP, and 11 g/kg crude fat. The rumen fluids, with solids, were collected before the morning feeding in pre-warmed (39.5 °C) thermal jugs, immediately transported to the laboratory, and filtered through a double-layered cheesecloth. The filtered rumen fluid from each heifer was pooled in equal amounts and mixed with two parts of a buffer solution, containing micro-and macrominerals as well as a redox agent (Menke et al., 1979). This mixture was maintained at 39.5 °C under anaerobic conditions by continuous flushing with CO_2 gas. Ninety mL of buffered rumen fluid was dosed into a prewarmed 100 mL Duran® glass bottle that contained a feed mixture of 0.1 g macroalgae and 0.4 g MS or 0.5 g of MS or no feed (blanks), where samples had been randomly assigned to bottles. After dosing, the bottles were directly flushed with N_2 gas (to ensure anaerobic conditions and to remove any residual CO_2 present before the microbial degradation of feed) and fitted with an automatic wireless ANKOM module (ANKOM Technology, Macedon, NY, USA). The bottles were then incubated in a thermoshaker (Gerhardt Analytical Systems, Germany) at 39.5 °C with an oscillation of 40 rpm for 48 h. Duplicates of each sample type were incubated, and the fermentation was repeated twice, producing a total of four replicates per sample. To evaluate the CH_4 production, headspace gas samples were collected in gas-tight evacuated sample bags (SKC, Flex Foil PLUS) that were attached to the vent valve tube of the ANKOM module.

2.5.2. Gas recordings and rumen feed degradability determination

The pressure generated by gases in the headspace of each fermentation bottle was recorded directly to a computer connected to the ANKOM^{RF} Gas Production System. The live time was set to 60 s, pressure readings were recorded at 10-min intervals, and global release pressure was set to 0.75 psi. The cumulative pressure readings of samples from 48 h of incubation were corrected for blanks and then converted to total gas production (TGP) volumes (mL) per gram OM of feed under standard temperature and pressure conditions using the ideal gas law.

At the end of the 48 h incubation period, the fermentation bottles were transferred into an ice bath. The fluid with undegraded feed residue in the bottles was thereafter filtered through an Ankom filter bag (F57, ANKOM Technology, Macedon, NY, USA, pore size: 25 μm) and final pH of the fermentation fluid was recorded. The DM and OM contents of the undegraded feed residues retained in the filter bags were determined gravimetrically. The organic matter degradability (OMD) of the feed samples was calculated from the OM in material initially added to incubation bottles subtracted by OM in the filtered residual. The values were corrected for the increased weight of blank bags containing microbial biomass from the rumen fluid added to incubation bottles.

2.5.3. Methane measurements

Methane concentration in the gas produced over the 48 h of *in vitro* fermentation was determined by gas chromatography (GC) (Agilent 7820A GC, Agilent Technologies, Santa Clara, CA, USA). The GC equipment consisted of a HPPLOT Q column (30 m × 0.53 mm × 40 μmm) and a thermal conductivity detector that was set to 250 °C. The column flow was maintained to 5 mL/min, whereas the reference and makeup flow was adjusted to 10 mL/min. Hydrogen was used as a carrier gas. From each gas bag, a 250 μL gas sample was injected into the GC machine and run for 3 min at an isothermal oven temperature of 50 °C, and this process was performed twice for each gas sample. To calculate the CH_4 percentage in gas samples, a calibration curve made from standards containing 1%, 2.5%, 5%, 10%, 15%, and 25% of CH_4 in N_2 gas (Mikrolab A/S, Aarhus, Denmark) was used. The total volume of CH_4 produced was thereafter calculated from the CH_4 percentage multiplied by TGP.

2.5.4. Analysis of volatile fatty acids (VFAs)

For VFA analysis, samples of the liquid flow-through were collected during filtration of the fluid and undegraded feed residues after fermentation, and it was immediately mixed with 25% metaphosphoric acid solution in a 5:1 ratio (v/v) and frozen at −80 °C. Volatile fatty acids concentrations in the liquid samples were analyzed by GC (System 7890A Agilent Technologies, Santa Clara, CA, USA) as described previously (Aryal et al., 2021) and the concentrations were normalized to the VFAs produced per gram of OM fermented.

2.6. Rumen microbiome analyses using Illumina 16S rRNA amplicon sequencing

Selected samples of the liquid flow-through obtained during filtration of fluid after fermentation were used for microbiome studies. Based on *in vitro* fermentation characteristics, the samples were from blank incubations (no MS or macroalgae added to the bottles), incubations with MS alone (the basal feed), and incubations with five different macroalgae representing different rumen degradability clusters (high, medium, and low) and phylum (brown, red, and green).

2.6.1. Sample collection and DNA extraction

First, during filtration of the contents of incubation bottles through Ankom filter bags by the end of the fermentation period (see above), about 5 mL of liquid flow-through was collected in a sterile test tube and immediately frozen at −80 ^{O}C. During genomic DNA extraction, the frozen samples were thawed in ice and 1.8 mL of fluid sample was transferred into a new sterile tube and centrifuged at 10000 × g_{av} for 5 min to get cell-rich pellets (Machado et al., 2018). DNA from the cell-rich pellets was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, California, USA), and further purified using Monarch® PCR & DNA Cleanup Kit (New England Biolabs Inc., Ipswich, MA, USA) following the manufacturer's protocols. The concentration and purity of the extracted DNA were tested with NanoDrop Lite UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.6.2. Library preparation

The bacterial primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R

(GGACTACHVGGGTWTCTAAT), along with the Illumina Nextera overhang adapters, were used to amplify the V4 region of the bacterial 16S rRNA gene (Caporaso et al., 2011). The first PCR (PCR1) for amplification of 16S rRNA gene products was carried out in technical duplicates and pooled before adding index combination. Thermocycler conditions were 95 ^{O}C for 5 min, 35 cycles of 95 ^{O}C for 30 s, 55 ^{O}C for 30 s, 72 ^{O}C for 1 min, and the final elongation at 72 ^{O}C for 5 min (SimpliAmp Thermal Cycler, Applied Biosystems, California, USA). Each PCR reaction of 25 μl consisted of 5xPCRBIO HiFi Buffer (5 μl) (PCRBiosystems, London, UK), 10 ng of DNA template, 0.5 unit of PCRBIO HiFi Polymerase (PCRBiosystems, London, UK), 0.2 mM of forward and reverse primers, and 100 ng bovine serum. After PCR1, a second PCR (PCR2) was used to add unique index combinations (i7and i5) and adaptors. For PCR2, Thermocycler conditions were 95 ^{O}C for 5 min, 13 cycles of 95 ^{O}C for 30 s, 58 ^{O}C for 30 s, 68 ^{O}C for 1 min, and the final elongation at 68 ^{O}C for 10 min. Subsequently, the amplicon product was cleaned using HighPrep™ magnetic beads (MagBio Genomics Inc. Gaithersburg, USA), according to the manufacturer's instructions. Finally, amplicons were pooled in equimolar concentration, and sequencing was carried out using the Illumina MiSeq platform. All the sequence files were deposited in the NCBI Sequence Read Archive (SRA) under the accession number: PRJNA780171.

2.6.3. Bioinformatics of sequencing data

The DNA reads obtained from the Illumina MiSeq run were analyzed using QIIME2 (Bolyen et al., 2019) mainly using the dada2 plugin (Callahan et al., 2016). In brief, paired-end reads were denoised, joined, dereplicated, forward and reverse primers trimmed, and finally filtered for chimeras using the 'dada2 denoise-paired' command. Following this, taxonomy to amplicon sequence variants (ASVs) was assigned via 'feature-classifier classify-consensus-vsearch' using the SILVA 132 database (Quast et al., 2012). To perform data analysis and data visualization, the ASV table and the taxonomy files were imported to the R version 4.0.3 (R Core Team, 2021). Diversity-based analysis was done using the vegan package ver. 2.5–7 (Oksanen et al., 2013) and the phyloseq package ver. 1.34 (McMurdie and Holmes, 2013).

2.7. Calculation and statistical analyses

All statistical analyses were performed using the R Foundation for Statistical Computing Platform, version 4.1.1 (R Core Team, 2021). Homogeneity of variance was evaluated by visual inspection of residual plots, and normality of residuals was tested by quantile-quantile plots. The environmental parameter data from two seasons were analyzed by unpaired *t*-test. Data for the chemical composition (ash, crude protein, NDFom, TPC, and minerals) of macroalgae, and *in vitro* rumen degradability characteristics (TGP, OMD, pH, VFA, and CH_4) were analyzed by two-way ANOVA, where fixed effects of season and species, and their interactions were used. The patterns of development of TGP during *in vitro* fermentation (broken down each hour of fermentation) were analyzed as repeated measures using a mixed effect model. The model included fixed effects of seasons, macroalgae species, incubation hours, and their interactions, while fermentation runs, and replicate numbers were used as random effects. For this, different correlation structures between measurements and heterogeneous variances were tested and the structure yielding the best fit (autoregressive first order) was chosen. In addition, the TGP data from each hour of fermentation was used for hierarchical clustering to generate a heatmap, where TGP values were center-scaled (z-transformation) across fermentation hours and the Euclidean distance matrix was generated. Then the "average" algorithm was conducted for hierarchical agglomerative clustering using ComplexHeatmap (Gu et al., 2016) and dendextend (Galili, 2015) R packages. In the end, a Spearman's correlation matrix was generated to evaluate the correlation among chemical composition, *in vitro* rumen fermentation characteristics, and rumen microbial compositions using the Corrplot package in R. Differences in the least square means (LS means) were compared by Tukey's multiple comparison test. The level of significance was set at $P < 0.05$.

3. Results

3.1. Seawater parameters during the sampling of macroalgae

The levels of salinity and dissolved oxygen were found to be similar across both harvesting seasons. The oxygen saturation levels were significantly higher in the spring as compared to those recorded in the autumn ($P < 0.0001$), whereas the water temperature levels were higher in the autumn compared to spring ($P < 0.0001$) (Suppl. Fig. S1).

3.2. Chemical composition of macroalgae

3.2.1. Ash and minerals

Brown ($P = 0.00029$) and green ($P = 0.023$) species had higher ash contents than the red species (Table 2). The ash contents were higher in the spring than in the autumn for all species ($P < 0.05$), except for opposite trends in *F. serratus*, *L. digitata* (stipe), and *C. crispus* ($P < 0.0001$, for all three). The largest seasonal differences were observed in the brown macroalgae, *S. latissima* ($P < 0.0001$) and *L. digitata* (blade) ($P < 0.0001$), and the red macroalga, *P. palmata* ($P < 0.0001$) with ~96%, 54.5%, and ~74%, respectively, higher ash concentrations in the spring compared to the autumn.

The contents of macrominerals were generally highest in brown species followed by green and red species. The most abundant macrominerals were either K (up to 9.27% DM, e.g., *L. digitata*, *S. latissima*, *H. elongata, P. palmata*) or Na (up to 5.9% DM, e.g., *A. nodosum., F. vesiculosus, P. canaliculata, C. crispus*) (Table 3), while Fe (28–223 mg/kg DM) was the most dominant micromineral in all macroalgae species (Table 4).

The effect of season on the studied mineral elements of macroalgae was species-specific. For instance, the level of K was found higher in spring ($P < 0.0001$) except for *F.serratus, P. canaliculata,* and *P. umbilicalis*, which had higher levels in autumn ($P < 0.0001$). Similarly, Na was present in greater concentration in spring for *A. esculenta, H. elongata, F. vesiculosus, S. latissima, P. umbilicalis* (~2 fold), and *U. lactuca* (3.5 fold) as compared to the autumn ($P < 0.05$), while the opposite was the case for *C. crispus* ($P = 0.025$). The largest seasonal variation in Ca content was noted for red species, *P. umbilicalis,* and *C. crispus*, with ~5 and ~2 fold higher contents, respectively, in autumn as compared to the spring ($P < 0.05$). The Mg content was 2.7 fold higher in autumn than spring for green macroalga, *U. lactuca,* whereas it was highest in spring or unaffected by season for other species. Similar to the macrominerals, the concentration of microminerals in macroalgae was also differentially affected by the harvesting season. Two brown species: *A. nodosum* and *P. canaliculata* had higher levels of Fe, Zn, and Mn in the spring than in the autumn ($P < 0.0001$, for both), while the opposite trend was evident for another brown macroalga, *H. elongata* ($P < 0.0001$), and green macroalga, *U. lactuca* ($P < 0.0001$).

3.2.2. Crude protein

Green ($P < 0.0001$) and red ($P < 0.0001$) macroalgae had similar CP contents, which were > 2-fold higher than the levels found in brown species. Crude protein concentrations were higher in the spring ($P < 0.0001$) than autumn for all studied species ($P < 0.0001$) (Table 2). The seasonal differences were most pronounced for the brown macroalgae belonging to the Fucaceae family: *F. vesiculosus*, *F. serratus*, *A. nodosum,* and *P. canaliculata* with 86%, 74%, 73%, and 66%, respectively, higher CP levels in the spring compared to autumn (interaction of season and species: $P < 0.0001$). The green macroalga, *U. lactuca*, and red macroalgae, *C. crispus, P. palmata* and *P. umbilicalis*, were the richest (13–20% of DM) in CP while the brown macroalgae *A. nodosum* and *F. vesiculosus* had the lowest (<8% of DM for both) contents.

Table 2
Chemical composition of macroalgae harvested in autumn and spring seasons.

Species	Ash% in DM		CP (g/kg DM)		NDFom (g/kg DM)		TPC (mg PGE/gDM)	
	Aut	Spr	Aut	Spr	Aut	Spr	Aut	Spr
A. esculenta	17.2 ± 0.09^{f}	$21.7 \pm 0.05^{g*}$	104.9 ± 0.64^{c}	$136.3 \pm 0.1^{c*}$	471.5 ± 10.75^{d}	487.9 ± 1.91^{de}	33.2 ± 1.6^{d}	22.3 ± 1.75^{de}
A. nodosum	20.9 ± 0.32^{d}	$24.3 \pm 0.34^{f*}$	41.1 ± 0.77^{h}	$71.3 \pm 0.38^{g*}$	579.2 ± 3.96^{a}	$537.5 \pm 6.47^{c*}$	111.9 ± 7.69^{b}	104.9 ± 2.2^{b}
F. serratus	25.2 ± 0.05^{c}	$19.5 \pm 0.08^{i*}$	60.6 ± 0.13^{ef}	$105.0 \pm 0.0^{de*}$	458.0 ± 5.02^{d}	$527.1 \pm 8.56^{c*}$	84.8 ± 5.08^{c}	94.5 ± 3.92^{b}
F. vesiculosus	18.8 ± 0.97^{e}	$24.4 \pm 0.23^{f*}$	44.0 ± 0.11^{gh}	$82.0 \pm 0.32^{f*}$	370.8 ± 5.83^{f}	$462.0 \pm 2.37^{e*}$	178.2 ± 0.99^{a}	$133.1 \pm 2.79^{a*}$
H. elongata	32.0 ± 0.06^{b}	$39.6 \pm 0.06^{a*}$	56.4 ± 0.53^{f}	$85.2 \pm 0.21^{f*}$	391.3 ± 9.63^{ef}	393.2 ± 8.07^{f}	38.1 ± 1.18^{d}	26.9 ± 0.45^{d}
L. digitata (blade)	19.6 ± 0.06^{e}	$30.3 \pm 0.04^{d*}$	65.7 ± 0.03^{de}	$98.7 \pm 0.48^{e*}$	474.3 ± 6.73^{d}	490.4 ± 2.65^{de}	7.1 ± 1.82^{ef}	6.6 ± 2.02^{f}
L. digitata (stipe)	33.7 ± 0.04^{a}	$32.2 \pm 0.02^{c*}$	57.0 ± 0.32^{f}	$70.4 \pm 0.08^{g*}$	546.9 ± 2.45^{b}	$664.1 \pm 1.2^{a*}$	6.5 ± 1.00^{ef}	6.9 ± 1.03^{f}
P. canaliculata	19.4 ± 0.13^{e}	$20.8 \pm 0.12^{h*}$	52.6 ± 0.05^{fg}	$87.3 \pm 0.16^{f*}$	507.9 ± 8.65^{c}	519.1 ± 3.07^{cd}	92.9 ± 2.7^{c}	$75.6 \pm 3.14^{c*}$
S. latissima	16.9 ± 0.19^{f}	$33.1 \pm 0.09^{b*}$	73.5 ± 0.42^{d}	$112.8 \pm 0.24^{d*}$	414.5 ± 2.02^{e}	517.4 ± 9.24^{cd}	16.5 ± 0.63^{e}	11.6 ± 0.43^{ef}
C. crispus	19.2 ± 0.12^{e}	$15.6 \pm 0.05^{j*}$	143.2 ± 0.5^{b}	$195.8 \pm 8.23^{b*}$	591.6 ± 5.12^{a}	$617.6 \pm 13.1^{b*}$	8.3 ± 1.45^{ef}	10.5 ± 2.26^{ef}
P. palmata	13.8 ± 0.03^{g}	$23.9 \pm 0.11^{f*}$	136.6 ± 0.05^{b}	$195.2 \pm 1.21^{b*}$	$363.1 \pm 3.7^{a*f}$	$321.6 \pm 0.97^{g*}$	6.5 ± 1.17^{ef}	8.2 ± 0.93^{f}
P. umbilicalis	10.5 ± 0.04^{h}	$13.5 \pm 0.16^{k*}$	143.8 ± 0.51^{ab}	$194.8 \pm 0.47^{b*}$	$599.3 \pm 3.93^{*a}$	$543.8 \pm 3.74^{c*}$	7.6 ± 0.75^{ef}	10.2 ± 1.44^{f}
U. lactuca	25.1 ± 0.05^{c}	$26.0 \pm 0.06^{e*}$	152.0 ± 0.19^{a}	$204.6 \pm 0.72^{a*}$	$271.7 \pm 5.1^{*g}$	$159.9 \pm 3.14^{h*}$	2.6 ± 0.06^{f}	4.1 ± 0.65^{f}
Maize silage	4.3 ± 0.01		80.2 ± 0.57		402.1 ± 3.4		9.4 ± 0.04	

Results are presented as mean ± standard errors of the mean of duplicate analyses. DM, Dry matter; Aut, Autumn; Spr, Spring; CP, Crude protein; NDFom, Ash corrected neutral detergent fiber; TPC, Total polyphenol content; PGE, Phloroglucinol equivalents. Species not sharing the same letters in the superscripts within a column are significantly different. Species with the * sign in the superscript within a row are significantly different from the sample of the same species from another harvesting season.

Table 3
Composition of macro minerals in macroalgae.

Species	K (% of DM)		Na (% of DM)		Ca (% of DM)		Mg (% of DM)	
	Aut	Spr	Aut	Spr	Aut	Spr	Aut	Spr
A. esculenta	4.10 ± 0.05^{bc}	$5.95 \pm 0.01^{d*}$	1.77 ± 0.02^{efg}	1.94 ± 0.05^{e}	1.01 ± 0.01^{cd}	0.93 ± 0.05^{bc}	0.54 ± 0.01^{fg}	$0.68 \pm 0.03^{e*}$
A. nodosum	1.72 ± 0.04^{g}	$2.21 \pm 0.05^{g*}$	3.16 ± 0.05^{bc}	3.62 ± 0.02^{c}	1.00 ± 0.02^{cd}	1.03 ± 0.01^{bc}	0.79 ± 0.02^{cd}	0.84 ± 0.01^{bc}
F. serratus	4.12 ± 0.07^{b}	$3.33 \pm 0.02^{e*}$	3.23 ± 0.05^{b}	2.80 ± 0.02^{cde}	1.10 ± 0.02^{c}	1.01 ± 0.00^{bc}	0.75 ± 0.01^{cd}	0.69 ± 0.01^{de}
F. vesiculosus	2.26 ± 0.00^{e}	$2.81 \pm 0.08^{f*}$	2.34 ± 0.01^{cdef}	$3.08 \pm 0.08^{cd*}$	0.89 ± 0.00^{cd}	$1.09 \pm 0.01^{bc*}$	0.63 ± 0.00^{ef}	$0.73 \pm 0.02^{cde*}$
H. elongata	4.22 ± 0.01^{b}	$7.56 \pm 0.01^{b*}$	4.72 ± 0.10^{a}	$5.90 \pm 0.00^{a*}$	2.16 ± 0.02^{a}	$1.12 \pm 0.01^{b*}$	0.98 ± 0.00^{b}	$1.07 \pm 0.01^{a*}$
L. digitata (blade)	3.85 ± 0.02^{b}	$7.57 \pm 0.10^{b*}$	3.06 ± 0.48^{bc}	3.14 ± 0.56^{cd}	1.09 ± 0.16^{c}	1.11 ± 0.11^{bc}	0.68 ± 0.06^{de}	0.69 ± 0.08^{de}
L. digitata (stipe)	9.27 ± 0.11^{a}	$8.41 \pm 0.00^{a*}$	3.17 ± 0.03^{bc}	3.33 ± 0.00^{c}	1.35 ± 0.01^{b}	$1.47 \pm 0.03^{a*}$	0.74 ± 0.03^{cd}	0.80 ± 0.00^{cd}
P. canaliculata	1.98 ± 0.00^{f}	$1.65 \pm 0.03^{h*}$	2.61 ± 0.00^{bcde}	2.94 ± 0.05^{cd}	1.05 ± 0.01^{cd}	$0.90 \pm 0.00^{c*}$	0.82 ± 0.00^{c}	0.81 ± 0.01^{c}
S. latissima	3.99 ± 0.04^{bc}	$6.99 \pm 0.07^{c*}$	2.03 ± 0.02^{defg}	$2.94 \pm 0.01^{cd*}$	0.84 ± 0.01^{d}	0.91 ± 0.00^{bc}	0.50 ± 0.00^{gh}	$0.73 \pm 0.00^{cde*}$
C. crispus	1.67 ± 0.02^{g}	1.53 ± 0.01^{h}	2.81 ± 0.01^{bcd}	$2.35 \pm 0.01^{de*}$	1.07 ± 0.00^{c}	$0.51 \pm 0.00^{d*}$	0.68 ± 0.00^{de}	0.68 ± 0.00^{e}
P. palmata	2.18 ± 0.05^{ef}	$2.59 \pm 0.00^{f*}$	0.19 ± 0.00^{k}	0.65 ± 0.00^{f}	0.20 ± 0.00^{f}	0.15 ± 0.00^{e}	0.10 ± 0.00^{i}	0.15 ± 0.00^{g}
P. umbilicalis	2.02 ± 0.02^{ef}	$1.28 \pm 0.01^{i*}$	1.55 ± 0.01^{h}	$3.01 \pm 0.28^{cd*}$	1.07 ± 0.01^{c}	$0.21 \pm 0.00^{e*}$	0.39 ± 0.00^{h}	$0.49 \pm 0.00^{f*}$
U. lactuca	2.74 ± 0.04^{d}	2.67 ± 0.03^{f}	1.30 ± 0.01^{g}	$4.62 \pm 0.00^{b*}$	0.52 ± 0.00^{e}	0.58 ± 0.00^{d}	2.62 ± 0.01^{a}	$0.96 \pm 0.01^{b*}$
Maize silage	1.28 ± 0.08		0.01 ± 0.00		0.18 ± 0.00		0.10 ± 0.01	

Results are presented as mean ± standard error of the mean for duplicate analyses. DM, Dry matter; Aut, Autumn; Spr, Spring. Species not sharing the same letters in the superscripts within a column are significantly different. Species with the * sign in the superscript within a row are significantly different from the sample of the same species from another harvesting season.

Table 4
Composition of microminerals in macroalgae.

Species	Fe (mg/kg DM)		Zn (mg/kg DM)		Mn (mg/kg DM)		Cu (mg/kg DM)	
	Aut	Spr	Aut	Spr	Aut	Spr	Aut	Spr
A. esculenta	57.6 ± 0.31^{f}	$114.8 \pm 9.11^{c*}$	42.1 ± 0.03^{c}	$33.3 \pm 2.57^{cde*}$	3.3 ± 0.13^{h}	$8.4 \pm 0.26^{fg*}$	2.6 ± 0.00^{cd}	2.2 ± 0.13^{ef}
A. nodosum	28.3 ± 0.60^{g}	$51.2 \pm 2.20^{hi*}$	17.5 ± 1.30^{gh}	$26.7 \pm 0.03^{f*}$	7.8 ± 0.25^{f}	$13.3 \pm 0.25^{e*}$	1.6 ± 0.00^{de}	1.9 ± 0.00^{ef}
F. serratus	86.5 ± 0.86^{de}	84.1 ± 0.90^{de}	63.5 ± 0.05^{b}	$85.0 \pm 0.53^{a*}$	65.4 ± 0.58^{a}	$59.7 \pm 0.02^{a*}$	6.8 ± 0.14^{a}	$9.81 \pm 0.04^{a*}$
F. vesiculosus	138.9 ± 0.89^{b}	$97.7 \pm 0.68^{cd*}$	18.3 ± 0.01^{g}	$44.5 \pm 0.01^{b*}$	44.1 ± 0.36^{b}	$46.7 \pm 0.90^{b*}$	2.1 ± 0.00^{de}	$4.1 \pm 0.65^{cd*}$
H. elongata	109.9 ± 1.98^{c}	$44.6 \pm 0.50^{i*}$	30.2 ± 1.42^{de}	$25.9 \pm 0.08^{f*}$	39.0 ± 0.54^{c}	$34.6 \pm 0.02^{c*}$	4.6 ± 0.11^{b}	$2.6 \pm 0.01^{ef*}$
L. digitata (blade)	79.3 ± 0.37^{e}	$45.3 \pm 0.64^{i*}$	31.5 ± 0.09^{d}	$34.5 \pm 0.02^{c*}$	3.3 ± 0.14^{h}	4.0 ± 0.00^{i}	1.6 ± 0.00^{de}	1.6 ± 0.00^{f}
L. digitata (stipe)	52.9 ± 0.80^{f}	$59.5 \pm 1.74^{gh*}$	13.3 ± 0.03^{h}	$29.2 \pm 0.14^{ef*}$	1.9 ± 0.00^{h}	2.1 ± 0.01^{j}	2.3 ± 0.14^{cde}	2.1 ± 0.28^{ef}
P. canaliculata	80.3 ± 2.28^{e}	$223.3 \pm 9.59^{a*}$	26.3 ± 0.07^{ef}	$33.6 \pm 0.03^{cd*}$	8.3 ± 0.11^{f}	$9.6 \pm 0.01^{f*}$	3.6 ± 0.12^{bc}	3.1 ± 0.00^{de}
S. latissima	98.3 ± 0.02^{cd}	$80.3 \pm 0.27^{ef*}$	15.6 ± 0.04^{gh}	$33.2 \pm 0.11^{cde*}$	3.0 ± 0.12^{h}	$6.7 \pm 0.02^{h*}$	1.0 ± 0.00^{e}	2.6 ± 0.01^{ef}
C. crispus	156.7 ± 0.75^{b}	162.8 ± 1.87^{b}	73.4 ± 0.20^{a}	$85.6 \pm 0.04^{a*}$	7.3 ± 0.02^{g}	7.0 ± 0.26^{gh}	6.7 ± 0.90^{a}	$4.4 \pm 0.26^{cd*}$
P. palmata	144.1 ± 0.13^{b}	$96.4 \pm 0.10^{cd*}$	22.9 ± 0.04^{f}	$34.2 \pm 1.27^{c*}$	5.6 ± 0.01^{f}	$9.1 \pm 0.00^{f*}$	4.8 ± 0.01^{b}	$4.2 \pm 0.13^{cd*}$
P. umbilicalis	62.1 ± 2.03^{f}	$70.2 \pm 7.53^{fg*}$	44.4 ± 1.08^{c}	$29.4 \pm 1.23^{def*}$	16.4 ± 0.31^{d}	$12.5 \pm 0.02^{e*}$	6.7 ± 0.10^{a}	$4.7 \pm 0.12^{c*}$
U. lactuca	214.8 ± 1.19^{a}	$189.7 \pm 5.05^{ab*}$	BDL	$11.7 \pm 1.28^{g*}$	10.2 ± 0.12^{e}	$15.2 \pm 0.36^{d*}$	4.7 ± 0.13^{b}	$6.4 \pm 0.12^{b*}$
Maize silage	154.6 ± 0.19		10.7 ± 0.06		25.7 ± 0.15		7.35 ± 0.18	

Results are presented as mean ± standard error of the mean for duplicate analyses. DM, Dry matter; Aut, Autumn; Spr, Spring; BDL, Below the detectable level. Species not sharing the same letters in the superscripts within a column are significantly different. Species with * sign in the superscript within a row are significantly different from the sample of the same species from another harvesting season.

3.2.3. Ash corrected neutral detergent fiber

The NDFom content was higher in brown and red species compared to green species ($P < 0.0001$ for both) (Table 2). However, a strong interaction between species and harvesting season was observed ($P < 0.0001$), since some species (*C. crispus*, *F. serratus, F. vesiculosus*, *S. latissima*) had higher NDFom contents in the spring ($P < 0.0062$, for all), whereas others (*A. nodosum, L. digitata* (stipe), *P. palmata, P. umbilicalis* and *U. lactuca*) had highest contents in the autumn ($P < 0.0001$, for all). The red macroalga, *C. crispus* (in spring) had the highest content of NDFom (62% of DM), while the lowest content (16% of DM) was found in green macroalga *U. lactuca* (in autumn).

3.2.4. Total polyphenol contents

As expected, brown macroalgae had higher TPC (10–20 times) as compared to red and green species, except for a low level in *L. digitata*, which was similar to those seen in the red species (season and species interaction: $P = 0.0002$; Table 2). The highest TPC contents were found in four species from the Fucaceae family: *A. nodosum, F. vesiculous, F. serratus,* and *P. canaliculata* (>75 mg PGE per gram DM). Seasonal variations in TPC were observed in two brown species, *F. vesiculosus* ($P < 0.0001$) and *P. canaliculata* ($P < 0.0001$), with higher values in the autumn. A similar trend was also observed for *H. elongata* ($P = 0.078$) and *A. esculenta* ($P = 0.083$), with up to 49% higher TPC levels in the autumn than in the spring.

3.3. In vitro gas production by pure (sole) macroalgae

The *in vitro* study, with pure macroalgal species fermented alone as a substrate, showed that macroalgae produce remarkably less total gas than the standard ruminant feed, maize silage (MS), regardless of species and harvesting time (Suppl. Fig. S2). *S. latissima* and *A. esculenta* had the highest TGP among the tested species during the 48 h of fermentation, however, such productions were ~40 and 47%, respectively, lower than the TGP of MS.

3.4. Effects of addition of macroalgae to maize silage on in vitro rumen fermentation characteristics

3.4.1. Organic matter degradability

When individual macroalgae were co-fermented with MS, no impact of the macroalgae harvesting season was observed on OMD of the feed mixture (individual macroalgae + MS), except for *A. esculenta* ($P = 0.0001$) and *A. nodosum* ($P = 0.017$) harvested in the spring that resulted in higher OMD than from the autumn (Table 5). However, when compared to the MS fermented alone, using the following four brown macroalgae as additives suppressed total OMD by 12–25%, regardless of harvesting season: *A. nodosum* ($P < 0.0001$), *F. vesiculosus* ($P < 0.0001$), *F. serratus* ($P < 0.0001$), *P. canaliculata* ($P < 0.0001$), and the red macroalga *C. crispus* ($P = 0.0037$) (Suppl. Fig. S3). In contrast, *P. umbilicalis* ($P = 0.023$) and *A. esculenta* ($P = 0.0001$) reduced total OMD by ~8–12%, but only when harvested in the autumn. Other brown species, *L. digitata, S. latissima,* and *H. elongata,* the red species, *P. palmata,* and the green species, *U. lactuca*, caused no or only marginal changes in total OMD.

3.4.2. Volatile fatty acid profiles

The addition of spring harvested *A. esculenta* ($P = 0.002$), *A. nodosum* ($P = 0.03$), and *F. vesiculosus* ($P = 0.003$), resulted in a higher total concentration of VFAs in the post-fermentation rumen fluid by the end of the 48-h fermentation period, as compared to the same macroalgae harvested in the autumn (Table 6). Such differences in total VFA were in agreement with differences in OMD except for *F. vesiculosus*. This was

Table 5
Impacts of addition of macroalgae (20% of total DM) to maize silage (1:4 ratio ~20% macroalgae DM in the mix) *in vitro* rumen fermentation characteristics (48 h).

Species	TGP (ml/gOM)		pH		CH_4 (% of TGP)		CH_4 (ml/gOM)		OMD (%)	
	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring
AE	150.3 ± 3.09^{bcde}	160.2 ± 2.97^{abcd}	6.9 ± 0.04	6.9 ± 0.02	7.4 ± 0.56^{ab}	8.3 ± 0.69^{a}	11.1 ± 0.85^{abc}	13.3 ± 1.41^{a}	64.1 ± 1.57$^{de\#}$	70.4 ± 0.53^{abc*}
AN	133.2 ± 1.71$^{ef\#}$	140.8 ± 3.13$^{de\#}$	7.0 ± 0.06	7.0 ± 0.04	5.7 ± 0.06^{ab}	6.8 ± 1.30^{ab}	7.5 ± 0.02^{bc}	9.5 ± 1.57^{ab*}	54.8 ± 0.89$^{g\#}$	59.3 ± 0.86$^{e*\#}$
FS	142.8 ± 4.11^{cde}	139.4 ± 2.99$^{de\#}$	6.9 ± 0.03	6.9 ± 0.03	6.0 ± 0.17^{ab}	7.0 ± 0.05^{ab}	8.5 ± 0.60^{abc}	9.8 ± 0.38^{ab}	60.6 ± 1.22$^{ef\#}$	61.5 ± 2.23$^{de\#}$
FV	113.2 ± 5.91f$^{\#}$	128.3 ± 2.35$^{e*\#}$	7.0 ± 0.04	7.0 ± 0.04	4.6 ± 0.80$^{b\#}$	7.0 ± 0.72^{ab*}	5.4 ± 1.09$^{c\#}$	8.9 ± 1.07^{ab*}	55.7 ± 2.00$^{fg\#}$	56.9 ± 0.82$^{e\#}$
HE	166.3 ± 5.38^{abc}	158.7 ± 3.00^{abcd}	6.9 ± 0.02	7.0 ± 0.03	8.2 ± 0.09^{a}	7.4 ± 0.18^{ab}	13.6 ± 0.18^{a}	11.3 ± 0.47^{ab}	67.8 ± 1.46^{bcd}	70.1 ± 0.24^{abc}
LD	173.6 ± 5.42^{ab}	163.2 ± 4.65^{abcd}	6.9 ± 0.04	6.9 ± 0.03	6.4 ± 0.0^{ab}	7.9 ± 0.25^{ab}	11.3 ± 0.52^{a}	12.8 ± 0.92^{ab}	72.8 ± 1.77^{ab}	70.9 ± 0.25^{ab}
LS	173.8 ± 7.86^{ab}	175.1 ± 3.50^{a}	6.9 ± 0.04	7.0 ± 0.04	7.6 ± 0.90^{ab}	7.9 ± 0.29^{a}	13.3 ± 0.96^{ab}	13.8 ± 0.03^{a}	71.6 ± 0.19^{ab}	73.7 ± 2.03^{a}
PC	142.3 ± 4.96^{de}	152.8 ± 4.03^{abcd}	7.0 ± 0.04	7.0 ± 0.05	7.2 ± 1.02^{ab}	7.8 ± 1.25^{ab}	10.4 ± 2.03^{abc}	12.0 ± 2.43^{ab}	60.1 ± 1.15$^{\#}$	61.7 ± 1.74$^{de\#}$
SL	163.4 ± 6.14^{abcd}	166.7 ± 4.59^{abc}	6.9 ± 0.02	6.9 ± 0.04	8.1 ± 0.22^{ab}	7.9 ± 0.75^{ab}	13.2 ± 0.11^{ab}	13.1 ± 1.24^{a}	70.1 ± 0.46^{abc}	70.6 ± 0.51^{abc}
CC	142.7 ± 3.3^{cde}	146.8 ± 1.01^{bcde}	7.0 ± 0.03	7.0 ± 0.06	8.5 ± 0.44^{a}	4.3 ± 0.35$^{b*\#}$	12.5 ± 1.25^{ab}	6.3 ± 0.52$^{b\#}$	64.2 ± 0.61$^{de*\#}$	65.5 ± 1.38$^{cd\#}$
PP	183.0 ± 7.25^{a}	169.3 ± 3.05^{ab*}	7.0 ± 0.06	7.0 ± 0.04	7.5 ± 1.29^{ab}	7.4 ± 0.83^{ab}	13.6 ± 1.71^{a}	12.6 ± 1.50^{ab}	73.5 ± 2.03^{a}	73.5 ± 1.42^{a}
PU	153.9 ± 8.61^{bcde}	143.3 ± 11.30^{cde}	7.0 ± 0.03	7.0 ± 0.01	6.7 ± 0.0^{ab}	5.6 ± 0.37^{ab}	10.3 ± 0.08^{abc}	8.2 ± 0.16^{ab}	66.4 ± 2.19$^{cd\#}$	68.5 ± 2.09^{bc}
UL	156.3 ± 2.40^{abcd}	155.9 ± 3.92^{abc}	6.9 ± 0.03	7.0 ± 0.03	5.7 ± 0.49^{ab}	6.3 ± 1.22^{ab}	8.9 ± 0.62^{abc}	9.8 ± 1.96^{ab}	72.6 ± 1.11^{ab}	72.1 ± 1.30^{ab}
MS	167.7 ± 5.00		6.9 ± 0.06		8.7 ± 0.49		14.5 ± 0.75		72.8 ± 2.39	

Results are presented as mean values ± standard error of the mean (n = 4 for OMD, TGP, and pH, and n = 2 for CH_4). AE, *Alaria esculenta*; AN, *Ascophyllum nodosum*; FS, *Fucus serratus*; FV, *Fucus vesiculosus*; HE, *Himanthalia elongata*; LD, *Laminaria digitata* (blade); LS, *Laminaria digitata* (stipe); PC, *Pelvetia canaliculata*; SL, *Saccharina latissima*; CC, *Chondrus crispus*; PP, *Palmaria palmata*; PU, *Porphyra umbilicalis*; UL, *Ulva lactuca*; MS, Maize silage. DM, Dry matter;/gOM, Per gram organic matter; TGP, Total gas production; OMD, Organic matter degradability. Species not sharing the same letters in the superscripts within a column are significantly different. Species with the * sign in the superscript within a row are significantly different from the sample of the same species from another harvesting season. Species with the # sign in the superscript are significantly different from maize silage (MS) fermented alone.

Table 6
Effects of macroalgae inclusion on concentrations of volatile fatty acids at 48 h of *in vitro* rumen fermentation.

Species	Total VFA		Acetic acid		Propionic acid		Butyric acid		Total Minor VFAs	
	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring
A. esculenta	33.4 ± 2.83^{abcd}	37.8 ± 0.97^{ab*}	20.4 ± 2.23^{abc}	24.2 ± 0.80^{a*}	9.7 ± 0.82^{ab}	8.3 ± 0.81*	2.6 ± 0.29$^{cde\#}$	4.0 ± 0.82*	0.70 ± 0.07^{cd}	1.35 ± 0.15^{abc*}
A. nodosum	28.1 ± 3.16$^{de\#}$	32.3 ± 0.84^{abc*}	18.2 ± 1.96^{cd}	21.1 ± 0.43^{abc*}	7.0 ± 1.73^{d}	7.5 ± 1.11	2.5 ± 0.64$^{de\#}$	3.1 ± 0.77	0.38 ± 0.06^{de}	0.56 ± 0.08^{ef}
F. serratus	30.8 ± 2.84^{cd}	31.2 ± 0.86^{bc}	19.4 ± 1.84^{bc}	19.5 ± 0.07^{bc}	8.0 ± 1.49^{abcd}	7.9 ± 0.49	2.9 ± 0.68$^{bcde\#}$	3.3 ± 1.26	0.53 ± 0.19^{de}	0.57 ± 0.01^{ef}
F. vesiculosus	23.1 ± 2.51$^{e\#}$	29.0 ± 0.19$^{c*\#}$	13.8 ± 1.45$^{d\#}$	18.6 ± 0.53^{c*}	7.1 ± 1.87^{d}	7.5 ± 0.82	2.1 ± 0.82$^{e\#}$	2.9 ± 1.17$^{*\#}$	0.17 ± 0.02^{e}	0.37 ± 0.02^{f}
H. elongata	36.4 ± 0.56^{abc}	34.3 ± 1.62^{abc}	23.7 ± 0.33^{ab}	22.2 ± 1.15^{abc}	8.0 ± 0.95^{abcd}	7.3 ± 1.04	3.7 ± 0.76^{abcd}	3.8 ± 0.67	0.98 ± 0.04^{bc}	1.04 ± 0.10^{cd}
L. digitata (blade)	39.4 ± 0.65^{a}	36.9 ± 1.48^{ab}	24.3 ± 0.54^{a}	22.6 ± 1.2^{abc}	9.8 ± 0.58^{a}	8.9 ± 0.85	4.0 ± 0.5^{ab}	4.1 ± 0.65	1.29 ± 0.04^{ab}	1.31 ± 0.08^{abc}
L. digitata (stipe)	36.8 ± 2.18^{abc}	37.1 ± 2.57^{ab}	23.3 ± 1.73^{abcd}	23.9 ± 1.92^{ab}	8.2 ± 0.99^{abcd}	8.1 ± 1.24	3.8 ± 0.49^{abc}	4.0 ± 0.73	1.35 ± 0.05^{ab}	1.20 ± 0.13^{abcd}
P. canaliculata	31.4 ± 0.15^{bcd}	34.2 ± 3.39^{abc}	19.9 ± 0.04^{abc}	21.6 ± 2.44^{abc}	7.7 ± 0.81^{bcd}	8.0 ± 1.51	3.3 ± 1.04^{abcde}	3.8 ± 0.73	0.54 ± 0.05^{de}	0.84 ± 0.16^{de*}
S. latissima	37.7 ± 1.81^{ab}	38.3 ± 0.30^{a}	23.2 ± 1.47^{ab}	24.2 ± 0.35^{a}	9.7 ± 0.56^{ab}	9.0 ± 0.46	3.7 ± 0.26^{abcd}	3.8 ± 0.53	1.19 ± 0.03^{ab}	1.32 ± 0.04^{abc}
C. crispus	32.9 ± 0.15^{abcde}	32.0 ± 1.56^{abc}	20.9 ± 0.42^{abc}	20.1 ± 1.15^{abc}	7.4 ± 0.8^{cd}	7.1 ± 0.91	3.5 ± 0.59^{abcd}	3.7 ± 0.61	1.11 ± 0.06^{abc}	1.13 ± 0.12^{bcd}
P. palmata	37.9 ± 1.84^{ab}	36.4 ± 1.93^{ab}	22.8 ± 1.54^{ab}	22.1 ± 1.76^{abc}	9.3 ± 0.7^{abc}	8.6 ± 0.5	4.3 ± 0.45^{a}	4.1 ± 0.41	1.44 ± 0.06^{a}	1.57 ± 0.08^{a}
P. umbilicalis	37.3 ± 1.85^{ab}	34.0 ± 1.20^{abc}	23.6 ± 0.84^{ab}	21.1 ± 0.46^{abc}	8.2 ± 1.42^{abcd}	7.5 ± 0.24	4.1 ± 0.3^{ab}	4.1 ± 0.92	1.40 ± 0.12^{a}	1.32 ± 0.04^{abc}
U. lactuca	34.9 ± 0.78^{abc}	35.6 ± 0.80^{abc}	21.8 ± 0.81^{abc}	22.2 ± 0.83^{abc}	7.9 ± 0.77^{abcd}	7.9 ± 0.94	4.0 ± 0.83^{ab}	4.0 ± 0.83	1.26 ± 0.04^{ab}	1.50 ± 0.15^{ab*}
Maize silage	37.0 ± 3.07		22.6 ± 2.18		8.7 ± 1.56		4.4 ± 0.77		1.32 ± 0.09	

The amounts of VFAs (Volatile fatty acids) are presented in mmol/L produced per gram of organic matter. At the end of fermentation, the fermentation medium was filtered, and fluid was mixed with 25% metaphosphoric acid at a 1:5 ratio (v/v) and VFAs were analyzed by gas chromatography. Results are presented as mean ± standard error of the mean (n = 2). Total Minor VFAs, a sum of isobutyric acid, valeric acid; isovaleric acid and hexanoic acid. Species not sharing the same letters in the superscripts within a column are significantly different. Species with the * sign in the superscript within a row are significantly different from the sample of the same species from another harvesting season. Species with the # sign in the superscript are significantly different from maize silage (MS) fermented alone.

ascribed to the higher production of acetic acid by all three species (*A. esculenta:* $P = 0.005$, *F. vesiculosus:* $P = 0.0015$, and *A nodossum:* $P = 0.025$), of butyric acid by *A. esculenta* ($P = 0.0004$) and *F. vesiculosus* ($P = 0.033$), and of minor VFAs (sum of isobutyric acid, valeric acid, isovaleric acid, and hexanoic acid) by *A. esculenta* when harvested in the spring ($P = 0.005$).

When compared with MS fermented alone, only two of the 12 macroalgae: *F. vesiculosus* ($P < 0.033$) and autumn harvested *A. nodosum* ($P = 0.01$), reduced total VFA concentrations in the post-fermentation rumen fluid namely by 37.5 and 21.6%, respectively (Suppl. Figure 3). With *F. vesiculosus*, this was associated with a sharp reduction in concentrations of acetic acid (autumn harvest only, $P < 0.0001$) and, in both species, by decreased concentrations of butyric acid (*F. vesiculosus:* $P < 0.03$; autumn harvested *A. nodosum:* $P = 0.002$). Butyric acid concentrations were, in general, most susceptible to change upon macroalgae addition to MS, and were also significantly decreased by autumn harvested *A. esculenta* ($P = 0.003$) and *F. serratus* ($P = 0.026$). However, none of the macroalgae addition reduced propionate production as compared to pure MS. Instead, the addition of autumn harvested *F. vesiculosus* resulted in a significantly higher propionate:acetate ratio as compared to the pure MS fermented alone ($P = 0.035$) (Suppl. Fig. S4). In addition, seasonal impacts were visible on the propionate: acetate ratio, but only in the autumn harvested species *F. vesiculosus* ($P = 0.038$) and *A. esculenta* ($P = 0.0002$) as compared to those from the spring harvest.

3.4.3. Total gas and methane production

Addition of *A. nodosum* ($P < 0.0001$) and *F. vesiculosus* ($P < 0.0001$), irrespective of the harvesting season, as well as spring harvested *F. serratus* ($P = 0.04$), reduced TGP per gram OM of the feed (macroalgae + MS). The reduction in TGP was most pronounced with autumn harvested samples of *F. vesiculosus* as compared to spring harvested samples ($P = 0.025$). In contrast, for the red macroalga *P. palmata*, there was a lower TGP with the addition of spring harvested material as compared to autumn harvested material ($P = 0.042$) (Table 5).

A significant reduction of CH_4 produced per gram OM of the feed (macroalgae + MS) was achieved with autumn harvested *F. vesiculosus* ($P = 0.0009$) and *A. nodosum* ($P = 0.023$), as well as for spring harvested *C. crispus* ($P = 0.0037$) with ~62.6%, ~48.2%, and ~56.5% reductions, respectively (Table 5, Suppl. Fig. S3). In terms of relative proportions of CH_4 in the produced total gas, the reductions were 50.4%, 47.3%, and 34.8% for *C. crispus* (spring), *F. vesiculosus* (autumn), and *A. nodosum* (autumn) respectively. A >30% numerical reduction in CH_4 production during fermentation was also observed for brown species *F. serratus* and the green species *U. lactuca*, irrespective of the harvesting season, and for spring harvested *P. umbilicalis*, but these reductions did not reach significance.

3.5. Hierarchical clustering of macroalgae based on effects on TGP production

The time course of development in TGP during fermentation suggested that the rate of gas production from MS with or without added macroalgae was minimal at the beginning of the fermentation, but an exponential increase occurred after approximately 15–22 h of fermentation, and a maximal TGP (asymptote) was reached after approximately 36 h (Figs. 2a and 3). When a center scaling across macroalgae species was undertaken followed by hierarchical clustering, macroalgae species clustered into four distinct groups based on their impact on TGP: high gas producer, medium-high gas producer, average gas producer, and low gas producer (Figs. 2b and 3). As observed with other parameters, autumn and spring harvested biomass of macroalgae clustered at the same region in the heatmap, except for *A. esculenta, C. crispus and H. elongata*, indicating a minimal effect of seasonality on TGP. Five

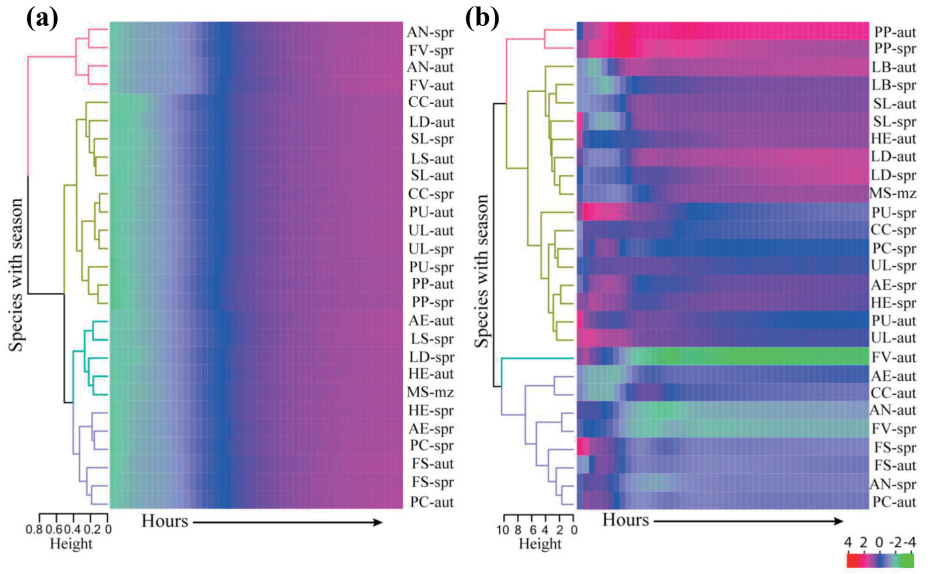

Fig. 2. Clustering of macroalgae based on their effects on total gas production when co-fermented *in vitro* with maize silage in buffered rumen fluid for 48 h. Two batches of *in vitro* fermentations were conducted to simulate rumen fermentation, where macroalgae were added to a standard feed, maize silage, in a 20%-to-80% ratio in dry matter and then incubated for 48-h in a buffered rumen inoculum. Each row represents a species from a specific season, and each column represents the accumulated total gas production after a specific duration (hr) of fermentation. (a) The total gas production values were center-scaled along time (row-wise) followed by a heatmap generation to evaluate gas production status for each hour of fermentation; (b) The total gas values were center-scaled across species (column-wise) followed by heat map generation to cluster macroalgae species. The color scale of the heat map denotes the center-scaled value in the form of color, in which red color indicates the maximum value and green color indicates the minimum value. Also, species from specific seasons were clustered together based on the agglomerative hierarchical clustering algorithm. Here, the values were clustered together row-wise (along time) based on similarity and were represented by a dendrogram. The height of the dendrogram shows the distance or dissimilarity between the two species. The unique colors of the dendrogram indicate the specific clusters. aut, Autumn; spr, Spring; MS-mz, Maize silage incubated alone without addition of macroalgae; AE, *Alaria esculenta*; AN, *Ascophyllum nodosum*; FS, *Fucus serratus;* FV, *Fucus vesiculosus*; HE, *Himanthalia elongata*; LD, *Laminaria digitata* (blade); LS, *Laminaria digitata* (stipe); PC, *Pelvetia canaliculate*; SL, *Saccharina latissima*; CC, *Chondrus crispus*; PP, *Palmaria palmata*; PU, *Porphyra umbilicalis*; UL, *Ulva lactuca*.

macroalgae species: *P. palmata* (high gas producer), *S. latissima* (medium-high gas producer), *U. lactuca* (average gas producer), *F. vesiculosus* (low gas producer), and *A. nodosum* (low gas producer) were selected to evaluate their effects on the rumen microbiome.

3.6. Effects of addition of macroalgae to maize silage on the rumen microbiome

The 16S rRNA amplicon sequencing of the rumen microbiome with 44 samples yielded a total of 987665 reads with 22447 ± 4756 clean reads per sample (mean ± standard deviation) with 3026 amplicon sequence variants (ASV). Amongst the 3026 detected ASVs, 3013 ASVs accounted for rumen bacteria, while the other 13 ASVs represented rumen archaea. The addition of macroalgae to MS differentially modulated the rumen microbial composition after 48 h of fermentation. The microbial Shannon α-diversity index was affected differently with the addition of macroalgae ($P = 0.033$, Kruskal Wallis test) (Fig. 4a), and autumn harvested samples tended to reduce this diversity as compared to the spring harvested macroalgae ($P = 0.055$, Kruskal Wallis test). Pairwise comparisons showed that addition to MS of the brown macroalga *F. vesiculosus*, lowered the species richness as compared to the addition of *P. palmata* ($P = 0.021$), *S. latissima* ($P = 0.029$), *U. lactuca* ($P = 0.029$), and also compared to MS fermented in pure form ($P = 0.067$). When macroalgae were clustered based on their effects on bacterial and archaeal β-diversity using PCoA plots, a clear difference among macroalgae was observed, which was in line with their impacts on the other observed characteristics during fermentation of the feeds (Fig. 4b). Relative abundance analyses at the phylum (Fig. 5) and genus (Fig. 6) level revealed that several cellulolytic bacteria belonging to the phylum Firmicutes: *Ruminococcus* 2, Ruminococcaceae UCG-010, Ruminococcaceae NK4A2, Lachnospiraceae XPB1014 groups, as well as Bacteriodetes: Rikenelaceae RC9 gut group (Suppl. Fig. S5a) were inhibited by the two macroalgae, *F. vesiculosus* and *A. nodosum*, which deteriorated fermentability of the basal feed. On the other hand, these two species promoted hemicellulolytic microorganisms, such as *Prevotella* 1, Prevotellaceae NK3B31 group (Bacteriodetes), and *Treponema* 2 (Spirochaetes) in comparison with the post-fermentation rumen fluid derived from the fermentation of MS alone or with the addition of other macroalgae species.

Interestingly, the relative abundance of phylum Euryarchaeota which consists of methanogenic archaea was lowered (Fig. 5), when these four macroalgae species were co-fermented with MS: *F. vesiculosus, A. nodosum, S. latissima,* and *U. lactuca*, and the most dominant methanogenic archaea, *Methanobrevibacter* spp., was significantly reduced by *F. vesiculosus* ($P < 0.025$) (Suppl. Fig. S5b). Macroalgae harvest season did not have any impact in this respect.

3.7. Correlation between chemical composition and rumen fermentation parameters

Overall, a strong inverse correlation was demonstrated between TPC contents of macroalgae and most of the fermentation parameters: OMD

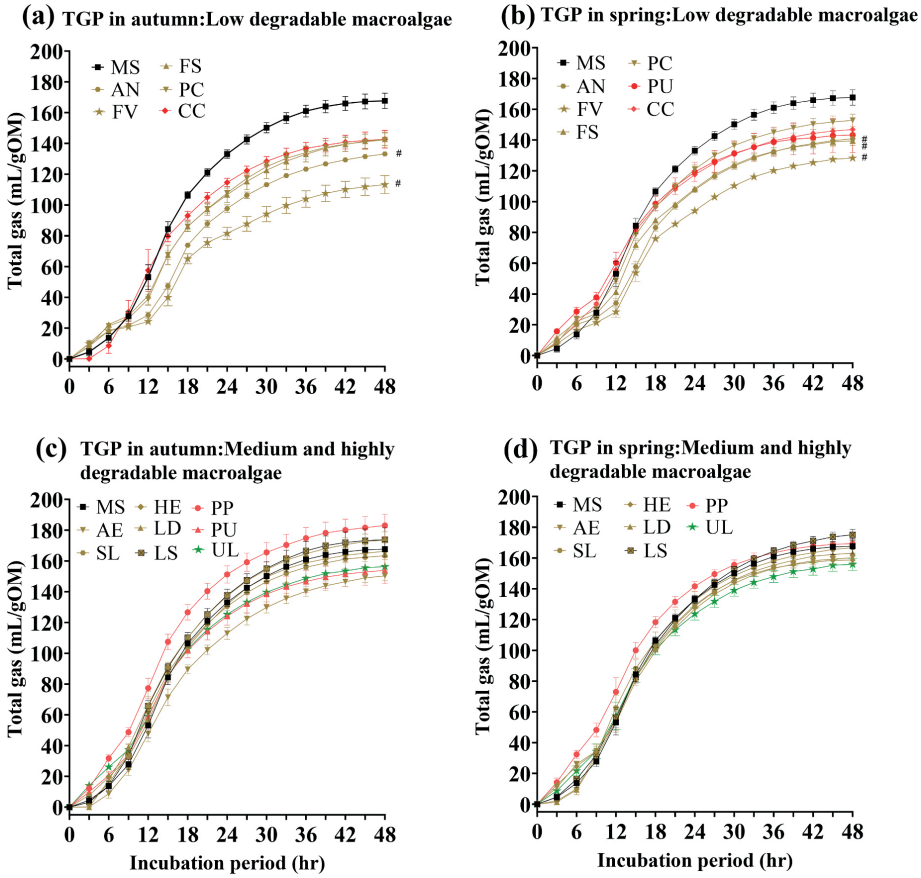

Fig. 3. Effect of co-fermenting macroalgae *in vitro* with maize silage in buffered rumen fluid for 48 h on total gas production. Red, brown, and green colored curves indicate the phylum (type) the macroalgal species belonged to, which was co-fermented with maize silage (MS) in a 20%-to-80% ratio (see legends in Fig. 2). #, Different from MS incubated without macroalgae addition; AE, *Alaria esculenta*; AN, *Ascophyllum nodosum*; FS, *Fucus* serratus; FV, *Fucus vesiculosus*;/gOM, Per gram organic matter; HE, *Himanthalia elongata*; hr, hours; LD, *Laminaria digitata* (blade); LS, *Laminaria digitata* (stipe); PC, *Pelvetia canaliculata*; SL, *Saccharina latissima*; CC, *Chondrus crispus*; PP, *Palmaria palmata*; PU, *Porphyra umbilicalis*; UL, *Ulva lactuca*.

(r = −0.85; $P < 0.001$), TGP (r = −0.79; $P < 0.001$), total VFA concentration (r = −0.78; $P < 0.001$) and CH_4 production (r = −0.53; $P < 0.03$). Crude protein content of macroalgae was moderately and positively correlated to concentrations of the minor VFAs, such as isobutyric acid, valeric, and isovaleric acid (r = 0.53 to 0.60; $P < 0.05$) in the fermented liquid and to OMD (r = 0.39; $P = 0.002$) (Fig. 7).

The Spearman's correlation analyses confirmed an expected negative correlation between TPC in macroalgae to the microbial Shannon Index (r = −0.46, $P = 0.029$) (Suppl. Fig. S5c) and abundance of fiber degrading microorganisms, such as Ruminococcaceae UCG-010 (r = −0.79, $P < 0.0001$), Rikenellaceae RC9 gut group (r = −0.71, $P < 0.0001$), and *Saccharofermentans* (r = −0.75, $P < 0.0001$), and a positive correlation to abundance of *Prevotella* spp. (r = 0.89, $P < 0.0001$), *Ruminobacter* (r = 0.76, $P < 0.0001$) and *Treponema* 2 (r = 0.70, $P = 0.0001$) (For details: Appendix A, Suppl. data SD1). However, no significant correlation of TPC against *Methanobrevibacter* spp. could be detected.

4. Discussion

The major hypotheses of this study were that a) Nordic macroalgae harvested in the spring are more suitable to be utilized as ruminant feed resources than those harvested in the autumn due to their more favorable nutritional values and influences on rumen degradability, and b) polyphenol-rich brown macroalgae are more capable of reducing enteric CH_4 production, irrespective of the harvesting season, due to the polyphenol-associated changes in the rumen microbiome and subsequent rumen fermentation characteristics.

The major findings of this study were that: a) macroalgae chemical compositions differed in a phylum-specific manner, but brown macroalgae possessed greater seasonal variability in ash, protein, and total polyphenol content than red and green species, b) impacts of addition of macroalgae to the standard feed MS on *in vitro* ruminal feed degradation and CH_4 production were species-specific, and only two brown species, *F. vesiculosus* and *A. nodosum*, harvested in autumn and a red species, *C. crispus*, harvested in spring appeared to have significant anti-methanogenic properties (~48–62.6% CH_4 reduction), and c) TPC

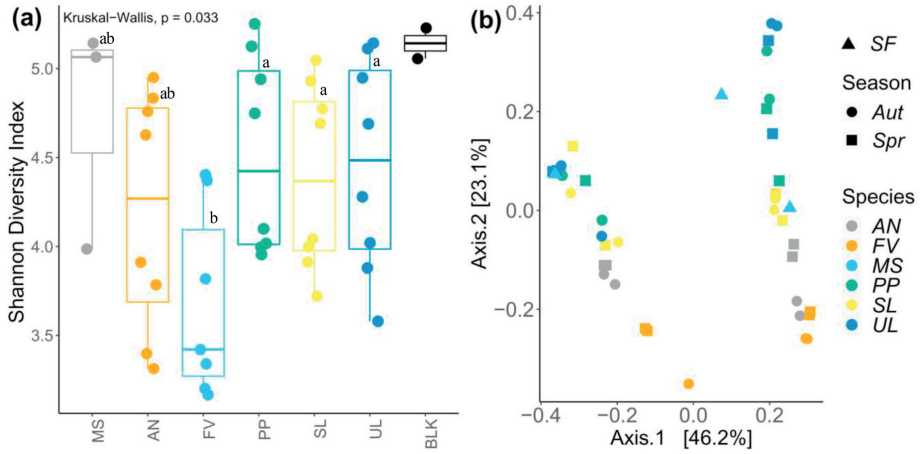

Fig. 4. Effects of co-fermenting macroalgae *in vitro* with maize silage in buffered rumen fluid for 48 h on the rumen microbiome. (a) Alpha diversity estimated by Shannon diversity matrices; (b) Beta diversity based on Bray-Curtis dissimilarity matrix visualized using Principal Coordinate Analysis. Macroalgae were co-fermented in buffered rumen fluid with the standard feed (SF) maize silage in a 20%-to-80% ratio on a dry matter basis (see legends to Fig. 2). AN, *Ascophyllum nodosum*; Aut, Autumn; BLK, Blank samples incubated with buffered rumen fluid alone without maize silage or macroalgae; FV, *Fucus vesiculosus*; MS, Maize silage incubated without macroalgae; PP, *Palmaria palmata*; SL, *Saccharina latissima*; Spr, spring; UL, *Ulva lactuca*. In Fig. 4 (a), blank samples are presented as a reference but were not included in the statistical analysis. Species not sharing the letters over the box plots are significantly different.

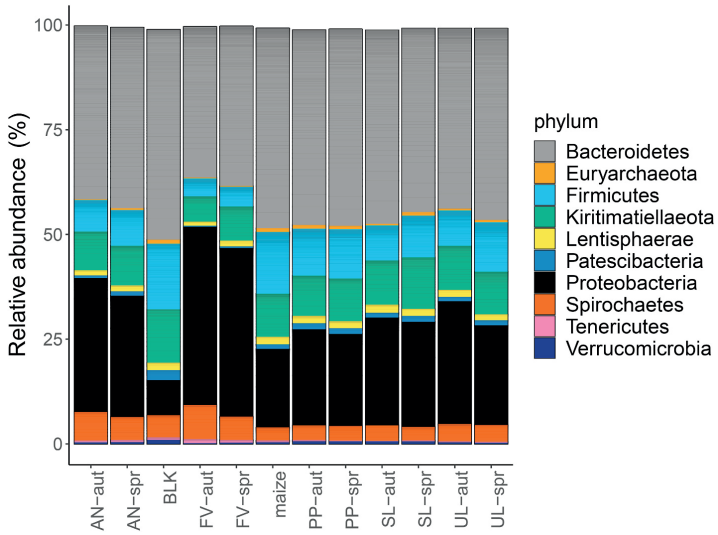

Fig. 5. Effects of co-fermenting macroalgae *in vitro* with maize silage in buffered rumen fluid for 48 h on the relative abundance of the rumen microbiome at the phylum level. AN, *Ascophyllum nodosum*; aut, Autumn; BLK, Blank samples incubated with buffered rumen fluid alone without maize silage or macroalgae; FV, *Fucus vesiculosus*; MS, Maize silage incubated without macroalgae; PP, *Palmaria palmata*; SL, *Saccharina latissima*; spr, spring; UL, *Ulva lactuca*.

content of macroalgae seemed to be a major determining factor behind the suppression of feed degradability and CH_4 production, directly affecting the diversity and abundance of rumen fibrolytic bacteria and indirectly the methanogens.

4.1. The potential of macroalgae as ruminant feed additives and their interspecies and seasonal variations

Our findings suggested that the chemical composition (CP, NDFom, mineral elements, and TPC) of macroalgae are predominantly determined by their phyla. Overall, red (*C. crispus, P. palmata,* and *P. umbilicalis*) and green (*U. lactuca*) species had the highest protein

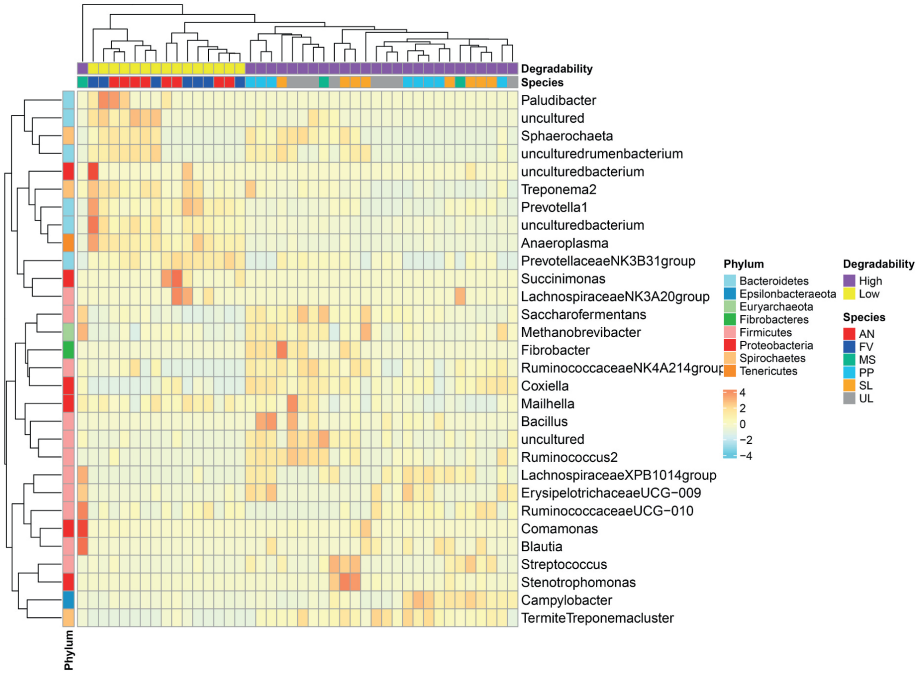

Fig. 6. Heat map showing abundance of significantly affected microbial taxa and genera of rumen microbiome after co-fermenting macroalgae with maize silage in buffered rumen fluid for 48 h *in vitro*. MS, Maize silage; AN, *Ascophyllum nodosum*; FV, *Fucus vesiculosus*; SL, *Saccharina latissima*; PP, *Palmaria palmata;* UL, *Ulva lactuca*; BLK, Blank; aut, Autumn; spr, Spring. See legends in Fig. 2 for further details.

contents (up to 20% CP of DM) in agreement with previous findings (Gaillard et al., 2018; Mæhre et al., 2014). Among brown macroalgae, a few species (e.g., *A. esculenta* and *S. latisima)* with a medium CP level (8–13% of DM) could have better value as protein feed resources than other brown species, such as fucoids. Macroalgal proteins have been characterized as quality proteins comprised of a high proportion of essential amino acids (~46% of total amino acids) (Angell et al., 2016a). The digestibility of macroalgal CP is generally found to be higher for red and green species (64–87%) than for brown species (55–82%), but there are large interspecies variations within phyla (Gülzari et al., 2019; Tibbetts et al., 2016). Certain red (*Porphyra* sp., *P. palmata*) and green (*Cladophora* sp., *Ulva* sp.) species have shown higher ruminal as well as total tract degradability of amino acids compared to brown species such as *L. digitata* in an *in sacco* study (Gaillard et al., 2018). Hence, large interspecies variations among macroalgae have been found *in situ* in dairy cattle with regards to total tract digestibility of CP, due to differences in both rumen degradability and intestinal digestibility of proteins escaping rumen fermentation (Tayyab et al., 2016). These findings suggest that in the search for novel protein feed resources, red and green macroalgae would be the most promising candidates due to favorable CP contents and amino acid compositions and highest digestibility.

In this research, a variable part of DM of macroalgae was identified as NDFom (16–62%), which generally represents material that cannot be degraded by enzymes produced by the animal itself. Hence, rumen degradability of NDF is a major determining factor for the overall digestibility and value of macroalgae as feeds for ruminants. There is limited information available from *in sacco* (goat) and *in vivo* (sheep) studies on this matter. These studies have suggested that only 16–29% of NDF from species such as *U. lactuca*, *Ulva rigida,* and *Gracilaria vermicophylla* is digestible to ruminants (Ana et al., 2017; Ventura and Castañón, 1998). Thus, the low digestibility of macroalgae fiber that results in overall low DM or OM digestibility could be one of the major bottlenecks of using larger proportions of the macroalgae in the feeds for high producing ruminants (Ana et al., 2017). However, in the present study, when certain red (*P. palmata* and *P. umbilicalis* from spring), brown (*S. latisima, L. digitata, A. esculenta* from spring, *H. elongata*) and green (*U. lactuca*) macroalgae were added to MS, no significant changes of the overall OMD *in vitro* occurred. This agrees with a previous *in vitro* 8-day rusitec fermentation study with 25% DM inclusion (as compared to 20% in the present study) of *G. vermiculophylla, S. latissima,* and *Ulva rigida* to a total mixed ration comprised of haylage, corn silage, wheat straw, and a commercial concentrate (Maia et al., 2019). The rest of the brown and red (*C. crispus*) species of macroalgae in the current study lowered the overall OMD by 12–25%, and these negative effects on OMD may be ascribed to their high contents of poorly degradable complex polysaccharides (alginates, fucoidan, carrageenan) (Williams et al., 2013) as well as the contents of other macroalgal compounds that may interfere with the bacterial fermentation.

Macroalgae are known to contain high levels of a range of minerals, and contents are generally higher than in terrestrial plants (Rupérez, 2002). In this study, the brown species *H. elongata,* and *L. digitata* were mainly enriched with the essential macrominerals Ca, Mg, Na, and K (also for *S. latissima)* compared to other species. Similarly, green species, *U. lactuca* was enriched with Na and Mg. Thus, macroalgae included in this study could be considered as potential sources of macrominerals for ruminants. The inclusion of intact macroalgae as a significant proportion of diets can, however, be of concern, as the dietary maximum tolerable levels of certain minerals, such as sodium chloride (1 g salt/kg body weight), iodine (50 mg/kg diet), arsenic, fluorine, cadmium, etc. may be exceeded (Bikker et al., 2020; NRC, 2005). Thus, specific post-harvest

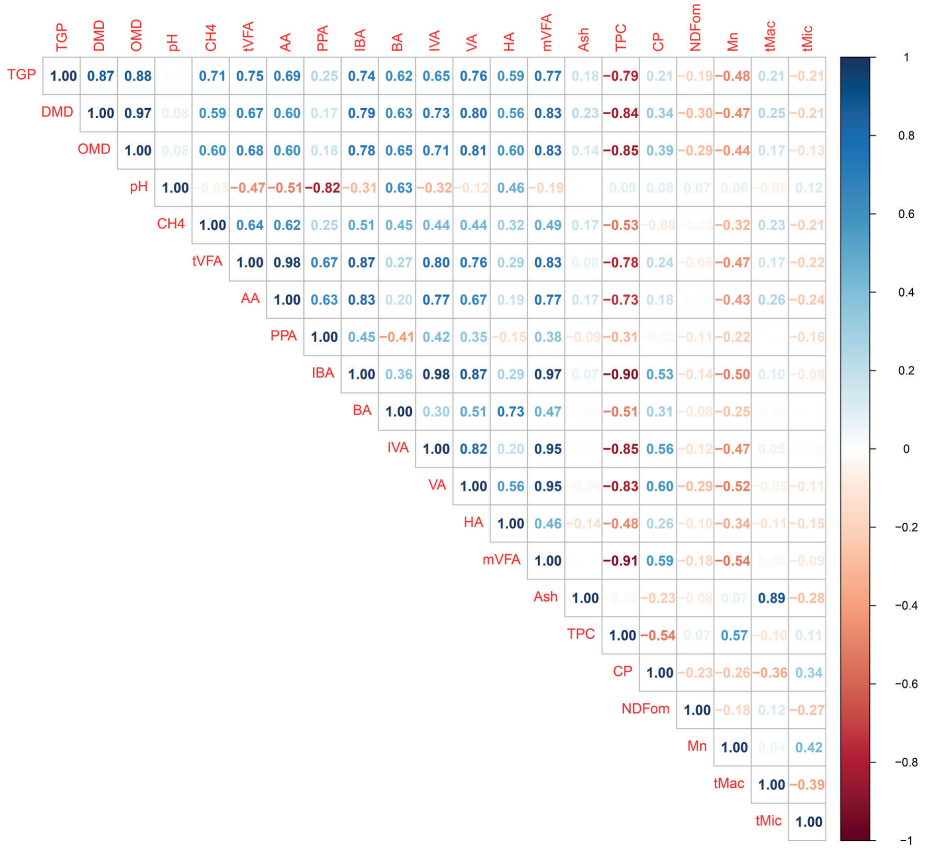

Fig. 7. Correlation matrix between the chemical composition of macroalgae and the *in vitro* fermentation characteristics after their co-fermentation with maize silage in buffered rumen fluid for 48 h. TGP, Total gas produced per gram of organic matter; DMD, dry matter degradability; OMD, Organic matter degradability; pH, Fermentation pH; CH_4, Methane; tVFA, Total volatile fatty acids; AA, Acetic acid; PPA, Propionic acid; BA, Butyric acid; IBA, iso-butyric acid; VA, valeric acid; IVA, isovaleric acid; HA, hexanoic acid; mVFA (sum of IBA,VA, IVA, and HA); TPC, Total polyphenol content; CP, Crude protein; NDFom, Ash corrected neutral detergent fiber; Mn, Manganese; tMac, Total macrominerals; tMic, Total microminerals. See legends in Fig. 2 for further details.

processing techniques, such as blanching, of macroalgae may be necessary to reduce the contents of critical minerals before their inclusions in the diets (Nielsen et al., 2020).

In addition to protein, fiber, and minerals, macroalgae are known to contain bioactive compounds, of which e.g. polyphenols have been associated with health-promoting effects (Ford et al., 2020; Gupta and Abu-Ghannam, 2011). Brown macroalgae, particularly species belonging to the Fucaceae family (*F. vesiculosus*, *A. nodosum*, *F. serratus,* and *P. canaliculata*), were found to be rich in such polyphenolic compounds, and they play an important role in the macroalgal defense against external stressors (Steevensz et al., 2012). Species-specific variations in macroalgal polyphenol contents can be linked to their growth stage, habitats, and exposures to certain biotic (epiphytes, herbivores, microbes, etc.) and environmental stressors (UV-light, salinity, tides) (Connan et al., 2004; Parys et al., 2009). Within brown macroalgae, the species that were collected from the upper (*P. canaliculata*) or middle (*A. nodosum, F. vesiculosus*, *F. serratus)* intertidal zones in Norway had higher TPC levels than species collected from the lower intertidal (*A. esculenta, H. elongata*) and subtidal (*L. digitata, S. latissima*) zones. On the other hand, intertidal zones and macroalgal polyphenol levels were not related in the red or green species, suggesting that they may rely on different biological mechanisms or chemical compounds to deal with external stressors (Roleda et al., 2019; Wada et al., 2015). Therefore, brown species, particularly the fucoids would be the species of interest as a source of natural bioactives for the health benefits of ruminants.

In agreement with previous studies including from the Nordic region (de la Moneda et al., 2019; Tayyab et al., 2016), seasonal changes in macroalgae compositions were observed also in this study. Specifically, spring harvested macroalgal biomass had the highest CP and mineral contents, whereas the highest TPC levels were found in autumn harvested biomass in some brown species. The seasonal variations were substantial for some species (54–96% higher contents of minerals in spring harvested: *S. latissima, L. digitata* (blade), and *P. palmata;* 66–86% higher CP contents in spring harvested: *A. nodosum, F. vesiculosus, F. serratus,* and *P. canaliculata*; 23–49% higher TPC contents in autumn harvested: *A. esculenta, P. cannliculata,* and *F. vesiculosus*). The higher CP contents in spring harvested macroalgae can be attributed to more favorable growing conditions in the spring, as evidenced by higher nitrogen and dissolved oxygen in seawater as well as increased light intensity, all of which can favor nutrient uptake and assimilation (Gaillard

et al., 2018; Rødde et al., 2004). In the autumn, more pronounced signs of biofouling and biomass deterioration were observed in the macroalgae, as also reported by others (Lüning, 1993; Stévant et al., 2017). This can probably explain, why some brown species had the highest level of TPC in the autumn, since polyphenols as mentioned earlier play an important part in the macroalgae defense against external stressors.

4.2. Impacts of inclusion of macroalgae on in vitro fermentation characteristics of feed and the role of total polyphenol contents

The present study implies that species-specific modulations of rumen fermentation characteristics (OMD, TGP, VFA, and CH_4) occur, when macroalgae are added to a standard feed (MS), and the level of TPC in macroalgae appears to be the principal explanatory factor behind such modulations. Hence, with the addition of species containing low TPC and generally high levels of CP (*P. palmata, L. digitata, S. latissima, H. elongata*, *A. esculenta,* and *U. lactuca*) to MS, the *in vitro* fermentation characteristics remained similar to those of MS fermented alone. This was irrespective of the harvesting season, except for *A. esculenta*, where VFA production and OMD were reduced upon the addition of autumn harvested material. This suggests that species showing no obvious negative impacts on rumen fermentation patterns could constitute up to 20% of a ruminant diet without undesirable effects on rumen fermentation. On the other hand, when brown macroalgae, especially from the Fucaceae family: *A. nodosum, F. vesiculosus, F. serratus,* and *P. canaliculata* were added to MS, a reduction of the overall OMD resulted. Additionally, *A. nodosum* and *F. vesiculosus* diminished TGP and VFA production (24–37.5%). These fucoid species are characterized by high contents of TPC and relatively low CP levels. The negative impacts of *F. vesiculosus and A. nodosum* on feed degradation parameters were more pronounced, when they had been harvested in the autumn, and this was associated with lowered production during fermentation of acetic acid and/or butyric acid, which are important parts of the energy supply to ruminant animals. Phlorotannins, the major constituents of polyphenols in brown macroalgae, form complexes with protein molecules by non-covalent bondings, thereby impairing the degradation of dietary CP and presumably also the activity of extracellular bacterial enzymes (Vissers et al., 2018; Wang et al., 2008). They may additionally inhibit microbial attachment to fiber materials, thus reducing the efficiency of NDF degradation (Makkar, 2003; Wang et al., 2008). Thus, brown species from the Fucaceae family are not suitable for inclusion in ruminant diets in as high proportions as used in this study due to the potential negative impacts of high polyphenol contents on feed utilization and associated animal productivity.

Our preliminary data (not provided) indicated that simple pretreatments of fresh macroalgal biomass can reduce the TPC content by up to 25% in species from the Fucaceae family. Such TPC-reduced material, when added (20%) to MS gave rise to ~25% higher TGP than the native material (Deepak et al., unpublished data). Therefore, extraction of polyphenols from TPC rich macroalgae via novel biorefinery approaches, such as microwave, ultrasound, or enzyme-assisted extraction, may be a feasible way to overcome the negative impacts of high TPC on feed degradability, while recovering the highest amount of bioactive polyphenols for other purposes (Filote et al., 2021; Marinho et al., 2016).

4.3. Impacts of Nordic macroalgae on the rumen microbiome and potential anti-methanogenic properties

The composition of the rumen microbial population plays an important role in the degradation of dietary plant components (Mizrahi et al., 2021). In this study, the rumen microbial compositions were differentially modulated by the addition of macroalgae species to MS. The effect of the macroalgae harvesting season in this respect was marginal. Some macroalgae species (*P. palmata, S.latissima,* and *U. lactuca*) that did not have apparent effects on rumen fermentation parameters or *in vitro* feed degradation, induced only minimal changes in microbial compositions in the post-fermentation rumen fluid as compared to MS fermented alone. In contrast, supplementation of *A. nodosum* and *F. vesiculosus* to MS was associated with an overall reduction in rumen microbial species richness (18–21%) and diversity (Shannon diversity index, 4.72 vs 3.67: MS vs *F. vesiculosus*). These two macroalgae species substantially inhibited the abundance of fiber degrading cellulolytic bacteria belonging to the taxa Firmicutes: *Ruminococcus* spp., Ruminococcaceae UCG-010, species in the Lachnospiraceae family, and Bacteriodetes: Rikenelaceae RC9 gut group. This had implications in terms of a reduced OMD, presumably in part due to reduced cellulose degradability, since the abundance of cellulolytic bacteria in this study was directly correlated with rumen fermentation parameters. In contrast to our results, a few *in vitro* (Belanche et al., 2016a) and *in vivo* (Zhou et al., 2018) studies have reported only minor changes in rumen microbial populations, when *A. nodosum* was added to a basal feed. This could probably be due to a lower level of macroalgal inclusion (≤5% DM) in the diet than in the present study. In this study, although cellulolytic bacteria were suppressed, the addition of *A. nodosum* and *F. vesiculosus* to MS, promoted hemicellulolytic bacteria, such as *Prevotella* spp. (Bacteriodetes), and *Treponema* 2 (Spirochaetes) indicating the microorganism-specific effects of macroalgae. These effects are analogous to the impacts of *A. nodosum*-derived phlorotannin on cellulolytic and non-cellulolytic rumen microorganisms as reported in a previous *in vitro* study (Wang et al., 2009). The promotion of rumen hemicellulolytic microorganisms by the polyphenol-rich macroalgal feed may be an indication of a compensatory enhancement of hemicellulolytic activity in response to poor cellulose degradation. However, further studies regarding the impacts of polyphenol-rich macroalgae on different fiber degradation rates are required to confirm this hypothesis.

The macroalgae species included in this study represented a range of CH_4 mitigation potential when they were added to the MS. Such mitigation was >30% per gram of fermented OM with brown species: *F. vesiculosus*, *A. nodosum,* and *F. serratus*; green species: *U. lactuca* regardless of harvesting seasons, and the red species: *C. crispus* and *P. umbilicalis* from the spring harvest. However, the most promising CH_4 mitigation was observed for poorly degradable fucoid species, namely 48.2% and 62.6% reduction, respectively, for *A. nodosum,* and *F. vesiculosus* from the autumn harvest, and ~56.5% for the red species, *C. crispus* from the spring harvest. To the best of our knowledge, such anti-methanogenic action of *F. vesiculosus* and *C. crispus* has never been reported. The potency of these Nordic macroalgae species encourages to further *in vivo* studies to explore their potential application as anti-methanogenic feed additives for ruminants.

The anti-methanogenic property of *A. nodosum* has previously been reported from an *in vitro* study that used the rusitec system. In that study, only a 15% reduction in CH_4 production per gram of fermentable OM was observed when 2 g L^{-1} *A. nodosum* was added to the fermentation medium (Belanche et al., 2016b). Two tropical red species, *Asparagopsis taxiformis* and *A. armata* are well documented to possess very potent anti-methanogenic actions, and they can almost entirely reduce CH_4 production from ruminant livestock both *in vitro* (Machado et al., 2014) and *in vivo* (Kinley et al., 2020; Roque et al., 2019b) at low levels of dietary inclusion (<5% of OM). In addition, variable anti-methanogenic properties *in vitro* have been ascribed also to the tropical brown macroalgae *Dictyota bartayresii* (Machado et al., 2014) and the red *Gracilaria vermiculophylla* and *Gigartina* sp., as well as the widely distributed green *Ulva* sp. (Maia et al., 2016). The CH_4 mitigating properties of selected brown macroalgae in our research were associated with a marked reduction in the abundance of the dominant CH_4 producing archaea, *Methanobrevibacter* (phylum Euryarchaeota) in the post-fermentation rumen fluid. These results are in agreement with previous studies that evaluated the impacts of *A. nodosum* (Zhou et al., 2018) and *A. taxiformis* inclusion on rumen methanogenic archaea (Machado et al., 2018; Roque et al., 2019a). Therefore, certain Nordic brown and red macroalgae species can mitigate enteric CH_4 formation from ruminants by inhibiting

the methane-producing archaea in the rumen.

The reduced abundance of rumen methanogens by the red macroalgae *Asparagopsis* spp. has been ascribed to direct inhibitory properties of halomethanes, such as bromoform and dibromochloromethane on the rate-limiting enzymatic process, where CH_4 is formed (Machado et al., 2018; Roque et al., 2019a). However, a recent study with 17 different red, brown and green species (including *A. nodossum*, *F. vesiculosus* and *C. crispus*) indicated that such halomethanes are not present in Nordic macroalgae (Nørskov et al., 2021). Thus, other secondary polyphenolic metabolites, such as phlorotannin and flavonoids, could be responsible for the CH_4 mitigating properties of brown (Vissers et al., 2018; Wang et al., 2008) and red species (*C. crispus*), respectively (Bodas et al., 2008). Surprisingly, no negative correlation between macroalgae TPC and rumen methanogens (*Methanobrevibacter* spp.) was evident in this study, suggesting that alternative bioactive compounds or mechanisms may be involved with a direct or indirect suppression of methanogenesis as well as the population of methanogens.

Rumen microorganisms exist in a complex system where the activities of different microbial species can be interconnected. Certain rumen methanogenic archaea live in a symbiotic relationship with ciliated protozoa where they utilize H_2 produced by the protozoa for methanogenesis and energy metabolism (Patra et al., 2017). The addition of 2 g L^{-1} A. *nodosum*, a phlorotannin-rich macroalga, in the fermentation medium suppressed the CH_4 production and rumen protozoa (by 23%) with a lowered acetate and butyrate production, presumably due to an anti-protozoal effect of phlorotannins (Belanche et al., 2016b). A reduction in the ciliated protozoa and CH_4 production without affecting rumen methanogens was observed in another *in vitro* batch fermentation study when ethanolic extracts of two brown macroalgae, *Undaria pinnatifida*, and *Sargassum fulvellum*, containing diverse phenolic compounds (flavonoids and polyphenols) were added to a control diet (Choi et al., 2021). Thus, the inhibition of rumen CH_4 production by polyphenol-rich macroalgae in this study may be associated with indirect modulation of the rumen environment potentially due to reduced rumen protozoal activity as supported by the observed reduction in acetate and butyrate production. The rumen protozoal population has also been found to be correlated positively with the concentrations of acetic acid and negatively with that of propionic acid (Zhou et al., 2018). We observed an increased propionate:acetate ratio with the addition of *F. vesiculosus* (autumn harvested) to MS, suggesting a potential reduction in protozoal activities and subsequently, contributing to CH_4 mitigation. However, studies directly evaluating the impacts of phlorotannins on the rumen protozoa and methanogens are lacking. Therefore, future studies should evaluate not only the specific effect of phlorotannins on methanogenesis and the populations of rumen methanogens and protozoa, but also investigate whether there are other active anti-methanogenic components (e.g. flavonoids, complex carbohydrates) that could be involved in the suppression of CH_4 production by these brown macroalgae.

To date, *Asparagopsis* spp. are the most potent macroalgae in terms of mitigating CH_4 emissions from ruminant livestock. However, scalable commercial cultivation of *Asparagopsis* spp. to produce sufficient biomass to be used in livestock farms is yet to be achieved due to its complex life cycle and requirements for optimal growth in an artificial environment (Zhu et al., 2021). Moreover, *Asparagopsis* spp. are not the native species in Nordic waters (Andreakis et al., 2004) and there are safety concerns due to the potential toxic properties of the major bioactive compound, bromoform, for animals and the environment (Muizelaar et al., 2021). In contrast, a large volume of biomass of the brown macroalgae: *A. nodosum* and *F. vesiculosus* are naturally available across the North Atlantic seacoasts all year around (Pereira et al., 2020; Stévant et al., 2017), although commercial cultivation is yet to be established for these species. Nonetheless, our findings suggest that certain Nordic macroalgae could serve as ingredients for the production of anti-methanogenic feed additives, with potentially safer bioactive compounds for CH_4 mitigation from ruminants.

Despite the promising CH_4 mitigation, *A. nodosum* and *F. vesiculosus* significantly impaired the OMD, TGP, and VFA production and that could lead to reduced animal productivity if the dietary inclusion rates are too high. Though the rate of CH_4 inhibition was greater than the inhibition of feed degradation, the positive correlation of CH_4 inhibition with the feed degradability inhibition indicates that a part of CH_4 mitigation was due to the reduced feed degradation. Thus, the anti-methanogenic potential of these macroalgae should be validated *in vivo* to design optimal strategies for implementation. In the future, it would be interesting to evaluate whether the anti-methanogenic properties of these macroalgae also persist at lower inclusion levels so that feed degradability is not or minimally compromised. Moreover, using a mixture of highly anti-methanogenic macroalgae species together with less potent species that do not depress rumen feed degradability could be another viable option. Future studies should also focus on the use of polyphenolic or other types of bioactivity enriched fractions, rather than whole biomass, to identify specific compounds directly responsible for anti-methanogenic properties and their mechanisms of action. Certain Nordic species, such as the green macroalga, *U. lactuca,* seem attractive candidates to achieve ~30% of CH_4 mitigation *in vitro* without affecting the feed digestion and animal productivity at 20% inclusion in the feed. Hence, this study points to the importance of certain Nordic macroalgae as potential anti-methanogenic feed additives and/or nutritional feedstuffs for ruminants.

5. Conclusions

Brown (*L. digitata, S. latissima, H. elongata*), red (*P. palmata*) and green (*U. lactuca*) species with low TPC contents could become valuable future feed sources for ruminants, particularly when harvested in the spring, where protein contents are highest. *In vitro,* they could constitute a significant part of the diet (20% of DM) without negative implications for rumen fermentability of the feed or the rumen microbiome. Macroalgae among the brown species with high levels of TPC: *A. nodosum, F. vesiculosus, F. serratus,* and *P. canaliculata,* seem unsuitable for ruminant feed applications at large inclusions due to negative impacts on OMD, fermentation patterns, and the rumen microflora, particularly when harvested in the autumn, where TPC levels are highest. The substantial depression of feed degradability by polyphenol-rich macroalgae: *A. nodosum* and *F. vesiculosus* was associated with depression of cellulose-degrading microorganisms, including *Ruminococcus* spp., Ruminococcaceae UCG-010, species in the Lachnospiraceae family, and Rikenelaceae RC9 gut group. Nevertheless, *A. nodosum* and *F. vesiculosus* could be promising as ingredients in feed additives to mitigate the enteric CH_4 emissions from ruminants through inhibition of methanogens (*Methanobrevibacter* spp.), an effect which could not solely be described by TPC. Future research should validate these findings via *in vivo* feeding trials with ruminants to discover a minimum effective dose of inclusion into the feed from both anti-methanogenic and digestibility perspectives. To optimize a strategy for implementation of macroalgae as feed additives to reduce enteric CH_4 emission, future studies should focus on enrichment of polyphenolic and/or other bioactivity enriched extractions. Therefore, macroalgal biorefinery platforms should identify efficient measures to cost-efficiently extract valuable and safe bioactive compounds that can be utilized for CH_4-mitigating purposes without impairing the digestibility of the diet and hence animal performance.

CRediT authorship contribution statement

Deepak Pandey: Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Hanne Helene Hansen:** Resources, Methodology, Data evaluation, Writing – review & editing. **Rajan Dhakal:** Methodology, Data curation, Writing – review & editing, evaluation. **Nabin Aryal:** Methodology, Writing – review & editing. **Surya Prakash Rai:** Visualization, Writing – review & editing. **Rumakanta Sapkota:** Formal analysis, Visualization,

Writing – review & editing. **Mette Olaf Nielsen:** evaluation, Methodology, Writing – review & editing. **Margarita Novoa-Garrido:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Prabhat Khanal:** Conceptualization, Resources, Methodology, Data evaluation, Writing – review & editing, Project administration, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Abbreviations/nomenclature

AE: Alaria esculenta
AN: Ascophyllum nodosum
AOAC: Association of Official Analytical Chemists
ASVs: Amplicon sequence variants
Ca: Calcium
CC: Chondrus crispus
CH_4: Methane
CP: Crude protein
Cu: Copper
DF: Dilution factor
DM: Dry matter
Fe: Iron
FS: Fucus serratus
FV: Fucus vesiculosus
GC: Gas chromatography
HE: Himanthalia elongata
K: Potassium
LD: Laminaria digitata (blade)
LS: Laminaria digitata (stipe)
Mg: Magnesium
Mn: Manganese
MS: Maize silage
Na: Sodium
NDF: Neutral detergent fiber
NDFom: Ash corrected neutral detergent fiber
OM: Organic matter
OMD: Organic matter degradability
PC: Pelvetia canaliculata
PCR: Polymerase chain reaction
PGE: Phloroglucinol equivalents
PP: Palmaria palmata
PU: Porphyra umbilicalis
rRNA: Ribosomal RNA
SL: Saccharina latissima
SV: Solvent volume
TGP: Total gas production
UL: Ulva lactuca
mVFA: Minor volatile fatty acids
VFA: Volatile fatty acids
Zn: Zinc

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This study revealed that selective green, red, and brown macroalgae species from the Norwegian coast could be good feeding ingredients for ruminant animals because of their sufficient crude protein, NDF, and (*in vitro*) digestibility when included (20%) in the ruminant feed. They carried higher nutritional value in spring, with greater protein and minerals than in autumn. Brown species, *Fucus vesiculosus*, and *Ascophyllum nodosum*, although had low nutritive value for animals, seemed excellent sources of polyphenols and mitigated up to 63% of enteric methane (in vitro) from ruminants. For monogastric species, a low dietary inclusion (5%) of those brown algae could improve animal health by inducing healthier gut microbiota and reducing the fat mass gain during high-fat diet exposure, as observed in our mice study. This may have implications for humans to alleviate diet-associated obesity.

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