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# Application of ensiled *Saccharina latissima* and *Alaria esculenta* as feed: ensilbility, digestibility and bioactivity

Ying Yen

FACULTY OF BIOSCIENCES AND AQUACULTURE

NORD University

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A thesis for the degree of
Philosophiae Doctor (PhD)

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Nord University
N-8049 Bodø
Tel: +47 75 51 72 00
www.nord.no

# Preface

This doctoral thesis is submitted in fulfilment of the requirements for the degree of Philosophiae Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University, Bodø, Norway. The studies included in this dissertation represent original research that was carried out over a period of 3 years from Feb. 2019 to Apr. 2022. This PhD research was supported by funds from multiple sources: 1) The *OpEnMac* project funded by the Nordland county (project nr. AF0083), 2) The *EnMac* project funded by the Research Council of Norway (project nr. ES 631289), and 3) The Stipendiatprogram Nord funded by the Norwegian ministry of education (project nr. KD-224000-140).

The project team consists of:

**Ying Yen**, MSc, FBA, Nord University: PhD candidate

**Margarita Novoa-Garrido,** Associate Professor, FBA, Nord University: Main supervisor

**Martin Riis Weisbjerg**, Professor, Department of Animal Science, Aarhus University: Co-supervisor

**Kiron Viswanath**, Professor, FBA, Nord University: Co-supervisor

**Prabhat Khanal**, Associate Professor, FBA, Nord University: Co-supervisor


Ying Yen

Oslo, October 2022

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# List of abbreviations

AA: amino acids

ADF: acid detergent fiber

bp: base pair

C: carbon

CP: crude protein

$CP_{ED}$: CP effective degradability calculated at 5 % $h^{-1}$ rumen passage rate

$CP_{TT}$: CP total tract digestibility

DM: Dry matter

$DM_{ED}$: DM effective degradability calculated at 5 % $h^{-1}$ rumen passage rate

$DM_{TT}$: DM total tract digestibility

DSS: dextran sodium sulfate

EU: European union

GHG: greenhouse gas

iL-1β: Interluekin-1 beta

iL-6: interleukin-6

iNDF: indigestible NDF

IRF: incubated ruminal fluid

IVOMD: *in vitro* organic matter digestibility

LAB: lactic acid bacteria

LFC: Log2FoldChange (1 LFC = 2 folds, 2 LFC = 4 folds, 3 LFC = 8 folds, etc.)

mcrA: methyl coenzyme-M reductase subunit A

N: nitrogen

NDF: neutral detergent fiber

NGS: next generation sequencing

PGE: phloroglucinol equivalents

RA: relative abundance

RF: ruminal fluid

RML: recommendation maximum level

SCFA: short chain fatty acids

SD rat: Sprague-Dawley rat

SPF: specific pathogen free

TNFa: tumor necrosis factor alpha

TPC: total polyphenol content

VFA: volatile fatty acids

WSC: water soluble carbohydrates

ZO-1: zonula occludens-1

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Yen, Y., Weisbjerg, M.R., Rautenberger, R., Fečkaninová, A. & Novoa-Garrido, M.

Improving fermentation of *Saccharina latissima* and *Alaria esculenta* silages with additives for preserving biomass and antioxidants. J Appl Phycol 34, 625–636 (2022). https://doi.org/10.1007/s10811-021-02628-4

**Paper II**

Yen, Y., Weisbjerg, M.R., Abdelhafiza, Y., Le Moine Bauerc, S., Kiron, V. & Novoa-Garridoa, M.

Feed characteristics and potential effects on ruminal bacteria of ensiled *Saccharina latissima* and *Alaria esculenta* for dairy cows.

*Manuscript*

**Paper III**

Yen, Y., Abdelhafiz, Y., Khanal, P., Kiron, V., Weisbjerg, M.R. & Novoa-Garrido, M.

The effect of *Lactobacillus* spp. ensiled *Alaria esculenta* on fecal bacterial composition and butyrate content in healthy- and DSS-induced-colitis rats.

*Manuscript*

# Abstract

Applying cultivated seaweeds as feed ingredients is of great interest for sustainable animal production. To date, the yearly aquaculture production of *Saccharina latissima* and *Alaria esculenta* is among the highest in countries, including Norway. However, one bottleneck for using seaweed as feed lies in the way to preserve seaweeds after harvest for their year-round availability. Furthermore, the digestibility and benefits of dietary seaweed inclusion to farm animals are still unclear.

This PhD work optimized the ensiling conditions for *S. latissima* and *A. esculenta* using various ensiling treatments, and determined the ensiled seaweed's nutrient digestibility and bioactivity using dairy cows and rats as animal model. The results suggest that seaweed biomass is preservable by ensiling, but seaweed silage is peculiar because the main fermentation product is acetate instead of lactate as normally found in land crop silages. The use of lactic acid bacteria and formic acid as ensiling additives facilitated silage acidification, but reducing moisture by oven drying led to phlorotannin and protein degradation in the seaweed silages. Moreover, ensiling had minor effect on these seaweed's chemical composition and their nutrient digestibility in ruminants, and ensiled *S. latissima* can potentially be applied as alternative forage-like ingredient. There was an unexpected absence of rumen degradable crude protein in *A. esculenta*, and the microbiome analysis revealed the importance of *Prevotella* spp. and other ruminal fibrolytic bacteria in digesting seaweeds. Finally, dietary inclusion of ensiled *A. esculenta* in rats showed no clear indication of prebiotic effect. However, some changes in the fecal bacterial composition might be of interest for controlling *Campylobacter* spp. infections in broiler chicken production.

Overall, this thesis confirmed the ensilability of cultivated *S. latissima* and *A. esculenta*, and provide insights of the nutritional value of these seaweed silages to ruminants and monogastric animals.

# Sammendrag på norsk

Å bruke dyrket tare som fôringredienser er av stor interesse for bærekraftig husdyrproduksjon. Til dags dato er den årlige akvakulturproduksjonen av *Saccharina latissima* og *Alaria esculenta* blant de høyeste i land inkludert Norge. En flaskehals for å bruke tare som fôr ligger imidlertid i veien for å bevare taren etter høsting for helårs tilgjengelighet. Videre er fordøyeligheten og fordelene ved inkludering av tare i kosten for husdyr fortsatt uklare.

Dette doktorgradsarbeidet optimaliserte ensileringsforholdene for *S. latissima* og *A. esculenta* ved ulike ensileringsbehandlinger, og bestemte den ensilerte tarens næringsfordøyelighet og bioaktivitet ved å bruke melkekyr og rotter som dyremodell. Resultatene tyder på at tarebiomasse kan bevares ved ensilering, men hovedfermenteringsproduktet i tareensilasje var acetat i stedet for laktat som normalt finnes i plantensilajer. Bruk av melkesyrebakterier og maursyre som ensilerings tilsetningsstoffer førte til tilstrekkelig forsuring, men redusert fuktighet ved ovnstørking førte til florotannin og proteinnedbrytning i tareensilasjene. Dessuten hadde ensilering lite effekt på tarens kjemiske sammensetning og næringsfordøyelighet hos drøvtyggere, og ensilert *S. latissima* kan potensielt brukes som alternativ fôrlignende ingrediens. Det var et uventet fravær av vomnedbrytbart råprotein i *A. esculenta*, og mikrobiomanalysen avdekket viktigheten av *Prevotella* spp. og andre ruminal fibrolytiske bakterier i å fordøye tare. Til slutt viste inklusjon av ensilert *A. esculenta* i fôret til rotter ingen klar indikasjon på prebiotisk effekt. Noen endringer i den fekale bakteriesammensetningen kan imidlertid være av interesse for å kontrollere *Campylobacter* spp. infeksjoner i produksjon av slaktekylling.

Totalt sett bekreftet denne oppgaven ensileringsevnen til kultiverte *S. latissima* og *A. esculenta*, og gir innsikt i ernæringsverdien til disse tareensilasjene for drøvtyggere og enmagede dyr.

# 1. Introduction

There is a worldwide interest to use the resource from ocean, which covers over 70 % of global surface, to provide energy, food, and feed for the growing human population. Seaweed, also called macroalga, is perceived as a sustainable resource because its cultivation can serve as a way to capture and store carbon to mitigate global warming. In addition, these algae can remove nutrients that leach from fish farming and agricultural operations and reduce coastal eutrophication (Alvarado-Morales et al., 2013; Hasselström et al., 2020). Furthermore, seaweed cultivation supports the livelihood in the coastal areas with otherwise limited economical resources, thereby stimulating the emergence and diversification of markets (Hasselström et al., 2020; Rimmer et al., 2021). To date, seaweed cultivation can provide larger volumes of brown seaweeds popularly known as kelps, thanks to its rapid growth rate and the specialized machinery for kelp harvesting that can reduce labor costs when compared to the green and red seaweed species. Among different kelps, the production volume of *S. latissima* and *A. esculenta* are two of the largest in Norway and other western countries (FAO, 2021; Fiskeridirektoratet, 2022). However, the product value chain of these kelps has not yet been established, partially constrained by the high cost of post-harvest preservation and the challenging chemical composition characteristic of this biomass (Stévant et al., 2017b; Biancarosa et al., 2018).

Animal products such as milk, meat, and eggs are high quality protein sources for humans, and are important elements for the global food security and nutrient demands (Smith et al., 2013). The demand for animal products is expected to grow along with the newly emerging economies (Brazil, Turkey, Russia, India, and China) (Ingram et al., 2012). However, the animal production sector is facing a great challenge to maintain its production without addressing its effects on global warming, food security, and antimicrobial resistance (Ingram et al., 2012; Rushton et al., 2014). In terms of global warming, the animal production activity accounts for 14 – 18 % of all

human-induced greenhouse gas emissions (GHG), with methane being the major GHG (Haque, 2018). Ruminant farming accounts for 80 % of total methane emission in the animal production sector and the ruminal enteric fermentation is one of the major methane sources (Haque, 2018). In terms of food security, half of the produced grains in the world is currently used as animal feed. The feed grain production directly competes with human food production for arable land and fresh water (Smith et al., 2013). Concerning the increasing antimicrobial resistance, in 2006, the EU banned the use of antibiotics as growth promoters, and is going to prohibit the use of veterinary drugs containing pharmacological doses of zinc oxide (3000 mg $kg^{-1}$ feed) in 2022. Nonetheless, both antibiotic growth promoters and zinc oxide are widely used in animal production systems, to enhance growth rate and reduce mortality in monogastric animals (i.e. poultry and swine). Alternatively, non-antibiotic feed or feed additives that can promote the animal's immunity and modulate gut microbiota are needed to build the animal's resilience after physiological challenges (i.e., weaning) and environmental challenges (i.e., pathogens) (Rushton et al., 2014; Liu et al., 2018).

There is a great interest to make use of seaweed both as an alternative nutrient source and as a bioactive feed additive for meeting the sustainable demands the animal production sector encounters (Evans & Critchley, 2014; Min et al., 2021). However, one of the prerequisites is to stabilize the chemical composition in seaweed after harvest (Stévant & Rebours, 2021). Traditionally, this is achieved by freezing or oven-drying in the western countries, nevertheless, such preservation contributes to the high production costs. Ensiling was first established to preserve land crop in the late $19^{th}$ century because it requires low energy and machinery input, and it can preserve different volumes of harvested biomass (McDonald et al., 1991; Weinberg & Ashbell, 2003). By transferring the current knowledge of ensiling to preserve seaweed biomass, the production costs in the seaweed value chain can be reduced, consequently making the use of seaweed as feed more feasible.

## 1.1 Seaweeds and seaweed aquaculture

Seaweeds (or macroalgae) are the primary producers in the ocean. Seaweeds acquire energy from sunlight and essential chemical elements (carbon, nitrogen, and phosphorus) as well as minerals from seawater. Seaweeds are multicellular eukaryotes and their size ranges from visible to over 60 m in length. Based on their pigments and characteristic colors, seaweeds have traditionally been classified into brown seaweeds (Phaeophyceae), green seaweeds (Chlorophyceae), and red seaweeds (Rhodophyceae). In general, brown seaweed species are larger than the green and red seaweeds, and are characterized by structures such as holdfast, stipe, and blades. The holdfast is at the base of seaweed that attaches itself to a surface (e.g., rock and shell), the stipe is equivalent to stem in plants, and the blades is where most of photosynthesis takes place. Otherwise, the morphology differs between different seaweed species within each class or division.

The global seaweed production experienced a vast growth from an annual production of less than 10 million wet tons in 1950 to 35 million wet tons in 2019, generating USD 275 billions and becoming the third-largest aquaculture product globally (FAO, 2021). Most of the seaweeds are produced (97 %) of seaweeds are produced in Asian countries, where the labor costs are low, and their consumption are deeply rooted in their culture. The rest of the seaweeds are produced in America (1.4 % of total volume), Europe (0.8 % of total volume), Africa (0.4 % of total volume), and Oceania (0.05 % of total volume) (Cai et al., 2021). The expansion of seaweed cultivation of brown seaweed species (16.4 million wet tons in 2019) and red seaweed species (18.3 million wet tons in 2019) has contributed to the growth in seaweed production in the last years (Cai et al., 2021). In the case of brown seaweeds, the temperate water species including *Saccharina japonica* (commonly known as makonbu in Japanese, dasima in Korean and haidài in Chinese), *Undaria pinnatifida* (wakame), *S. latissima* (earlier known as *Laminaria saccharina* and commonly known as sugar kelp in English), and *Alaria esculenta* (winged kelp) are the most extensively farmed species,

and the farming activity is mainly in the northern Atlantic Ocean and northern Pacific Ocean (Grebe et al., 2019; Cai et al., 2021). In the case of red seaweeds, the mostly farmed species are the warm-water species *Kappaphycus* and *Gracilaria* that are farmed in Southeast Asia, South Europe, and Africa as well as the temperated water species *Porphyra* that are grown in the Northeast Asia (Cai et al., 2021).

However, seaweed cultivation outside Asia is not yet well developed and most of the seaweed products in Europe come from wild-harvested seaweeds (i.e., 163,000 wet tons in Norway in 2019); mainly *Laminaria hyperborean* (tangle or cuvie), *Laminaria digitata* (oarweed), and *Ascophyllum nodosum* (rockweed) that are harvested with different types of mechanical harvesting equipment (Stévant et al., 2017b; Monagail & Morrison, 2020). The major seaweed producing countries in Europe are Norway, France, and Ireland which produced 57.5 %, 17.5 %, and 10.3 % of the European seaweed production in 2019, respectively (Cai et al., 2021). There are concerns about over-exploitation of wild seaweeds (Rebours et al., 2014) and the ecological consequences, because the wild kelp forests are habitat and shelter for other marine organisms. Also, mechanically harvesting the wild seaweed populations can disrupt the marine food chain and destroy the seabed, and thus potentially affect other coastal production activities like fishing. Moreover, the harvested biomass is often a mix of different seaweed species and can be contaminated by other organisms which leads to reduced price besides making for downstream processing difficult (Rebours et al., 2014; Halat et al., 2015).

Seaweed cultivation under good management can avoid the above-mentioned concerns. Therefore, upscaling seaweed cultivation has been of great interest in Norway due to its long coastline and the above-mentioned environmental and socioeconomic motivations (Stévant et al., 2017b). The cultivated seaweed production almost tripled during the period 2019 to 2020 from 117 wet tons to 336 wet tons that generated USD 0.8 million, with the brown seaweeds such as *S. latissima* and *A. esculenta* being the most cultivated species (Fiskeridirektoratet, 2022). Some

seaweeds have more complex reproductive cycles that make their domestication challenging. Furthermore, small-scale production of *U. pinnatifida*, and the red seaweeds *Palmaria Palmata* and *P. umbilicalis* are undertaken in European countries. In Norway, the cultivated seaweed production was projected to be 20 million tons in 2050 with focus on large kelps particularly *S. latissima* and other brown seaweed species (i.e. *A. esculenta*) (Broch et al., 2019). The upscaled production volume can potentially lower the production costs per unit and facilitate the application of cultivated seaweed biomass as feed ingredient.

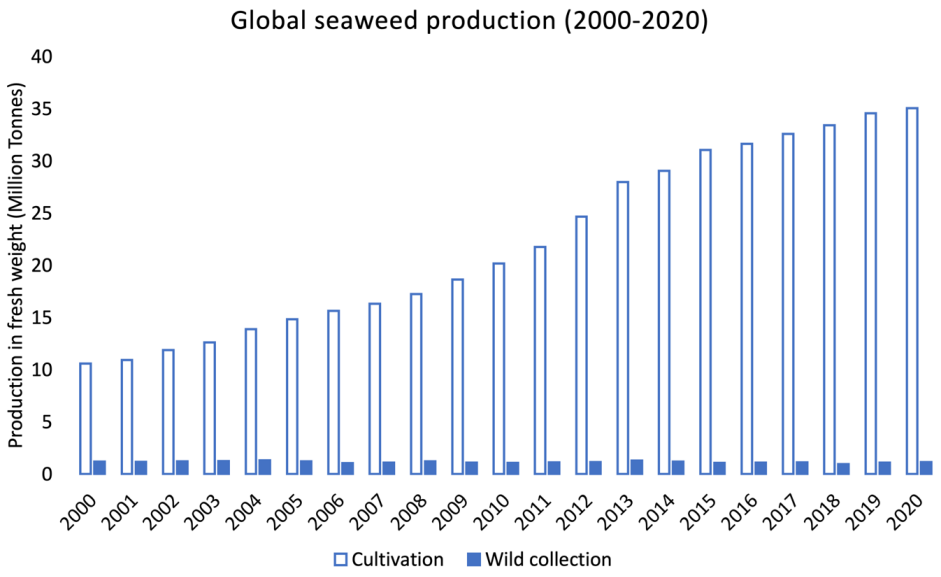


**Figure 1.** Global seaweed production (2000-2020). Source: FAO statistic updated Mar. 2022

## 1.2 Chemical composition of brown seaweeds

The moisture content of brown seaweeds at harvest ranges from 80 - 95 % (Schiener et al., 2015). The biomass of brown seaweed is characterized by high level of carbohydrates (25 - 60 % DM) and ash (13 - 25 % DM), medium to high level of phlorotannins (5 – 50 mg PGE $g^{-1}DM^{-1}$), medium to low level of protein (5 - 15 % DM), and low level of lipids (< 1 % DM). Nevertheless, the lipid fraction is rich in the n-3 polyunsaturated fatty acids that are of great interest due to their health benefits

(Maehre et al., 2014; Schiener et al., 2015; Roleda et al., 2019; Olsson et al., 2020; Saifullah et al., 2021). In addition, brown seaweeds are known to accumulate iodine and heavy metals such as lead (Pb), cadmium (Cd), Mercury (Hg), and arsenic (As) from the seawater that can pose risk when consumed by human or animals (Roleda et al., 2018; Roleda et al., 2019). However, there are differences between the chemical composition of different species of seaweeds, in addition to the changes caused by the growing location and harvest season (Steevensz et al., 2012; Schiener et al., 2015; Roleda et al., 2019). In Norway and other northern European countries, brown seaweed harvesting is normally done in the Spring or early Summer, depending on the latitude/location, in order to avoid biofouling by epiphytic organisms which regularly takes place when water temperature increases (Rebours et al., 2014; Stévant et al., 2017a). Therefore, spring is often the season of choice to harvest cultivated seaweed, and the spring-harvested seaweed has a lower carbohydrate content and higher CP content, compared to the summer- and autumn- harvested seaweed (Schiener et al., 2015). Hence, in this section, I focus on the chemical composition and properties of cultivated *S. latissima* and *A. esculenta*, mainly harvested in spring in Northern Europe. Nevertheless, results from wild harvested biomass or from other locations are used for discussion.

*Carbohydrate*

Carbohydrates are the dominant components in brown seaweeds (Davis et al., 2003). Among all, alginate and fucoidan are the structural components present in the cell wall of brown seaweeds, and the mannitol (monomer) and laminarin (polymer) are the main energy reserves of brown seaweeds (Davis et al., 2003). However, the fucoidan content was found to be less than 1.5 % DM in both *S. latissima* and *A. esculenta*, and both seaweeds are not suggested to be the main source for obtaining fucoidan (Stévant et al., 2017a; Sørensen et al., 2021). The alginate content is between 20 – 30 % DM and the mannitol content is 10 – 20 % DM in *S. latissima* and *A. esculenta.*

The laminarin content in *S. latissima* (0 - 5 % DM) is lower than in *A. esculenta* (10 – 15 % DM) (Schiener et al., 2015; Stévant et al., 2017a).

The proportion of alginate and mannitol appears to be the largest among the carbohydrates and they might play an important role in the fermentation properties of these seaweeds during ensiling and in the microbial fermentation in the digestive tract of the animals. As shown in Figure 2, alginate is a polysaccharide made of polymer complex of *D*-mannuronic and *L*-guluronic acids linked through the 1,4-glycosidic bond and is widely used in both pharmaceutical (e.g., hydrogel), nutraceutical (e.g., dietary fiber) and food (e.g., gelling agent) industries (Liu et al., 2019). Mannitol is a fermentable sugar alcohol derived from D-mannose (Figure 2). Also, it is possible for laminarin to release mannitol or glucose because laminarin consists of beta-glucans linked by (1,3) and (1,6) glycosidic bonds and with a glucose or mannitol attached to one end (Figure 2). Finally, the glucose content that ranges between 5 – 10 % was reported in the biomass of both seaweed species.

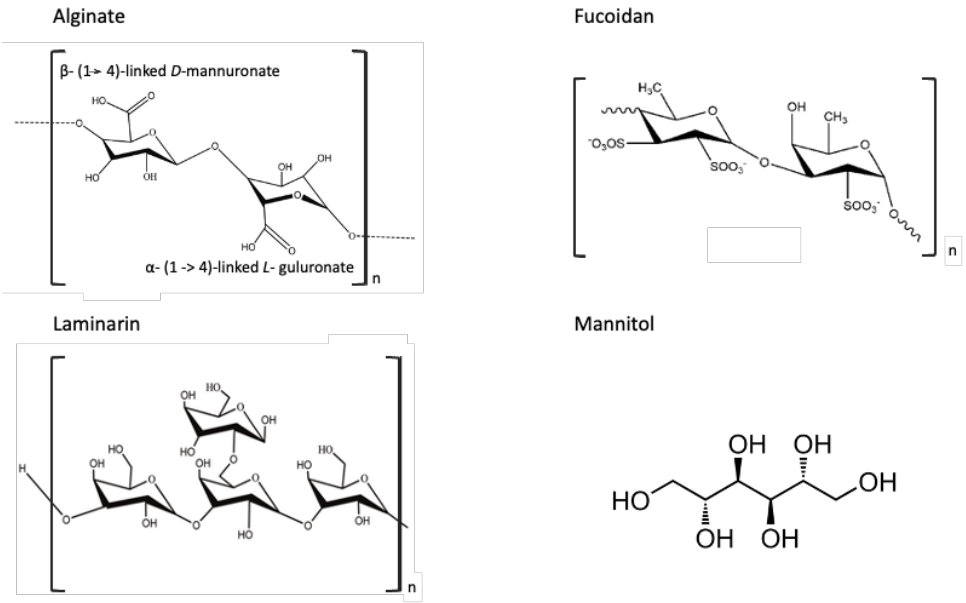


**Figure 2.** The molecular structure of main structure components (alginate & fucoidan) and main energy reserves (laminarin & mannitol) commonly found in brown seaweeds.

*Protein*

Protein content in brown seaweeds is generally lower than in green and red seaweeds (Maehre et al., 2014; Tayyab et al., 2016). The common CP content found in Spring-harvest *A. esculenta* and *S. latissima* is around 10 % DM, which is comparable to the common feed ingredient corn meal (ca. 8 - 9 % DM) (Stévant et al., 2017a). The main amino acid (AA) in *S. latissima* and *A. esculenta* is Glutamic acid (ca. 20 g $kg^{-1}$ DM seaweed) that majorly accounts for the umami taste (Maehre et al., 2014; Sharma et al., 2018). Furthermore, one third of total AA in *A. esculenta* and *S. latissima* was reported to be the essential amino acids, which is not synthesizable by vertebrates, and thus the animals must get them through their diet (Maehre et al., 2014; Sharma et al., 2018).

*Phlorotannins*

Phlorotannins are polyphenolic compounds that can be found only in brown seaweeds (Wang et al., 2009a) and are oligomers or polymers of phloroglucinol units (1,3,5-trihydroxybenzene) with various chemical structures and molecular weights (Steevensz et al., 2012). The phlorotannin content in *A. esculenta* (ca. 3 % DM) appears to be several times higher than in *S. latissima* (ca. 0.7 % DM) (Stévant et al., 2018). Some phlorotannins possess wide range of bioactivity including antioxidant, anti-inflammatory, anticancer that are of interest for the pharmaceutical industry and can potentially have high market value (Sanjeewa et al., 2016). The presence of phlorotannin may influence the bacterial fermentation in silages and in the digestive tract of animals due to its antimicrobial activities (Eom et al., 2012). The phenolic groups in the phlorotannins can bind with the amide groups in proteins and enzymes and cause anti-nutritional activity.

*Iodine and heavy metals*

The iodine content in brown seaweeds is high. Seaweeds can therefore be used as a natural source of dietary iodine. However, high levels of iodine content (2000 -

4600 mg $kg^{-1}$ DM) has been found in *S. latissima* (Biancarosa et al., 2018; Roleda et al., 2019) and it can lead to thyroid dysfunction due to excess iodine intake when consumed as feed or food. Iodine concentrations in *A. esculenta* is of less concern because it is in the range 300 – 1300 mg $kg^{-1}$ DM (Biancarosa et al., 2018; Roleda et al., 2019).

Heavy metals are toxic to animals and humans and therefore the EU has set a maximum level (RML) for heavy metals such as Pb, Cd, Hg, and As in seaweed feed and food products (OJEU L78/16, 2018). The concentration of Hg and Pb is below the RML in seaweeds, but the Cd and As level is of great concern (Biancarosa et al., 2018; Afonso et al., 2021). As is abundant in seawater and can accumulate in seaweeds. The accumulated As is converted to less toxic organic As molecules like arsenosugar by seaweeds, and the organic As often contributes to over 95 % of total As content in seaweeds (Ma et al., 2018). As in seaweeds can be moved using hot water, citric acids, and *Lactobacillus* spp. (Wang et al., 2022). However, it is unclear whether the organic As can mineralize during the storage period of drying, freezing or ensiling. Finally, the Cd content in *A. esculenta* (2.5 mg $kg^{-1}$ DM) and *S. latissima* (0.59 mg $kg^{-1}$ DM) was higher than RML and should be of concern when making feed or food products with these seaweeds (Biancarosa et al., 2018).

## 1.3 Ensiling preservation for brown seaweeds

The objective of ensiling preservation is to stabilize the biomass composition and to prevent the growth of undesirable spoilage microbes through anerobic fermentation (Figure 3). In general, the ensiling process can be divided into four phases: 1) The aerobic phase - after the silo is sealed, the plant cells (respiration), plant enzymes and aerobic bacteria continue their activities contributing to the degradation of plant protein and sugar. The carbon dioxide, water, and heat are the end products of this phase. 2) The lag phase – when the oxygen is depleted, the anaerobic bacteria start to grow. 3) The fermentation phase – the anaerobic bacteria (i.e. lactic acid bacteria (LAB)) ferment sugars into organic acids that decrease silage pH. 4) The stable

phase - the silage acidity and osmotic pressure are sufficient to arrest the microbial growth, and the biomass composition can be preserved if the anerobic environment is maintained (i.e. the silo is sealed). Silages typically reach the stable phase after 2 weeks of ensiling.

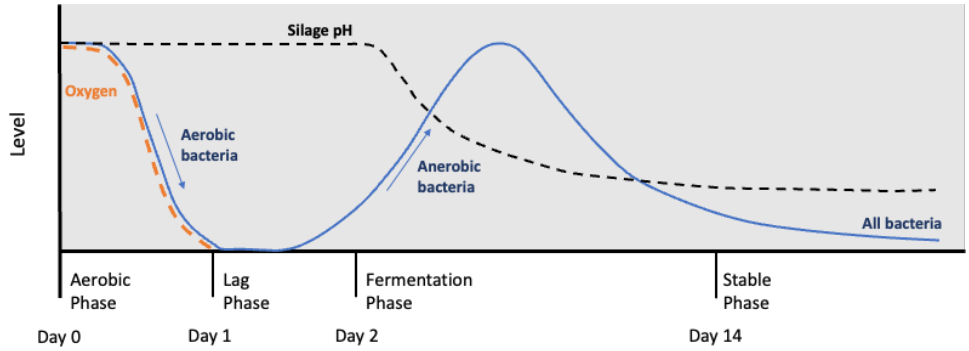


**Figure 3.** The silage fermentation phases. Modified from Van Soest (1994).

The DM content, the amount of fermentable carbohydrate, and the presence of LAB are three of the most important factors to produce good quality silages. If the LAB fails to develop, other bacteria such as enterobacteria and clostridia can take over the silage fermentation and result in high DM losses in the form of carbon dioxide and hydrogen during ensilage (Borreani et al., 2018). Also, the LAB fermentation in the fermentation phase can be limited by low content of fermentable carbohydrate in the biomass.

In the case of land crops (especially corn, grass, and alfalfa), the effects of moisture reduction and silage additives on silage quality have been extensively documented (Borreani et al., 2018; Kung et al., 2018; Muck et al., 2018). Reducing the moisture content to around 30 % DM facilitates silage stability because of the increased osmotic pressure and reduced water activity (McDonald et al., 1991). Moreover, moisture reduction prevents the formation of effluent and the consequent loss of components (Gebrehanna et al., 2014). In terms of silage additives, formic acid is used as a chemical additive to stimulate acidification that preserves the sugar and

improves protein quality by suppressing the growth of Clostridia (e.g., *C. perfringens*) in silages, one of the undesired spoilage bacteria mentioned earlier (Muck et al., 2018). Moreover, although LAB is abundant in land crops, it is not uncommon to use both homo- and hetero- fermentative LAB inoculants (Muck et al., 2018). The use of homofermentative LAB inoculant promotes lactate fermentation during ensiling and some heterofermentative LAB such as *Lactobacillus buchneri* can convert lactate to acetate that improves the aerobic stability after the silage is opened (Muck et al., 2018). Since there is a low or absent amount of epithetic *Lactobacillus* spp. found in seaweed biomass (Black, 1955; Uchida et al., 2004; Herrmann et al., 2015), the use of LAB inoculant can play a critical role in promoting lactate fermentation in seaweed silages. In addition, the high moisture content, low fermentable sugar content in seaweed biomass can lead to insufficient acidification and nutrient losses during ensiling (Herrmann et al., 2015). Therefore, both moisture reduction and silage additives can potentially improve silage fermentation in seaweeds. Nevertheless, optimization of these practices is essential before adopting a particular procedure (Black, 1955; Sandbakken et al., 2018; Novoa-Garrido et al., 2020; Sørensen et al., 2021).

## 1.4 Seaweed as ruminant feeds

The human society has a long history of domesticating and farming ruminant animals i.e., cows, goats, sheep, because of the ability of their digestive organ reticulo-rumen that can convert plant fiber, that is undigestible and with low nutritional value for humans, to high quality protein products such as meat and milk (Ajmone-Marsan et al., 2010). In ruminants, the reticulo-rumen is the largest organ in the digestive tract. The water-filling capacity of the reticulo-rumen in an adult cow was reported to be around 90 L (Tulloh & Hughes, 1965). There is a continuous anerobic fermentation activity in the rumen performed by a group of microorganisms, namely bacteria, protozoa, archaea and fungi, present in large numbers in the ruminal fluid (Van Soest, 1994). These microorganisms attach to the surface of feed particles, produce extracellular enzymes to break down polysaccharides (commonly cellulose and

hemicellulose, structural components of the primary cell wall in green plants) to monomers. These monomers are further fermented to volatile fatty acids (VFA), carbon dioxide, and metabolic hydrogen (H) by the ruminal microorganisms (Van Soest, 1994). Then, the majority of the produced VFA is absorbed through the ruminal wall and serves as the main energy source for the animal. The ruminal microorganisms degrade feed CP to N as well as utilize the available N in the rumen to replicate and thus synthesize the microbial protein. The rumen contraction takes place regularly in healthy adult cows (ca. 1-3 times per min) and plays an important role in mixing the undegraded feed particles with rumen microorganisms and moving the ruminal content containing microorganisms and unfermented feed particles to the lower digestive tract (Tulloh & Hughes, 1965; Van Soest, 1994).

Rumen nutrient degradation is affected by several factors including feed characteristics i.e., chemical composition, particle size, chewing time, and the rumen retention time (Åkerlind et al., 2011). In terms of feed characteristics, feed fiber content influences the rumen retention time, and the rumen retention time is negatively correlated to feed and energy intake due to the limited rumen capacity (Van Soest, 1994). Furthermore, feed carbohydrate components affect rumen degradation efficiency with the highest to lowest as follows: water soluble carbohydrates (WSC) > hemicellulose > cellulose > lignin (Åkerlind et al., 2011). Therefore, the fiber determination analysis using neutral- or acid- detergents was developed for land crops and are routinely performed to estimate the feed's rumen degradation pattern and energy yields. The neutral detergent fiber (NDF) is a parameter for hemicellulose, cellulose, and lignin, which account for most of cell wall components. The acid detergent fiber (ADF) is a parameter for the less degradable cellulose fraction and undegradable lignin. In land crops, a high NDF and low ADF content indicate a high hemicellulose content and often result in a shorter rumen retention time, thus higher feed intake and energy yield.

Previous studies that evaluated seaweed fiber content have reported that the NDF content range 9 – 12 % DM in brown seaweeds (*S. latissima* and *A. esculenta*) and 40 – 50 % DM in red seaweeds (*Porphyra umbilical* and *Palmaria palmata*); the ADF content is less than 10 % DM and the lignin content is very low in both red and brown seaweeds (Tayyab et al., 2016; Novoa-Garrido et al., 2020). The NDF and ADF content of brown seaweeds are low in comparison with forages used in cattle feed (i.e., alfalfa hay, wheat straw or corn silage) that have 40 – 60 % DM NDF content and 10 – 20 % DM ADF content (Soufizadeh et al., 2018). However, as mentioned in section 1.2, the carbohydrate composition of *S. latissima* and *A. esculenta* differ greatly from land crops, thus the interpretation of the fiber determination analysis must be made with caution.

The *in vitro* organic matter digestibility (IVOMD) estimates the content of organic matter that can be degraded by the ruminal microorganisms. The IVOMD of brown seaweeds was reported to be between 30 and 65 % DM which is lower compared to maize silage (ca. 80 % DM) and grass-clover silage (ca. 73 % DM), but comparable to some selected alternative feed of tropical grasses and legumes (ca. 35 – 70 % DM) (Mlay et al., 2006; Tayyab et al., 2016; Kragbæk Damborg et al., 2019).

## 1.5 Methane and seaweed's potential in methane mitigation in ruminants

Methane ($CH_4$) is the second most concerning greenhouse gas (GHG) after $CO_2$, both in terms of its abundance and contribution to the increasing global temperature (Hogan et al., 1991). Due to its molecular structure, methane is known to be 20 - 80 times more powerful in trapping the heat compared to $CO_2$, but it has a shorter lifespan (12- 20 years) than $CO_2$ (over 100 years) (Hogan et al., 1991). Hence, methane emission reduction is likely to have a more instant effect on stabilizing global temperature compared to other GHGs. As mentioned above, the enteric methane produced by ruminants is one of the major concerns in the livestock sectors as it accounts for 80 % of livestock methane emission.

The rumen enteric methane is mainly produced by methanogenic Archaea (hereafter called methanogens), following the chemical equation below:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

In the rumen, carbohydrates are fermented mainly into acetate, propionate, and butyrate, and the hydrogen is produced (see equation below). By producing methane, the methanogens remove the accumulated hydrogen that lower or suppress microbial fermentation pathways through negative feedback mechanisms (Knapp et al., 2014).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2 \text{ acetate } (CH_3COOH) + 2CO_2 + 4H_2$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2 \text{ propionate } (CH_3CH_2COOH) + 2H_2O$$

$$C_6H_{12}O_6. \rightarrow \text{ butyrate } (CH_3CH_2CH_2COOH) + 2CO_2 + 2H_2$$

However, when carbohydrates are fermented to propionate, there will be a net hydrogen use, and thus lower the enteric methane (Pereira et al., 2022). This can be achieved by replacing the fibrous ingredients (e.g., forage) with ingredients rich in starch (e.g., concentrates) in the diet (Benchaar et al., 2001; Haque, 2018) and supplementation of key microorganisms in the propionate fermentation pathway (Pereira et al., 2022). Another approach is to use specific substance aiming to directly reduce the abundance of methanogens Archaea and protozoa where the methanogens are attached to (Knapp et al., 2014).

Some red seaweed species such as *Asparagopsis* spp. possess halogenated compounds such as bromoform which can directly reduce methanogens and is reported to efficiently decrease methane production (by over 99 % with 2 % organic matter dosage rate) in *in vitro* rumen fermentation studies (Machado et al., 2018). However, the development of large-scale cultivation of *Asparagopsis* spp. has just begun, and there is a lack of understanding in the reproduction method, making it challenge to produce enough for livestock application (Wright et al., 2022). In addition, bromoform is highly toxic to animals and humans and there are also environmental

concerns since it may enter the food web via the feed and get excreted to the environment (Glasson et al., 2022).

Brown seaweed species also have methane mitigation potential. However, the methane mitigation efficiency is low compared to *Asparagopsis* spp., likely due to the different active compounds (de la Moneda et al., 2019; Pandey et al., 2022). The active components in brown seaweeds are the polyphenolic compound phlorotannins that can potentially inhibit methanogens proliferation and the sulfated polysaccharides that may alter the rumen fermentation characteristics (Min et al., 2021). Considering brown seaweed's fast growth rate and low content of bioactive compounds that are of concern to human and animal health, the ideal strategy will be to find very efficient brown seaweeds for enteric methane production mitigation.

## 1.6 Seaweeds as prebiotics in monogastric animals

The latest definition of prebiotic from FAO is "*A nonviable food component that confers a health benefit on the host associated with modulation of the microbiota*" (Pineiro, 2008 #26). Bioactive extracts or whole biomass of brown seaweed has been found to modulate the gut microbiota in monogastric animals (Charoensiddhi et al., 2017; Cherry et al., 2019; You et al., 2020). The brown seaweed polysaccharides (alginate, laminarin, and fucoidan) are fermentable by bacteria in large intestine and can promote the growth of beneficial bacteria (Charoensiddhi et al., 2017; Cherry et al., 2019; You et al., 2020). The non-digestible but fermentable fraction of polysaccharides is defined as dietary fiber and accounted for 30 – 75 % DM in brown seaweeds (Lahaye, 1991).

Beneficial bacteria including *Bifidobacterium* spp. and *Lactobacillus* spp. that are known producers of lactate and short chain fatty acids (SCFA) including acetate, propionate, and butyrate can impart anti-inflammatory, anti-bacterial, and other beneficial effects to the health of monogastric animals (Tan et al., 2014). In the large intestine of monogastric animals, lactate suppresses the growth of pathogens by

reducing the intestine pH, the acetate and propionate regulate the metabolic activity, and butyrate maintains the gut barrier integrity and provides energy to the intestinal epithelial cells (Tan et al., 2014).

Phlorotannins in brown seaweeds also have prebiotic potential and possess antibacterial activity against some of the known pathogenic bacteria such as *Salmonella* and *E. coli* in monogastric animals (Eom et al., 2012). The antibacterial action is through deconstructing bacteria's cell membrane and binding with specific bacterial protein that caused bacteria to lysis as described in Shannon & Abu-Ghannam (2016). Interestingly, phlorotannins that bind to dietary fiber are released when the dietary fiber is fermented in the large bowel of monogastric animals, and these compounds are also called the macromolecular antioxidants (Sanz-Pintos et al., 2017).

## 1.7 Objectives

*Knowledge gap*

Cultivation of the popular seaweed species such as *S. latissima* and *A. esculenta* can ensure the availability of large volumes of their biomass in the northern hemisphere. The use of the whole thalli (without further extraction processes) as feed ingredients is an economically viable option. However, optimal ensiling conditions must be established for seaweeds. Furthermore, the seaweed silage's chemical composition nutrient digestibility, bioactivity, and safety must be investigated if they have to be incorporated in feed formulations.

*Objectives*

Therefore, the objective of this PhD project was to develop an optimized ensiling treatment for cultivated *S. latissima* and *A. esculenta*, and explore their potential to be used as feed ingredients. To achieve this the following subgoals were defined:

- Study the effect of moisture reduction and silage additives on ensiling seaweed (Paper I), and the replicability of the observed effects (Paper I & II).
- Study seaweed silage's nutrient degradability and digestibility in dairy cows (Paper II).
- Study seaweed silage's impact on rumen bacterial composition (Paper II).
- Study seaweed silage's impact on fecal bacterial composition and short chain fatty acids in a monogastric (Paper III).

*Hypothesis*

We hypothesized that the silage additives and moisture reduction can improve seaweed silage fermentation, and ensiling can improve its rumen nutrient degradability. We also hypothesized that the bioactive components in ensiled seaweed can modulate bacteria fermentation in the colon of monogastric animals through their prebiotic effects.

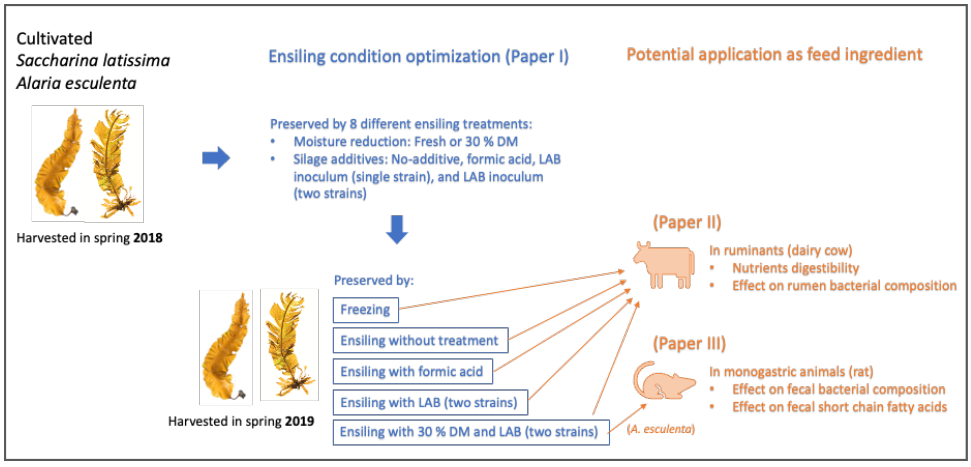


**Figure 4.** Overview of the study design in the thesis

## 1.8 Methodology

*Seaweed biomass*

The future growth of seaweed market depends on the use of cultivated biomass that is produced sustainability. Brown seaweed *S. latissima* was chosen because it is the most cultivated species in Norway and other western countries. *A. esculenta* was chosen because it is one of the seaweed species of interest for cultivation and its production is the second highest after *S. latissima* in Norway.

The parental material was collected locally at the same site for cultivation (N68, E15) and sent to Hortimare AS for preparing seeding material, in this way avoiding the spreading of non-native genetic material. The seaweed biomass for the experiments was harvested in 2018 and 2019. Aiming to minimize the location- and season- based variations in chemical composition, biomass was harvested from the same location, Austre Vågan, in the Lofoten islands in northern Norway. In both years, the seeded ropes were deployed in the sea in October and harvested in June (8 months cultivation).

The harvested biomass was rinsed on the vessel with seawater, packed in Styrofoam boxes, and transferred cold (4 - 7 °C) to the research station with in 24 hours. To mitigate potential degradation, the seaweed biomass was transferred to large water tanks (600 L) with running seawater set to $7 \pm 1$ °C and continued aeration until further processing.

*Ensiling treatments and the use of vacuumed bag as lab silo*

Before ensiling, the seaweed biomass was rinsed to remove impurities and attached organisms. The biomass was washed sequentially in three water baths with decreasing salinity 100 % seawater, 70 % seawater, and fresh water following a procedure that has been standardized in earlier investigations with the aim to reduce the effect of osmotic shock and the consequent losses of valuable components as well as to reduce the ash content in the seaweed biomass (Novoa-Garrido et al., 2020). After rinsing, the seaweed was chopped into 1 – 4 $cm^2$ pieces, using a commercial

butcher's cutter, for easy homogenization and to increase the surface area for the additives to act during ensiling.

The bacterial inoculants (*Lactobacillus plantarum* and *Lactobacillus fermentum*) and chemical additive (formic acid) were chosen because they are the most common silage additives and are widely available for future applications. Non-commercial additives were used in these studies. For moisture reduction, a water content of up to 30 % DM was targeted, as this is the highest limit recommended for the production of land crop silages (Gebrehanna et al., 2014). An oven with fan was used for drying, and the drying temperature was set at 37 °C to minimize changes in chemical composition caused by heating.

In laboratory conditions, vacuumed bags were often applied as lab silos because it can remove the air to create the anaerobic environment, and it allows the emission of $CO_2$ and $H_2$ during silage fermentation. The ensiling of the target biomass is commonly optimized using lab silos, prior to upscaling trials where a large volume of biomass is required. The silage pH, fermentation products (i.e., lactate, acetate, butyrate, and branched acids such as iso-butyrate and iso-valerate), $NH_3$, and ethanol are generally measured to investigate silage quality. In **paper I**, we tested 8 different ensiling treatments using 1 kg silo bags. In **paper** II, three of these ensiling treatments were selected based on their silage quality results and for easiness in handling including the wet biomass with LAB inoculants (two strains), the wet biomass with formic acid, and the prewilted biomass with LAB inoculants (two strains), using the 2 kg lab silos.

### 1.8.1 Nutrient accessibility for dairy cows

Seaweed biomass preserved by 1) freezing 2) ensiling without treatment 3) the selected ensiling treatments (mentioned above) was subjected to feed evaluation for ruminants. Here, the standardized methods were applied aiming to compare the results with common- and alternative- forages.

The estimation of feed nutritional value (i.e., protein and energy) is important as feed is the major expense in dairy farming. The complex dynamics of rumen makes it problematic to track the individual feed ingredients' nutrient- degradation, transformation, and absorption in the animal-based trials (*in vivo*) (Tamminga & Chen, 2000). Moreover, the animal's physiological states (i.e., growing, pregnancy, lactating, or maintenance) can affect digestion, leading to different outcomes in feed nutritional estimation. In addition to that, the *in vivo* trials are expensive and require a large quantity of the test feed.

Therefore, the *in vitro* and *in situ* (nylon bags, *in sacco*) techniques are often applied in the nutritional evaluation of feed ingredients. These techniques are important for feed nutritional estimation in ruminants because they allow measurements under standardized conditions, unaffected by digestive activities that occur in animals. In this way, the results are relatively consistent, and they are comparable in between studies which follow the same practice (i.e., the Nordic feed evaluation system).

*In vitro organic matter digestibility (IVOMD)*

The IVOMD analysis is performed by incubating the feed samples with ruminal fluids (RF) in closed flasks/vials. The feed's organic matter digestibility in the rumen can be estimated in a reproducible manner if the RF is collected from cows in a certain physiological state and fed with the corresponding diet. In present study, we used RF collected from three non-lactating (called "dry") cows fed a standardized ration at maintenance level (diet details stated in **Paper II**).

*In situ rumen degradability*

The *in situ* techniques, as described in Åkerlind et al. (2011), were used to determine rumen degradation of nutrients, aiming to make the results from different experiments comparable by standardizing the important factors. In principle, the rumen DM and CP degradation estimation is performed using rumen fistulated cows

and the Dacron bags. The fistulated cow is not lactating and fed at maintenance level with a diet containing forage and concentrate (see **Paper II**), aiming for a normal rumen fermentation activity and motility. A sample of the feed ingredient (i.e., 1 g) is sealed inside a Dacron bag with pores (38 µm) that allow the microorganisms to enter and degrade the feed particles. Also, the pores allow the fermentation products to be washed out. It is assumed that the small amount of feed ingredient does not influence the fermentation activity and motility in the rumen. The bags with feed samples are incubated inside rumen, and the residual DM and N content (CP = N*6.25) are measured at 8 different time intervals (0, 2, 4, 8, 16, 24, 48, 96 h). The results from these measurements are used in the established equations to calculate the necessary parameters to determine the effective DM- and CP- degradability as described in Equation 1 shown below (Hvelplund & Weisbjerg, 2000; Åkerlind et al., 2011). The indigestible NDF (iNDF) content in feed ingredient is measured by incubating the Dacron bags in the rumen for 288 h and measuring the residual NDF content.

Effective DM- and CP- degradability = a + b [c/c+k] ----------(Equation 1)

*a* = immediately degradable (soluble) fraction

*b* = insoluble but rumen degradable fraction

*c* = the fractional rate of degradation of fraction *b* ($h^{-1}$)

k = fractional outflow rate from the rumen ($h^{-1}$)

*In situ total tract digestibility*

In addition to the ruminal degradability, the total tract digestibility (TT) of DM and CP was estimated, using smaller Dacron nylon bags with smaller pores (12 µm) as mobile bags, and both the rumen fistulated cows and the duodenum cannulated cows (Hvelplund & Weisbjerg, 2000; Åkerlind et al., 2011). Consecutively, the mobile bag is incubated in the rumen for 16 h, thereafter treated with *in vitro* gastric digestion using pepsin-HCl, then inserted in the duodenum, and later these bags were recovered from

the feces. The residual DM and CP content in the mobile bag is used to determine the total tract digestibility of DM and CP.

### 1.8.2 Microbiota studies

The animal model is essential to investigate the interaction between diet and gut microbiota due to the complex nature of gut microbiota. Laboratory rodents are the main domesticated mammalian species for research purpose, while the rat model is used for human biomedical research as well as to reveal the mechanisms of bioactive ingredients for monogastric animals (Tomas et al., 2012).

The interspecies similarity of gut bacterial composition between rodents and the targeted monogastric (i.e., poultry and swine) is a critical factor when choosing animal models, as the modulation of gut microbiota is the interested mechanism for the usage of seaweeds as prebiotic. Ideally, the animal experiment using the target animal species is preferred as it can provide species-specific outcomes. However, **Paper III** is a pilot study needed for finding indications for further applications than can later be tested in *in vivo* trials using the targeted animal species. The rat is chosen over mice, because of the larger quantity of biological sample available, especially a larger fecal pellet that can be subjected to both bacterial composition analysis and SCFA content analysis.

*Sprague-Dawley (SD) rat*

The SD rat, one of the most extensively used rat strains, is a hybrid albino outbred line characterized with calm temperament and easy handling. The specific pathogen free (SPF) animals, which contain an undefined microbiota but are free of specific microorganisms and parasites, are recommended for biomedical research by the Federation of Laboratory Animal Science Associations. The SPF SD rat purchased from the same breeding facility are expected to possess close phenotype (i.e., weight, feed intake, water intake etc.), and the inter-individual variations in gut microbiota are expected to be minimized by mixing the bedding material of each cage during their

acclimatization period, as practiced in the present project (Miyoshi et al., 2018). Although there is genetic variation in the outbred rodent line, the gut microbiota has been reported to be more responsive towards diet shifts, particularly towards the change of fat and carbohydrate content of the diet, than to the genotype of the rodent lines (David et al., 2014; Carmody et al., 2015). Moreover, the change of gut microbiota occurred 3 days after receiving a treatment diet, and the reproducibility of such change was confirmed by continuously switching the diet between control diet and treatment diet (Carmody et al., 2015).

*Bowel inflammation induced by dextran sodium sulfate (DSS)*

Bowel inflammation (colitis) caused by virus and bacterial infections is a major reason for loss in monogastric animal production because it often leads to diarrhea, poor growth rate, and high mortality. DSS is a water-soluble chemical that is toxic to gut epithelial cells and compromises the gut barrier function when consumed by mammals (Solomon et al., 2010). By providing DSS in the rodent's drinking water, colitis (inflammation of the inner lining of the colon) can be induced at different degrees depending on the DSS concentration administered (2 - 5 %), the duration of each DSS treatment (more than 3 days), and the number of treatments, with established protocols (Kim et al., 2012; Wirtz et al., 2017). The DSS induced colitis model has the advantages of simple operation without the need of force-feeding or anesthesia and the colitis is reproducible even at low doses (Solomon et al., 2010; Samanta et al., 2012). Therefore, the DSS-induced colitis rodent model has been widely used to investigate the preventive and therapeutical measures for bowel inflammation, including the use of prebiotic as feed additives (Guarner, 2007; Ferenczi et al., 2016; Silveira et al., 2017; Li et al., 2021). This PhD project investigated the preventive effect of dietary seaweed inclusion in mild colitis induced by a low concentration of DSS (2 – 3 % in drinking water).

### 1.8.3 Microbiome composition analysis

Two types of samples were subjected to the microbiome composition analysis: the ruminal fluids after anaerobic incubation (48 h) with different seaweed samples (**Paper II**), and the feces of rats under different treatments for 25 days (**Paper III**).

The microbiome composition analysis was performed using the amplicon sequencing of the Prokaryotic 16S ribosomal RNA (16S rRNA) gene (V3-V4 region), following the Illumina protocols (Illumina, 2013). The 16S rRNA gene is the DNA sequence corresponding to the 16S rRNA and it exists in the genome of all bacteria. The length of 16S rRNA is approximately 1500 base pair (bp) with 9 variable regions (V1-V9) separated by the highly conserved regions (Figure 5). The sequence in the variable regions differs in each bacterium, and thus, can be used as barcode to identify the phylogenetic classification of bacteria. Using the next generation sequencing (NGS) technology to amplify the 16S rRNA allows us to study the unculturable bacteria which otherwise cannot be studied. However, the length of whole 16S rRNA gene exceeds the sequencing limit of 600 bp in the common sequencing platforms that have high sequencing reads output and high accuracy with the incorrect base call probability being 1 in 1000 base call (e.g., Illumina MiSeq, Illumina NovaSeq). Hence, the different regions are selected for the 16S rRNA amplicon sequencing, and the V3-V4 (ca. 460 bp) and the V4 (254 bp) are the most common region selected.

We chose this method because of its affordable cost, available bioinformatic tool for downstream analysis, and large database for taxonomic identification (e.g., Greengenes, Silva, EzBioCloud). The outcomes provide valuable information on the change of microbial composition in the biological samples caused by seaweed. However, there are certain limitations that must be kept in mind when using this method. First, it does not provide the species and strain information as many bacteria can only be classified to the genus level. Second, it does not provide the information on metabolic pathways. Third, the datasets collected from the 16S rRNA amplicon sequencing are compositional (Gloor et al., 2017), and this should be acknowledged

when choosing the bioinformatic tools especially for the differential abundance analysis. Also, unlike the plate-counting method, the molecular methods cannot determine the amount of living bacteria at the time of sampling.

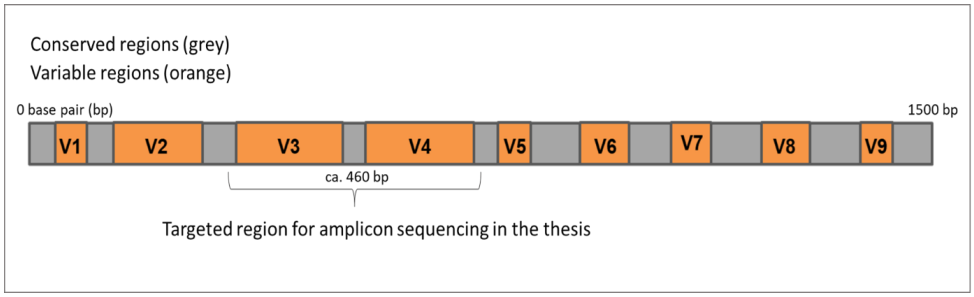


**Figure 5.** The structure of 16S ribosomal RNA gene

# 2. Summary of papers: Main findings

**Paper I: Improving fermentation of *Saccharina latissima* and *Alaria esculenta* silages with additives for preserving biomass and antioxidants.**

*J Appl Phycol 34, 625-636 (2022)*

Rapid deterioration of harvested macroalgal biomass is a challenge for macroalgal industry and can be overcome with the inexpensive ensiling preservation. To improve silage quality, *Saccharina latissima* and *Alaria esculenta* biomass was subjected to ensiling conditions following a 2 × 4 factorial design, with 2 prewilting treatments (no-prewilting / prewilted to 300 g DM $kg^{-1}$ fresh biomass) and 4 additive treatments (no additive, formic acid, single and two species of *Lactobacillus* inoculant), and ensiled for 3 or 12 months at 15 °C. Acetate was the main fermentation product in these seaweed silages. Prewilting reduced the acetate, mannitol, and $NH_3$ content in silages. In *S. latissima* silages without additives, prewilting led to less acidification (pH = 5.7). Also, prewilting caused protein and phlorotannin degradation. When treated with formic acid, the silage pH was below 4 regardless of the moisture content of the biomass. The use of *Lactobacillus* spp. inoculants was essential for lactate production in seaweed silages, and it significantly lowered silage pH in *S. latissima* and prewilted *A. esculenta* compared to silages with no additives. A high level of the phlorotannin content was preserved (> 90%) in the 3-month *A. esculenta* silages without prewilting. However, major reduction of antioxidant activity was observed in 12-month silages of both seaweed species. In conclusion, ensiling is a viable method for preserving Alaria and Saccharina biomass. Prewilting restricted silage fermentation, and both formic acid and bacterial additives facilitated silage acidification. The preservation of antioxidant activity in silages was not improved by either prewilting or additives treatment.

**Paper II: Feed characteristics and potential effects on ruminal bacteria of ensiled *Saccharina latissima* and *Alaria esculenta* for dairy cows.**

*Manuscript*

Seaweed silage has potential to be an alternative feed ingredient for dairy cows. This study aims to investigate seaweed and seaweed silages´ nutrient digestibility and their impact on the ruminal bacterial composition. The cultivated *S. latissima* and *A. esculenta* were preserved by freezing at - 40 °C or ensiling in four ensiling treatments (16 °C, 3 months). The nutrient digestibility was estimated using standard feed evaluation procedures. The bacterial composition in ruminal fluid after 48 h *in vitro* anaerobic incubation with seaweeds and common feedstuffs was analyzed using 16S rRNA amplicon sequencing (V3-V4) and qPCR. The results suggest that ensiled *S. latissima* can be included into the ruminant diet as an alternative forage-like ingredient with the potential of methane mitigation. The rumen DM degradability of *S. latissima* was comparable to common perennial and corn forage, however, the total tract CP digestibility of *S. latissima* (460 g $kg^{-1}$ CP) was lower than common forages (620 – 820 g $kg^{-1}$ CP), and was not improved by ensiling. There was a lack of insoluble but rumen degradable CP in *A. esculenta*, making it unsuitable as nutrient ingredients for dairy cows. The ruminal bacterial composition changed according to the different feed substrate. The dominant bacterial taxa when incubated with *S. latissima* belonged to the genus Prevotella (relative abundance: 79 – 93 %), known for its ability to degrade polysaccharides in various ecosystems, and the fibrolytic bacteria *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* were > 2.5 Log2FoldChange higher when incubating with *S. latissima* than with *A. esculenta*. These bacterial taxa may play an important role in the *in vitro* organic matter digestibility, noted as 2 times higher in *S. latissima* compared to *A. esculenta*. Potential methane mitigation was observed through the qPCR results, with a significantly lower gene copies of Archaea 16S rRNA and methyl coenzyme-M reductase subunit A genes when the ruminal fluid was incubated with seaweeds.

**Paper III: The effect of *Lactobacillus* spp. ensiled *Alaria esculenta* on fecal bacterial composition and metabolites in healthy rats and rats with DSS-induced colitis.**

*Manuscript*

*Alaria esculenta* is an edible brown seaweed, and it is increasingly included as wholefood in the diet due to its flavor and potential prebiotic benefits. On the other hand, the seaweed's high ash and undigestible fiber content is a concern when consumed as food. The present study aimed to investigate the effect of dietary inclusion of *A. esculenta* silages at 4 % (w/w) on gut health in rats with or without DSS-induced-colitis (2-3 % of DSS in drinking water, 10 days) as indicated by the fecal bacterial composition and butyrate content. In total 32 rats were randomly assigned to one of the four treatment groups (n=8): control diet without or with DSS, or seaweed diet without or with DSS for the whole experimental period of 25 days. The fecal bacterial composition was profiled using 16S rRNA amplicon sequencing (V3-V4 regions), and the fecal SCFA content was analyzed using gas chromatography. Seaweed supplementation neither aggravates the DSS-induced-colitis nor show preventive effect indicated by the disease-associated parameters. For fecal bacterial composition, seaweed supplementation enriched Bacteroidota phylum in all rats. In healthy rats, seaweed supplementation enriched the *Muribaculum* spp., positively correlated to the fecal butyrate content, and reduced the *Campylobacter* spp. and *Butyricimonas* spp. In DSS-induced-colitis rats, seaweed supplementation reduced four bowel-inflammation-associated genera (Elsenbergiella, Parasutterella, Erysipelatoclostridium, and Frisingicoccus). However, seaweed supplementation reduced the presence of known SCFA producers including *Bifidobacterium* spp. and *Lactobacillus* spp., and reduced fecal lactate and acetate content, but not the fecal butyrate content. Although seaweed supplementation does not lead to major phenotypic changes, but it leads to changes in microbiota composition associated with the fecal SCFA content. Further study is required to investigate the contribution of the observed microbiological change to the host health.

# 3. Discussion

Seaweeds as animal feed ingredients is gaining interests among food industries and researchers because they not only improve the growth and health of farmed animals but also help in reducing methane emissions and provide food for the fast-growing population. This study aimed to develop an optimized ensiling treatment for the cultivated brown seaweed species *S. latissima* and *A. esculenta*, and to explore their potential to be used as feed ingredient. More specifically, the use of seaweed silage as forage for ruminants (i.e. cows) and as prebiotic feed additive for monogastrics (i.e. poultry and swine).

## 3.1 Silage preservation

Preserving as silage is a common practice that is adopted for land crops. The known constraints in making seaweed silages include its high moisture and ash content, low fermentable carbohydrate content, and low epiphytic LAB that was reported to be less than $10^3$ CFU $g^{-1}$ wet biomass by Uchida et al. (2004). These constraints contribute to inadequate acidification as well as nutrient and effluent losses during ensiling (McDonald et al., 1991; Herrmann et al., 2015). Similar challenges have also been encountered when making other types of crop silages despite the LAB is abundant in land crops. Silage treatments including additives and moisture reduction have been developed as common practices to improve silage quality in land crops (Muck et al., 2018; Kung et al., 2018; Borreani et al., 2018). Many of these treatments were tested on seaweeds in the last couple of decades, and some of the most tested treatments are acid additives (Black, 1955; Sandbakken et al., 2018; Novoa-Garrido et al., 2020; Gallagher et al., 2021), LAB inoculum (Cabrita et al., 2017; Sørensen et al., 2021), and moisture reduction by oven-drying or pressing (Novoa-Garrido et al., 2020; Gallagher et al., 2021). However, it is challenging to determine the role of these silage treatments, because of the different and sometimes contrasting results reported from previous studies, likely caused by the various ensiling conditions and differences in seaweed

species. **Paper I** is the first study, within the author's knowledge, that has tested the effect of different combinations of the above-mentioned ensiling treatments simultaneously on seaweed silages. In this way, the optimum silage treatment conditions for cultivated *S. latissima* and *A. esculenta* could be determined. As expected, our results showed insufficient acidification (pH > 4.5) in seaweed silages without additives (Figure 6). Moreover, the concentrations of propionate, butyrate and $NH_3$ (grouped into "Others" in Figure 6) were the highest in silages without any treatment, indicating a higher risk of spoilage.

Ensiling with 0.4 % (v/w) formic acid (FA) facilitates silage acidification to around pH 3.6 in both wet- and prewilted- seaweeds (Figure 6), and this is in accordance with what is known from ensiling land crops (Snyman & Joubert, 1996), and with what has been reported when ensiling wild-harvested *S. latissima* (Novoa-Garrido et al., 2020). The same outcome was confirmed in silages produced in the following year (**Paper II**). Therefore, the observed effects of FA on silage can be considered constant and repeatable in preserving seaweed biomass. The addition of FA restricts fermentation activity in silages as indicated by a lowered concentration of total fermentation product. This may preserve more nutrients during ensiling.

The main fermentation product in seaweed silages is acetate which is a weaker acid than lactate. Ensiling with LAB inoculum are essential to promote lactate fermentation, and in this way, facilitate sufficient acidification in seaweed silages (Figure 6). In **Paper I**, LAB inoculum made of both single strain (*L. plantarum*) and two strains (*L. plantarum* and *L. fermentum*) were tested. In general, *L. plantarum* follows an homofermentative pathway with lactate as main product, whereas *L. fermentum* follows a heterofermentative pathway with lactate, acetate, and potentially ethanol as main products. In grass silages ensiled with *L. plantarum*, a numerically higher lactate content was reported compared to ensiled with *L. fermentum* (Jalč et al., 2009). In seaweed silages, similar lactate content was found in silages with one or two LAB,

indicating that the combined application did not further facilitate lactate production (Figure 6).

In addition to FA, prewilting seaweed biomass to ca. 30 % DM also restricts fermentation activity as indicated by a lower content of total fermentation products in silages (Figure 6). In this way, nutrients are better preserved due to a lower bacterial activity. However, the acetate content of prewilted silages was below 1 %, which may increase the risk spoilage by secondary fermentation once the silage is open (Danner et al., 2003). Also, the silage pH of *S. latissima* was above 5 when ensiled with prewilted biomass without additive, increasing the risk of spoilage caused by unfavorable bacteria. Drip (pigmented) loss from seaweed during the prewilting process may explain the reduced phlorotannin content in the prewilted ensiled seaweed (**Paper I**). Moreover, protein degradation occurred only in the prewilted silages as indicated by the detectable content of Iso-butyrate and Iso-valerate (**Paper I**).

Based on the observations in **Paper I**, it was decided to continue with the use of FA and LAB additives on the wet seaweed biomass, and the treatment groups were termed SFA and SLAB, respectively in **Paper II**. Yet another treatment was included – prewilted biomass with LAB inoculum – to ensure silage acidification, due to its potential advantage in preserving more nutrients as well as its practicality in real farm operations due to the lower water content (i.e. easier for packing and transporting); this treatment group was termed SLABp in **Paper II**.

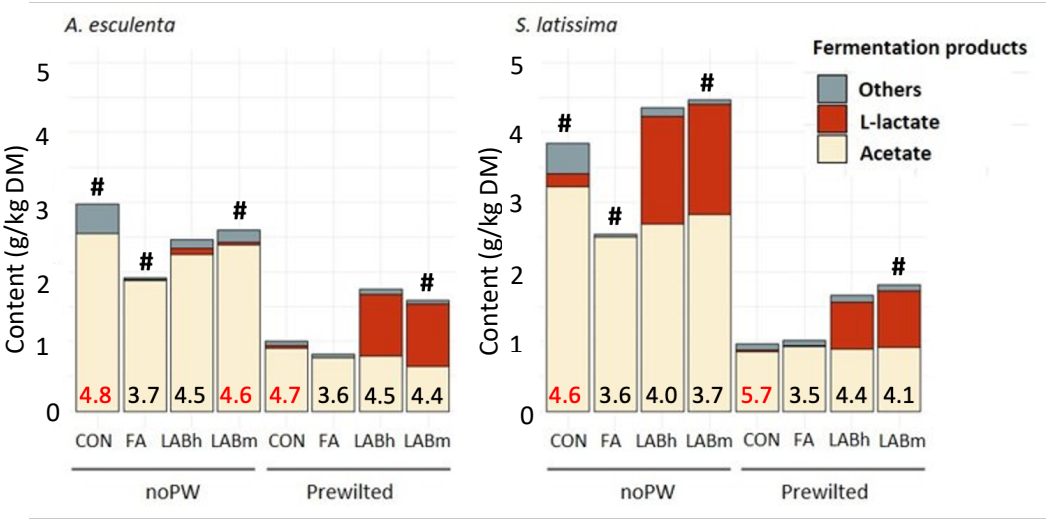


**Figure 6.** The fermentation products and pH value after 3 months ensiling of *A. esculenta* and *S. latissima* (16 °C, dark room). The numbers on the bar graphs are pH, and red font indicates a pH higher than 4.5 as recommended in crop silages. CON: ensiled without additive, FA: ensiled with 0.4 % (v/w) formic acid, LABh ensiled with 5 x $10^9$ CFU *L. plantarum* $kg^{-1}$ wet seaweed, LABm ensiled with 2.5 x $10^9$ CFU L. plantarum and 2.5 x $10^9$ CFU *L. fermentum* $kg^{-1}$ wet seaweed. noPW: ensiled as wet seaweed (DM: ca. 8-12 %), Prewilted: ensiled as ca. 30 % DM. Fermentation products (Others): Sum of propionate, butyrate, caproate, valerate, iso-butyrate, iso-valerate and $NH_3$. #: treatments selected for testing in Paper II.

## 3.2 Application for ruminant feed

Feeding seaweeds to ruminants (i.e. goats, sheep, and cattle) is a documented practice in coastal areas (Evans & Critchley, 2014), including northern Norway (personal communication). However, the characteristics of seaweed as feed ingredients have not been investigated thoroughly. *In situ* studies on the degradability of seaweed in rumen have been performed earlier using wild-harvested biomass of brown seaweed species such as *L. digitata*, *A. esculenta*, and *Pelvetia canaliculata* (Tayyab et al., 2016; Gaillard et al., 2018). In **Paper II**, cultivated *S. latissima* and *A. esculenta* were preserved by freezing at - 40 °C (Frozen) or ensiling with no additives (as control, SCON) and ensiling with three selected ensiling treatments (SFA, SLAB, and SLABp; **Paper I**). Chemical composition, and most importantly, their rumen degradability and total tract digestibility in dairy cows were determined employing an

*in situ* technique. Silage fermentation pattern in **Paper II** was in accordance with **Paper I**: Acetate is the main fermentation product, LAB is required for lactate fermentation, and the use of FA can bring the silage pH below 4. In addition, compared to the freezing preservation, ensiling leads to DM loss. The average DM content in silages (SCON, SFA, SLAB) was 17% lower in *S. latissima* and 8 % lower in *A. esculenta* when compared to the frozen biomass. The DM loss is likely due to the silage fermentation of easily fermentable carbohydrates and the evaporation of volatile fermentation products, as indicated by the lowered C content in silages (**Paper II**). Although there was a loss in DM, ensiling with formic acid (SFA) had the highest organic matter digestibility (IVOMD) and lowest indigestible fiber (iNDF) for both seaweed species, likely due to the hydrolysis of the biomass during ensiling.

The ash, C, and N content were similar between the two seaweed species, and between the different preservation treatments. The ash content was high as expected (Ash: 235 – 260 g $kg^{-1}$ DM) and it was approximately three times higher than grass- or grass-clover- silages (Ash: 80 g $kg^{-1}$ DM) (Jalč et al., 2009; Kragbæk Damborg et al., 2019). The C content ranged 330 – 360 g $kg^{-1}$ DM in both seaweed species (**Paper II**), and the fiber analysis showed a high NDF content of 681 g $kg^{-1}$ DM in frozen *S. latissima* (unpublished data), and a low NDF content of 385 g $kg^{-1}$ DM in frozen *A. esculenta* (de Evan et al., 2022). Furthermore, the *in situ* digestibility analysis showed that the indigestible fiber content (iNDF) was two times higher in *A. esculenta* (165 – 191 g $kg^{-1}$ DM) than in *S. latissima* (79 – 98 g $kg^{-1}$ DM) (**Paper II)** with a comparable iNDF level to grass- or grass-clover- silages (87 g $kg^{-1}$ DM) (Jalč et al., 2009; Kragbæk Damborg et al., 2019). Accordingly, the IVOMD was about two times higher in *S. latissima* compared to *A. esculenta* (**Paper II**). Previous studies have shown that the microbial community in rumen can be affected by the total phenolic content (TPC) in brown seaweeds (Wang et al., 2009b; Lee et al., 2019). Consequently, this may lead to the above-mentioned difference in digestibility, as the TPC content in frozen *A. esculenta* was 2.5 to 5 times higher than in *S. latissima* (**Paper I** and unpublished results in **Paper II**). In addition, ensiling degraded 50 % of TPC and likely contribute to the 1.4x - 1.7x higher IVOMD in

*A. esculenta* silages than in their frozen counterpart (**Paper II**). As for wild *A. esculenta*, the TPC is higher in biomass collected in autumn than in spring, and that might contribute to the reported lower IVOMD in feed containing 20 % of autumn collected *A. esculenta* than those collected in spring (Roleda et al., 2019; Pandey et al., 2022).

To gain more insights on their varied level in digestibility, we analyzed the bacterial composition in ruminal fluids after 48 h incubation with seaweed samples and conventional feedstuffs including blank and whole crop silages of maize, wheat, and barley. The result showed a very different bacterial composition in the incubated ruminal fluids (IRF) of No-seaweed group and the seaweed groups – *A. esculenta* and *S. latissima*. In *S. latissima*, the IRF was dominated by *Prevotella* spp. with a relative abundance of 80 – 90 % of the community. Interestingly, the *Prevotella* spp. is known for its ability to degrade polysaccharides in various types of ecosystems, including in the rumen of seaweed-fed sheep (Williams et al., 2013; Accetto & Avguštin, 2015). Moreover, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, the documented fibrolytic rumen bacteria, had a higher relative abundance (> 2 Log2FoldChange, LFC) in the *S. latissima* IRF than in the *A. esculenta* IRF. The *Prevotella* spp. and fibrolytic bacteria likely contributed to the substantially higher digestibility of *S. latissima* than *A. esculenta*. On the other hand, the community of dominant bacteria in *A. esculenta* IRF varied more with the genera Pseudobutyrivibrio, Pseudomonas, and an unclassified_Pseudomonadaceae, and the relative abundance of *Prevotella* spp. in *A. esculenta* IRF (10 - 30 %) was low comparing to that in *S. latissima* IRF (**Paper II**).

The CP content and CP digestibility are important when determining the potential of seaweeds as alternative ruminant feed. The N content ranged 17 – 18.5 g $kg^{-1}$ DM in both seaweed species, equivalent to the CP (N x 6.25) content of average 110.8 ± 3.1 g $kg^{-1}$ DM (**Paper II**). The CP content is higher than conventional maize silages (CP: 60 – 80 g $kg^{-1}$ DM) and comparable to some primary growth grass silages (CP: 137 ± 18.5 g $kg^{-1}$ DM), but ca. 30 – 50 % lower than conventional grass-clover or clover silages (Krizsan & Huhtanen, 2013; Kragbæk Damborg et al., 2019). Moreover, the CP level in

**Paper II** was comparable to the Spring harvest wild *S. latissima* and *A. esculenta* collected in northern Norway (Pandey et al., 2022). On the other hand, despite the same harvest season in Spring, the reported CP content of *S. latissima* varied from 81 g $kg^{-1}$ DM reported by Tibbetts et al. (2016) to 152 g $kg^{-1}$ DM reported by Samarasinghe et al. (2021). As mentioned in section 1.2, the variation can be caused by factors including the different cultivation sites, and additionally by the different analytical approach as well as the different N to CP convention factors (Angell et al., 2016; Gaillard et al., 2018).

The CP digestibility varied greatly between the two seaweed species. In *S. latissima*, rumen degradation profile showed a continuous degradation during rumen incubation (Figure 7), and can supply high fraction of insoluble but rumen degradable CP (CP*b*: 640 – 884 g $kg^{-1}$ CP). The CP in conventional forage is commonly degraded in the first 48 h of rumen incubation (Hvelplund & Weisbjerg, 2000). However, the CP in *S. latissima* degraded slowly with an average of 24 % of rumen degradable CP being degraded during 48 to 96 h of rumen incubation (Figure 7). Moreover, the immediately degradable (soluble) fraction in both frozen *S. latissima* (CP*a*: 144 g $kg^{-1}$ CP) and its silages (CP*a*: 116 – 250 g $kg^{-1}$ CP) were substantially lower compared to wild collected brown seaweeds (CP*a*: 300 - 500 g $kg^{-1}$ CP) (Tayyab et al., 2016) and the conventional forages (CP*a*: 355 - 840 g $kg^{-1}$ CP) (Hvelplund & Weisbjerg, 2000). The low CP*a* and the delayed degradation in *S. latissima* contributed to the lower effective CP degradability ($CP_{ED}$: 271 – 460 g $kg^{-1}$ CP) compared to common forages ($CP_{ED}$ > 700 g $kg^{-1}$ CP) (Hvelplund & Weisbjerg, 2000). Also, the total tract CP digestibility in *S. latissima* ($CP_{TT}$: 414 – 537 g $kg^{-1}$ CP) was lower than the conventional forages ($CP_{TT}$ > 900 g $kg^{-1}$ CP) (Hvelplund & Weisbjerg, 2000). However, the difference in $CP_{TT}$ and $CP_{ED}$ suggested that *S. latissima* can supply 100 – 200 g $kg^{-1}$ CP, accounted for ca. 30 % of $CP_{TT}$, to the small intestine of dairy cows.

On the other hand, there is a lack of CP*b* in *A. esculenta* (Figure 7), which was unexpected and in contrast with the previous study on wild *A. esculenta* (CP*b*: 462 g

$kg^{-1}$ CP) collected in Spring from location near Bodø with nearly 30 % higher CP content than the cultivated *A. esculenta* in **Paper II** (Tayyab et al., 2016). Interestingly, a low CP*b* of 42 g $kg^{-1}$ CP was also found in the wild *A. esculenta* collected in autumn (Tayyab et al., 2016). It seems like the CP in neither young, cultivated *A. esculenta* (8 months growth period) nor in wild *A. esculenta* collected in autumn is available for rumen microbes. Moreover, $CP_{TT}$ indicated that the rumen undegradable protein was not available in the small intestine either (**Paper II**). Other than being affected by the TPC content, it is possible that the indigestible CP may be bound to polysaccharides that cannot be degraded by rumen microbes. The lack of CP digestibility limited the application of cultivated *A. esculenta* as feed ingredients for ruminants.

The rumen DM degradability plays an important role in the voluntary DM intake of the ruminants (Shem et al., 1995). Both seaweed species had similar rumen degradable DM fraction (DM*a* + DM*b*) that was comparable to common perennial and corn forage ranging between 600 – 900 g $kg^{-1}$ DM (**Paper II**) (Hoffman et al., 1993; González et al., 2010). However, *S. latissima* was characterized with a higher insoluble but rumen degradable fraction (DM*b*) and *A. esculenta* with a higher immediately soluble fraction (DM*a*) (**Paper II**). Also, the delayed degradation as seen in CP was observed in DM degradation as well (**Paper II**), and in other *in vitro* fermentation study based on the gas production (Novoa-Garrido et al., 2020). Thus, the $DM_{ED}$ was determined to be 440 – 620 g $kg^{-1}$ DM, in accordance with other investigation of wild brown seaweeds (*Alaria* and *Laminaria* genera) (Tayyab et al., 2016). The $DM_{ED}$ is comparable to the forage harvested at heading or flowering stage (380 – 600 g $kg^{-1}$ DM) (Elizalde et al., 1999), and there was no further DM degradation in small intestine as suggested by $DM_{TT}$ **(Paper II)**. Finally, the highest $DM_{TT}$ was found in the prewilted *S. latissima* silages (SLABp, $DM_{TT}$: 595 g $kg^{-1}$ DM), and was comparable to a reported maize silages ($DM_{TT}$: 611 g $kg^{-1}$ DM) (Třináctý et al., 2003).

Overall, the DM and CP digestibility suggest that *S. latissima* and its silages can potentially be used as alternative forage for dairy cows (**Paper II**). However, the CP

digestibility is low, and the delayed DM degradation can reduce DM intake which is undesirable especially in high-producing animals with high energy demand. Further studies are needed to investigate whether this lag in degradation can be shortened by placing an adaptation period in feeding trials that allows the rumen microbes to adapt to seaweed biomass.

In addition, the qPCR analysis in the incubated ruminal fluid demonstrates that the gene copies of Archaea 16S rRNA and methyl coenzyme-M reductase subunit A (mcrA) were significantly lower in seaweed IRF than in No-seaweed IRF, and there is no significant difference in the gene copies of Bacteria 16S rRNA (**Paper II**). The mcrA gene is a common biomarker for investigating methane emission because it encodes the enzymes required in methanogenesis and can be found in all methanogens (Friedrich, 2005). The positive correlation between mcrA gene copies and methane production has been reported in anaerobic digestor (Wilkins et al., 2015) as well as in the ruminal fluid of dairy cows (Aguinaga-Casañas et al., 2015). Thus, the reduction of mcrA gene copies may indicate reduced abundance of methanogens and consequently potential methane mitigation effects. However, as described in section 1.5, methane production is the result of carbohydrate degradation and fermentation in the rumen, and no correlation between mcrA gene copies and methane emission have been reported in the *in vitro* fermentation system for evaluation of seaweed as feeds (Molina-Alcaide et al., 2017; Lee et al., 2019). In addition, it is unknown how much methane reduction might occur at the expense of its organic matter digestibility. Further studies are needed to measure the methane and other VFAs produced during the *in vitro* fermentation and *in vivo* assessing are required to answer these issues.

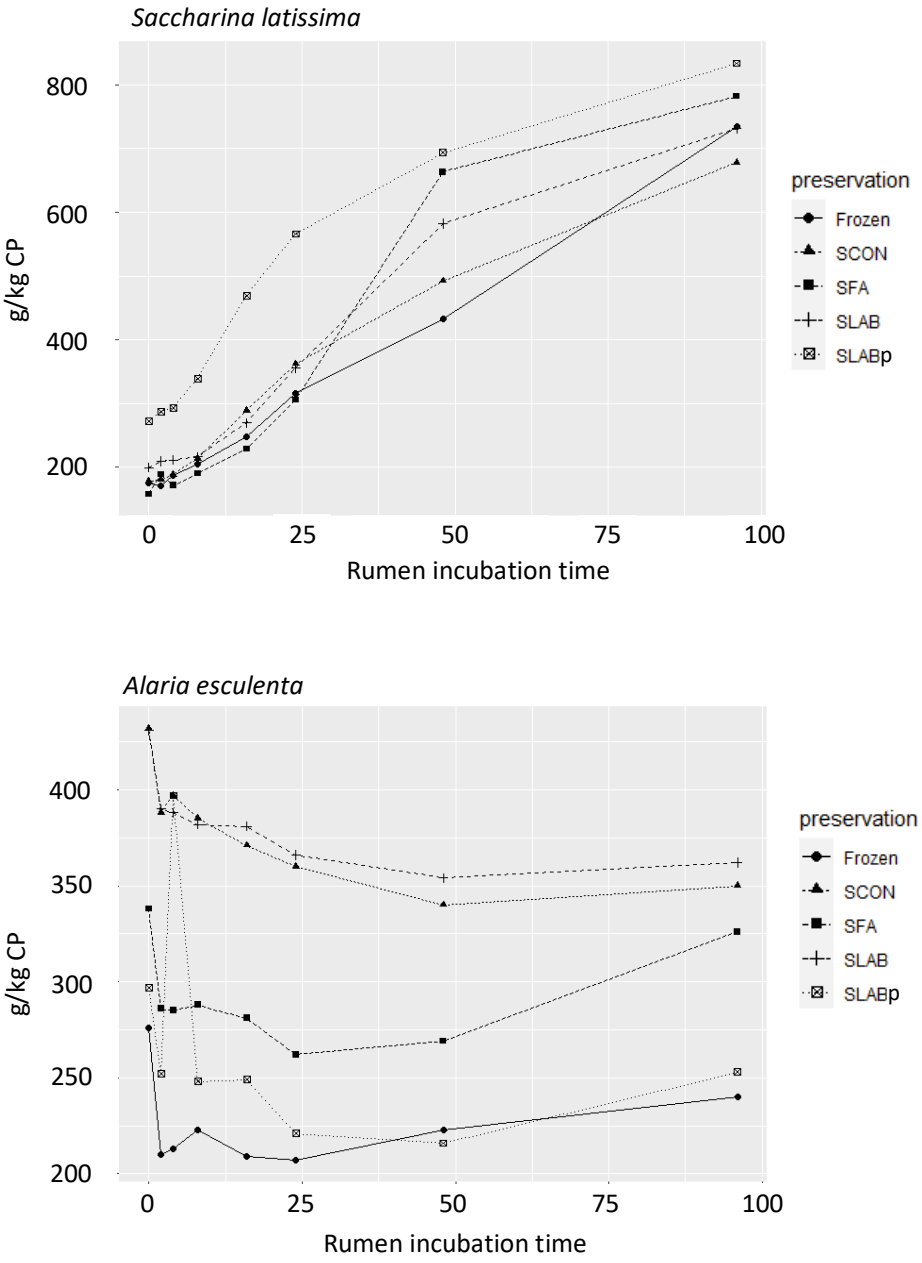


**Figure 7.** The crude protein degradability in the rumen after 0, 2, 4, 8, 16, 24, 48, 96 h incubation of *Saccharina latissima* and *Alaria esculenta* preserved by freezing (-40 °C, Frozen) or ensiling with no additives (SCON), with 0.4 % (v/w) formic acid (SFA), with lactic acid bacteria inoculum (SLAB), and with lactic acid bacteria using prewilted biomass with 30 % DM (SLABp).

## 3.3 Application for monogastric animal feed

For the prebiotic application to monogastric farmed animals such as poultry and swine, an *in vivo* pilot study was conducted using Sprague-Dawley (SD) rat. Fiber and phlorotannin are two of the important bioactive ingredients in brown seaweeds that is likely to modulate the gut microbiota as described in section 1.6. In this pilot study, the ensiling treatment SLABp was employed because it was easier to handle, compared to difficulty in generating the required amount of the freeze-dried material from the wet seaweed samples, and the loss of phlorotannin if the oven-drying option was considered. *A. esculenta* was selected because of its higher content of phlorotannin (*A. esculenta*: 7.4 g $kg^{-1}$ DM; *S. latissima*: 3.3 g $kg^{-1}$ DM) (unpublished data).

The present results showed that the supplementation of 4 % (w/w) ensiled *A. esculenta* for 25 days significantly increased the relative abundance (RA) of phylum Bacteroidota at the expense of Firmicutes in the fecal bacterial composition (Table 1), in line with the observation in the other dietary fiber supplementation study using rats (Ferrario et al., 2017). The differential abundance analysis (DAA) showed that seaweed supplementation led to a decreased RA of *Campylobacter* spp. reaching a level comparable to those reported for other feed additives based on probiotic, prebiotic, plant extract and acids in broilers (Guyard-Nicodème et al., 2016). Furthermore, the seaweed supplementation increased the RA of *Lachonospiraceae GCA-900066575*, reported to be associated with better growth performance and immunity response in broilers (Mohamed et al., 2022). This emphasizes the possibility of using ensiled *A. esculenta* as a feed additive in broiler feed. However, there is a lack of clear prebiotic effect of seaweed supplementation as hypothesized. Specifically, seaweed supplementation neither facilitated colonic butyrate production nor stimulated the growth of the traditionally known beneficial bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp.

Regarding the DSS-induced colitis, seaweed supplementation led to different fecal bacterial composition, and some of the differentially abundant genera were

associated with counteracting bowel inflammation (**Paper III**). However, the results from health parameters showed no difference in between rats provided with control or seaweed supplemented diets. The healthy parameters examined were limited to DAI, colon length, and interleukin 1 beta (iL-1β) concentration in the colonic tissue, and it is likely that they were not sensitive enough to detect the changes caused by seaweed supplementation, if there was any such change. One cannot discount the fact that the relatively short experimental period with one cycle of mild DSS challenge might not have triggered the expected outcome. Initially, the plan was to measure the gene expression of inflammation cytokine (TNFa, iL-1β, and iL-6) and the tight junction protein (Occluding-1, ZO-1 and, Claudin) in colon tissue using qPCR. However, I failed to quantify these gene expression because the qPCR efficiency of samples in the DSS group (CONDSS and SEADSS) was constantly lower than in the no DSS group (CON and SEA). Further investigation on serum samples, collected after 2 days of DSS challenge and on the termination day, will be undertaken, aiming to determine the rat's hepatic function (Duan et al., 2020) and superoxide dismutase level (Baba et al., 2009) at these time points.

**Table 1.** The average relative abundance of Phylum Bacteriodota, Firmicutes and Proteobacteria in the fecal samples from rats of four treatment groups[1]

| | CON | SEA | CONDSS | SEADSS |
|---|---|---|---|---|
| **Phylum** | | | | |
| Bacteroidota | 4.42 e-1 | **5.36 e-1** | **4.39 e-1** | **6.81 e-1** |
| Firmicutes | 5.06 e-1 | **4.35 e-1** | **5.48 e-1** | 2.94 e-1 |
| Proteobacteria | 2.41 e-3 | **2.61 e-3** | **7.65 e-3** | **9.61 e-3** |

[1] CON: no seaweed supplementation, no DSS-exposure; SEA: seaweed supplementation, no DSS-exposure; CONDSS: no seaweed supplementation, DSS-exposure; SEADSS: seaweed supplementation, DSS-exposure. The bold number indicated a significant difference when compared to the CON group (ANCON-BC, $p < 0.05$).

# 4. Conclusion

This PhD project examined the nutritional- and bioactive- value of cultivated *S. latissima* and *A. esculenta* ensiled in different methods as potential feed sources. Ensiling is a viable method to preserve these seaweeds, and had only small effect on the biomass's nutrient content, however it does not improve the digestibility for the ruminants. The conventional additives including formic acids and LAB inoculums can improve silage fermentation in seaweeds. This finding provides a less energy-demanding practice than the traditional freezing or drying to preserve seaweeds that is critical for the future development of seaweed products. Furthermore, ensiling preservation for seaweeds is relevant for not only feed application, but it also enables further biochemical treatments directly on wet silages to avoid losing bioactive content due to drying (i.e. phlorotannin).

The present thesis provided baseline knowledge about the feed characteristics of these seaweed silages for ruminants. Based on DM and CP digestibility, the *S. latissima* silages have a potential to be used as alternative forage-alike ingredients. Therefore, to incorporate *S. latissima* silages in feed, future studies are needed to address the delayed degradation of DM and CP in the rumen, and in this way increase their digestibility. On the other hand, there was a lack of rumen degradable CP in *A. esculenta*, making it unsuitable as a feed ingredient.

Finally, the microbial profiling showed that seaweed inclusion affects the bacterial composition in the ruminal fluid as well as in the digestive tract of monogastric animal. Some changes in the bacterial composition may convey beneficial outcomes and could be further developed as feed additives for ruminants to mitigate methane production and for broiler chicken to suppress the growth of *Campylobacter* spp.

# 5. Future perspectives

Kelps have potential for wide applications in feed and food. The present thesis provides important knowledge for preserving kelps through ensilage. Depending on the desired applications, the tested ensiling treatments can be adopted, and the ensiling quantity can be expanded. I hope this knowledge is helpful for the future product development in the kelp value chain.

In the present thesis, I aimed to test the potential of such seaweed silages as feed to the farm animals. The results suggested that rumen microbes can degrade DM in both kelps, but can only degrade CP in *S. latissima*. Therefore, the use of *S. latissima* silages as feed is possible, and this thesis provides some essential parameters for diet formulation. However, further studies are needed to test the viable inclusion rate, and the effect of these seaweed silages on animal's productivity as well as methane production. In addition, further studies should also consider other silage additives and mechanical treatments for improving the seaweed's nutrient digestibility. For monogastric animals, the results suggested no clear benefits for diet supplemented with *A. esculenta* silages. Therefore, further studies for applying seaweeds as functional feed additives should focus on their bioactive extracts or on the other seaweed species.

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Paper I

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# Improving fermentation of *Saccharina latissima* and *Alaria esculenta* silages with additives for preserving biomass and antioxidants

**Ying Yen[1] · Martin Riis Weisbjerg[2] · Ralf Rautenberger[3] · Adriána Fečkaninová[4] · Margarita Novoa-Garrido[1]**

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**Abstract**
Rapid deterioration of harvested macroalgal biomass is a challenge for macroalgal industry and can be overcome with the inexpensive ensiling preservation. To improve silage quality, *Saccharina latissima* and *Alaria esculenta* biomass was subjected to ensiling conditions following a 2 × 4 factorial design, with 2 prewilting treatments (no-prewilting and prewilted to 300 g DM $kg^{-1}$ fresh biomass) and 4 additive treatments (no additive, formic acid, single and two species of *Lactobacillus* inoculant), and ensiled for 3 or 12 months at 15 °C. Acetate was the main fermentation product in these seaweed silages. Prewilting reduced the acetate, mannitol, and $NH_3$ content in silages. In *S. latissima* silages without additives, prewilting led to less acidification (pH = 5.7). Also, prewilting caused protein and phlorotannin degradation. When treated with formic acid, the silage pH was below 4 regardless of the biomass's moisture content. The use of *Lactobacillus* spp. inoculants was essential for lactate production in seaweed silages, and it significantly lowered silage pH in *S. latissima* and prewilted *A. esculenta* compared to silages with no additives. A high level of the phlorotannin content was preserved (> 90%) in the 3-month *A. esculenta* silages without prewilting. However, major reduction of antioxidant activity was observed in 12-month silages in both seaweed species. In conclusion, ensiling is a viable method for preserving *Alaria* and *Saccharina* biomass. Prewilting restricted silage fermentation, and both formic acid and bacterial additives facilitated silage acidification. However, there was no clear benefit of these treatments in preserving the antioxidant activity.

**Keywords** Macroalgae · *Saccharina latissima* · *Alaria esculenta* · Phlorotannin · Chemical composition · Antioxidant activity

## Introduction

Brown marine macroalgae or seaweeds are characterized by their fast growth and their high contents in carbohydrates (e.g., alginate), minerals, and phlorotannin, which are valuable components for feed, food, pharmaceuticals, and biofuels application (Penalver et al. 2020). In particular, large amounts of brown seaweeds are processed into bioactive extracts for food and pharmaceuticals industry due to its antioxidant properties (Cherry et al. 2019; Penalver et al. 2020). In feed, seaweed extracts are included in the diets of monogastric animals for health benefits, and the dairy industry is exploring the use of intact seaweeds as alternative feed ingredients (Makkar et al. 2016; Gaillard et al. 2018). Ecologically, macroalgae aquaculture is a sustainable production of biomass with the advantages of not requiring fertilizer, arable land, or freshwater. The commercial cultivation of seaweeds is a globally growing industry, which accounted with 32.3 million tonnes of fresh weight for 28% of the entire aquaculture sector worldwide (FAO 2020). In Norway, there is a growing aquaculture activity for the two brown macroalgae *Saccharina latissima* (thereafter called *Saccharina*) and *Alaria esculenta* (thereafter called *Alaria*), with an estimated potential of producing 150–200

✉ Margarita Novoa-Garrido
margarita.novoa-garrido@nord.no

[1] Animal Science, Production and Welfare Division, Faculty of Biosciences and Aquaculture, Nord University, Post box 1490, 8049 Bodø, Norway

[2] Department of Animal Science, AU-Foulum, Aarhus University, 8830 Tjele, Denmark

[3] Division of Food Production and Society, Norwegian Institute of Bioeconomy Research (NIBIO), Postbox 115, 1431 Ås, Norway

[4] Department of Pharmaceutical Technology, Pharmacognosy and Botany, University of Veterinary Medicine and Pharmacy in Košice, Kosice, Slovakia

t fresh biomass per hectare per year (Broch et al. 2019; Fiskeridirektoratet 2020). Meanwhile, the rapid post-harvest deterioration of macroalgal biomass is a known limitation to its further utilization as nutrients and bioactive ingredients.

Ensiling is a common agricultural method to preserve forage for livestock. During ensilage, the freshly harvested biomass is preserved by anerobic fermentation in which epiphytic lactic acid bacteria (LAB) convert sugars into lactate ($pK_a$ of 3.86) and decrease the pH. The increased acidity and osmotic pressure arrest the microbial activities, and the nutrient content is preserved. Ensiling requires low mechanical and energy inputs. This is of great advantage for the preservation of harvested seaweeds in countries with climate conditions unfavorable for sun-drying and high labor costs (e.g., Norway). Moreover, ensiling potentially enables a year-round supply with biomass, batch process, and the possibility to avoid drying in several downstream processes which are designed to use wet biomass (Alvarado-Morales et al. 2013; Bach et al. 2014).

However, studies have shown difficulties in reaching adequate acidification of silage with the brown seaweeds *Ascophyllum nodosum* and *Fucus vesiculosus* due to the high moisture content, low fermentable carbohydrates, and lack of the natural epiphytic LAB (Black 1955; Herrmann et al. 2015; Campbell et al. 2020). For *Saccharina*, the high carbon to nitrogen ratio (C:N) of the biomass supports an adequate fermentation activity to reach a low silage pH, but nutrients losses were reported during ensilage (Herrmann et al. 2015; Cabrita et al. 2017; Campbell et al. 2020). Hence, common management strategies of moisture reduction and the use of silage additives were recommended to improve silage quality (Herrmann et al. 2015).

So far, there are only a few studies available that used either chemical or LAB additives in *Saccharina* silages (Cabrita et al. 2017; Campbell et al. 2020; Novoa-Garrido et al. 2020). However, it is challenging to compare these results due to the differences in seaweed biomass and ensiling conditions. Besides, the silage production of *Alaria* has not been studied yet despite this alga's commercial significance. Moreover, the effects of additives were found to differ in silages made of fresh and prewilted seaweed biomass (Gallagher et al. 2021).

The aim of the present study was to increase our knowledge of ensiled seaweed biomass with respect to its quality (fermentation pattern), antioxidant activity, and chemical composition in the different ensiling conditions, managed by means of moisture reduction (prewilting) and the use of common ensiling additives, in order to make a decisive approach towards ensiling. One hypothesis tested was that LAB additive will promote the lactic fermentation process in seaweed biomass. A second hypothesis tested was that lower moisture content will substantially affect the fermentation pattern.

## Material and method

### Seaweed cultivation

*Saccharina latissima* and *Alaria esculenta* were grown at the commercial floating aquaculture facility of Lofoten Blue Harvest in Austre Vågan on Lofoten, Norway (N68, E15). The seeding material was prepared from locally collected parental material by Hortimare AS (Bergen, Norway). Ropes were seeded before deployed to the sea in October 2017, and the macroalgae were allowed to grow for 8 months until they were harvested in June 2018. The biomass was washed with seawater on the vessel right after harvest and then packed in Styrofoam boxes to be transported (4–7 °C) to the Research Station of Nord University in Bodø. Upon arrival and within 24 h after harvest, the macroalgae were transferred to 600 L water tanks with running seawater set to 7 ± 1 °C and constant aeration to mitigate potential degradation. The macroalgae were maintained at 150 µmol photons $m^{-2}$ $s^{-1}$, measured at the water surface of the tanks, until further treated for silage preparation within 48 h.

### Experimental design

Ensiling treatments followed a 2 × 2 × 4 factorial design with two prewilting treatments (no-prewilting and prewilted to 300 g DM $kg^{-1}$ fresh biomass), two chopping times (20 s and 1 min), and four additive treatments (no additive, formic acid, single and two species of *Lactobacillus* inoculant) ($n = 3$). However, since the chopping time did not have an effect on our results, this factor was removed, and the results were pooled for statistical analysis as a 2 × 4 factorial design ($n = 6$).

### Silage preparation

The silage preparation framework is shown in Fig. 1. Macroalgal biomass was washed in three sequential water baths with decreasing salinity: 100% seawater, 70% seawater and freshwater (10 s at each step). Some of the excess water was drained by hand squeezing. Pre-ensiling samples were collected before washing ($n = 1$) and after draining ($n = 1$) and stored at − 40 °C until further analysis. For the prewilting treatment, half of the washed and drained biomass was processed with its original moisture content (noPW). The other half of the washed and drained biomass was oven-dried at 37 ± 4 °C (using a fan) to reach approximately 300 g dry matter per kg biomass (PW). The PW and noPW biomass were then chopped using a

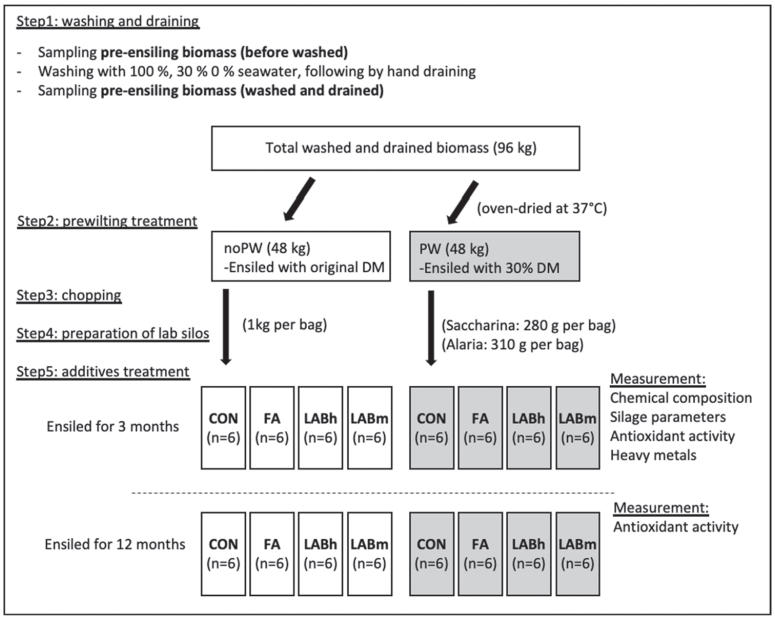


**Fig. 1** The silage preparation framework per seaweed species

butcher's cutter (TONDO5, ADE Germany GmbH, Germany) in batches of 3 kg to a particle size of about 1–4 $cm^2$.

After chopping, 1 kg of noPW biomass and the equivalent weight of PW biomass (*Alaria*: 310 g, *Saccharina*: 280 g) were placed in vacuum plastic bags as small-scale lab silos (Lavezzini, Fiorenzuola d'Arda, Italy; dimentions 20 × 60 cm). Different additives were added to each lab silos under the following treatments, CON: no additives = control, FA: 4 g formic acid per bag (WVR, Norway), LABh: $5 \times 10^9$ CFU *Lactobacillus plantarum* R2 Biocenol™ (CCM 8674) per bag, and LABm: $2.5 \times 10^9$ CFU *L. plantarum* (CCM 8674) and $2.5 \times 10^9$ CFU *Lactobacillus fermentum* R3 Biocenol™ (CCM 8675) per bag. Both LAB strains were isolated from the intestinal content of farmed healthy juvenile rainbow trout (Fečkaninová et al. 2019). The LAB inoculants were prepared fresh prior to ensiling. After adding the additives, the lab silos were gently massaged by hand to homogenize the macroalgal material with the ensiling additives, and vacuum-sealed using a heat-sealing mechanism. All silages were ensiled in the dark in a temperature-controlled room at 15 ± 1 °C for 3 or 12 months, simulating summer temperature conditions in Norway. At each sampling time, six lab silos per treatment were opened to terminate fermentation, and the biomass was transferred to another bag and stored at − 40 °C until further analysis.

## Chemical composition analysis

Seaweed samples were extracted by blending 80 g of biomass with 750 mL of $dH_2O$ twice for 40 s with an interval break of 40 s in a 4-L blender with stainless steel container (Warning Commercial, USA). The blended juice was poured into two 50-mL tubes and centrifuged at 2300 × *g* for 20 min at 10 °C. Silage pH was determined by the average pH of supernatant from the two tubes using pH meter (PHM240; Radiometer Medical ApS, Denmark) for each sample. For fermentation product analysis, 8 mL of supernatant was mixed with 2 mL of 25% meta-phosphoric acid (MPA) and stored at − 20 °C. Short-chain fatty acids (SCFA) including acetate, propionate, butyrate, valerate, iso-butyrate, iso-valerate, and caproate were measured by the gas chromatography methods described in Kristensen et al. (1996). Ammonia ($NH_3$) content was measured using the commercial kit based on glutamate dehydrogenase (AM 1015; Randox Laboratories Ltd., UK) and the Cobas Mira auto-analyzer (Roche Diagnostics, Switzerland). L-lactate content was measured using YSI 2900 Biochemistry Analyzer (YSI Inc., USA) with membrane-immobilized substrate specific oxidases (L-lactate oxidase). To measure mannitol content, the same supernatant used for measuring pH was further extracted with 1:2 water-EtOH solution under constant stirring. After centrifugation, the supernatant was analyzed with an enzymatic fluorimetric method, equivalent to the method used for determination of glutamic acid (Larsen

and Fernández 2017), to determine D-mannitol concentration after reaction with mannitol dehydrogenase. Dry matter (DM) content was determined by freeze drying the frozen samples (− 82 °C, 0.77 mbar). Dried samples from the same treatments were pooled together and milled to pass 1.0 mm filter for ash, nitrogen (N), carbon (C), and neutral detergent fiber (aNDF) analysis. Crude ash was determined after incinerated at 525 °C for 6 h (AOAC International, 2000). N and C content was measured by the Dumas method (Hansen, 1989), using Vario MAX CN (Elementar Analysesysteme GmbH, Germany). The aNDF content was analyzed using neutral detergent extraction according to Mertens (2002) with a Fibertec M6 System (Foss, Denmark) using heat-stable amylase and corrected for ash. The iodine (I) and heavy metal contents including lead (Pb), cadmium (Cd), mercury (Hg), arsenic (As), and inorganic As (iAs) were measured in a commercial laboratory by the inductively coupled plasma (ICP-MS) method. To make extracts, 1 g of freeze-dried samples were mixed with acid solution (39% $HNO_3$ + 1.8% HCl) followed by a pressured microwave digestion (up to 235 °C, 1.5 to 2 h). For iodine, the extraction was carried out with tetramethylammonium hydroxide. The iAs was determined using hydride generation atomic absorption spectrometry (HG-AAS).

### Antioxidant activity analysis

Pre-ensiling and ensiled seaweed samples (3 and 12 months) were freeze dried at − 55 °C, milled to pass 1.0-mm screen using a cell mill (Cyclotec 193 Sample Mill; Tecator, Sweden), and extracted by mixing 50–150 mg of milled powder with 1 mL of 70% (v/v) aqueous acetone with constant shaking at room temperature for 60 min, followed by centrifugation (10,000 × *g*, 6 min, 4 °C). The extraction step was repeated four to six times to extract more than 95% of the soluble phlorotannins from the macroalgal samples according to Koivikko et al. (2005). The collected supernatant was used to measure the total soluble phlorotannins (TSP) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, photometrically using Multiskan Sky microplate spectrophotometer (Thermo Scientific, USA). The absorbance was read at 730 nm for TSP assay and 520 nm for DPPH assay. The TSP contents were analyzed following a modified protocol described by Rautenberger et al. (2015), using phloroglucinol to set up the standard curve. Briefly, the extract was incubated with freshly prepared 1 N Folin-Ciocalteu phenol reagent (Merck KGaA, Germany) for 5 min, and then, the 20% (w/v) $NaCO_3$ was added to the mixture and incubated for 60 min at room temperature. The DPPH assay was performed following the Rautenberger et al. (2015), modified from Fukumoto and Mazza (2000). Briefly, the extract was diluted to different concentrations (1:1–1:160) using 70% (v/v) acetone and incubated with freshly prepared 165 µM DPPH (Sigma-Aldrich, Germany) for 18 h in dark at room temperature. The 50% inhibition of DPPH radical reduction (DPPH-$IC_{50}$) was calculated and expresses as mg DM seaweed $mL^{-1}$ DPPH.

### Statistics

Data was subjected to two-way analysis of variance with the fixed effects of silage additive treatment, prewilting treatment, and their interactions using the analysis of variance model (aov) program of R studio (Version 1.2.5033, RStudio, Inc., USA). As mentioned above, the fixed effect of chopping time (20 s, 1 min) from the original design was omitted from the original model, and there were 6 replicates per treatments. For TSP and DPPH-$IC_{50}$, the storage time (3 and 12 months) was included as the third fixed effect, without their interaction in the model. Effects were considered significant when $p$ value $\leq 0.05$, and a trend when $0.05 \leq p$ value $\leq 0.10$. Differences between means within the separated level of the prewilting treatments were tested using the Tukey's multiple comparison test ($p < 0.05$). The data are presented as mean ± standard deviation from the biological replicates unless otherwise stated.

## Result

### Chemical characteristics of pre-ensiling seaweeds

The average DM content ($n = 3$) was 93.1 ± 6.8 g $kg^{-1}$ fresh matter in *Alaria*, and 84.3 ± 4.9 g $kg^{-1}$ fresh matter in *Saccharina* before washing. The pH was neutral, and the C and mannitol contents were similar in both seaweed species (Table 1). In *Alaria*, the N and aNDF contents were 2 times higher, the TSP content was 4 times higher, and the DPPH radical scavenging capacity was 18 times stronger compared to *Saccharina* (Table 1). Washing and draining numerically reduced the ash content from 33.1 to 29.9% DM in *Alaria*, and from 28.7 to 23.9% DM in *Saccharina*.

### Silage pH, fermentation products, and mannitol content in 3-month silages

The silage pH and fermentation products were significantly affected by prewilting and silage additives (Tables 2 and 3). The total SCFA content was lower in PW-*Alaria* (noPW: 24.8 ± 4.3 g $kg^{-1}$ DM, PW: 12.9 ± 4.5 g $kg^{-1}$ DM), and PW-*Saccharina* (noPW:37.9 ± 8.8 g $kg^{-1}$ DM, PW: 13.6 ± 4.3 g $kg^{-1}$ DM). This reduction of total SCFA content led to a higher pH in *Saccharina*-CON (noPW: 4.56, PW: 5.71), but not in *Alaria*-CON (noPW: 4.84, PW: 4.68). The mannitol content was also lower in the PW-*Alaria* (noPW: 112.2 ± 14.2 g $kg^{-1}$ DM, PW: 46.0 ± 9.2 g $kg^{-1}$ DM) and

**Table 1** Chemical composition of pre-ensiling *Alaria esculenta* and *Saccharina latissima* ($n=1$)

| Seaweeds | pH | Ash (% DM) | C | N | aNDF | Mannitol (g $kg^{-1}$ DM) | TSP | DPPH-$IC_{50}$ (mg DM $mL^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| *Alaria esculenta* | | | | | | | | |
| Before washed | 7.25 | 33.1 | 32.0 | 1.60 | 15.9 | 119 | 28.0 | 2.24 |
| Washed and drained | 6.86 | 29.9 | 32.9 | 1.58 | 16.6 | 137 | 27.9 | 2.21 |
| *Saccharina latissima* | | | | | | | | |
| Before washed | 6.73 | 28.7 | 30.8 | 0.74 | 7.7 | 165 | 7.03 | 39.3 |
| Washed and drained | 6.90 | 23.9 | 33.4 | 0.88 | 4.5 | 112 | 7.36 | 36.1 |

*aNDF* amylase neutral detergent fiber, *TSP* total soluble phlorotannins, *DPPH-$IC_{50}$* the 50% inhibition of DPPH radical reduction

**Table 2** Characteristics of *Alaria esculenta* silages[1] ($n=6$)

| | Without prewilting (noPW) | | | | Prewilted (PW) | | | | | *p-value* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | CON | FA | LABh | LABm | CON | FA | LABh | LABm | *SEM* | Additive | Prewilting | Interaction |
| DM[2] | 112a[3] | 123b | 120ab | 113a | 301 | 302 | 291 | 288 | 3.54 | 0.015 | < 0.001 | 0.080 |
| Parameters in silage extracts at 3 months (g $kg^{-1}$ DM) | | | | | | | | | | | | |
| pH | 4.84b | 3.69a | 4.54b | 4.62b | 4.68c | 3.55a | 4.46b | 4.42b | 0.072 | < 0.001 | 0.006 | 0.866 |
| Total SCFA[4] | 29.6b | 19.2a | 24.6ab | 25.9b | 10.0a | 8.22a | 17.5b | 15.9b | 1.09 | < 0.001 | < 0.001 | < 0.001 |
| Acetate | 25.5c | 18.8a | 22.5b | 23.8bc | 9.04b | 7.66ab | 7.88ab | 6.38a | 0.602 | < 0.001 | < 0.001 | < 0.001 |
| L-lactate | 0.019a | 0.031a | 0.807b | 0.461ab | 0.311a | 0.015a | 8.83b | 8.96b | 0.345 | < 0.001 | < 0.001 | < 0.001 |
| Propionate | 3.5 | n.d.5 | 0.921 | 1.07 | n.d | n.d | n.d | n.d | 0.763 | 0.141 | 0.015 | 0.141 |
| Butyrate | n.d | n.d | n.d | n.d | n.d | n.d | 0.211 | n.d | 0.075 | 0.403 | 0.323 | 0.403 |
| Caproate | 0.564 | 0.332 | 0.347 | 0.552 | 0.205 | 0.1 | 0.137 | 0.14 | 0.061 | 0.022 | < 0.001 | 0.286 |
| Valerate | n.d | n.d | n.d | n.d | n.d | n.d | n.d | n.d | - | - | - | - |
| Iso-butyrate | n.d | n.d | n.d | n.d | 0.225 | 0.222 | 0.232 | 0.19 | 0.014 | 0.455 | < 0.001 | 0.455 |
| Iso-valerate | n.d | n.d | n.d | n.d | 0.227 | 0.225 | 0.233 | 0.192 | 0.014 | 0.453 | < 0.001 | 0.453 |
| $NH_3$ | 0.126b | 0.004a | 0.041a | 0.041a | 0.001 | n.d | n.d | n.d | 0.009 | < 0.001 | < 0.001 | < 0.001 |
| Mannitol | 123.9 | 110.4 | 105.4 | 109.1 | 50.7 | 50.5 | 41.7 | 41.1 | 4.52 | 0.018 | < 0.001 | 0.499 |
| Total soluble phlorotannins and antioxidant activity at 3 months | | | | | | | | | | | | |
| TSP (g $kg^{-1}$ DM) | 27.1b | 24a | 25.6ab | 25.4ab | 14.5 | 13.6 | 12.5 | 13.1 | 0.689 | 0.024 | < 0.001 | 0.211 |
| DPPH-$IC_{50}$[6] | 3.29a | 5.51b | 6.36b | 6.6b | 37.0 | 25.7 | 44.8 | 36.7 | 4.04 | 0.118 | < 0.001 | 0.157 |
| Total soluble phlorotannins and antioxidant activity at 12 months | | | | | | | | | | | | |
| TSP (g $kg^{-1}$ DM) | 7.26b | 4.9a | 5.76a | 5.51a | 4.08 | 3.53 | 3.51 | 3.69 | 0.229 | < 0.001 | < 0.001 | 0.002 |
| DPPH-$IC_{50}$ | 10.3a | 38c | 21.8b | 29.5bc | 59.7b | 27.4a | 66.4b | 61.5b | 5.49 | 0.056 | < 0.001 | < 0.001 |

[1]CON: no additive = control; FA: 4 g of formic acid per silo bag; LABh: $5\times10^9$ CFU *Lactobacillus plantarum* per silo bag; LABm: $2.5\times10^9$ CFU *L. plantarum* and $2.5\times10^9$ CFU *Lactobacillus fermentum* per silo bag

[2]Dry matter = g $kg^{-1}$ fresh matter

[3]a,b,c—Mean values with different superscripts differed in PW or noPW treatment, respectively ($p<0.05$)

[4]Total SCFA = Ac + Llac + Pr + Bu + Cap + Val + IBu + Ival

[5]n.d.: not detected, entered in the analysis with the value zero

[6]DPPH-$IC_{50}$: the 50% inhibition of DPPH radical reduction (mg DM seaweed $mL^{-1}$ DPPH)

PW-*Saccharina* (noPW: $236.8\pm27.1$ g $kg^{-1}$ DM, PW: $208.5\pm23.4$ g $kg^{-1}$ DM). The iso-butyrate and iso-valerate, potentially derived from degraded protein (valine and leucine), were only detected in the PW silages in both seaweed species.

In noPW-*Alaria*, the pH was similar in CON, LABh, and LABm, and the FA reached the lowest pH (3.69) with a significantly lower content of total SCFA, acetate, propionate, and $NH_3$ compared to CON. In PW-*Alaria*, the pH was significantly lower in FA, LABh, and LABm, and the L-lactate content was significantly higher in LABh and LABm which led to a higher content of total SCFA. In noPW-*Saccharina* silages, the pH and $NH_3$ contents were significantly lower in FA, LABh, and LABm, and

**Table 3** Characteristics of *Saccharina latissima* silages[1] ($n=6$)

| Item | Without prewilting (noPW) | | | | Prewilted (PW) | | | | *SEM* | *p-Value* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CON | FA | LABh | LABm | CON | FA | LABh | LABm | | Additive | Prewilting | Interaction |
| DM[2] | 85a[3] | 99.9b | 97.0ab | 92.3ab | 286.3 | 271.5 | 279.8 | 272.8 | 5.24 | 0.746 | < 0.001 | 0.050 |
| Parameters in silage extracts at 3 months (g $kg^{-1}$ DM) | | | | | | | | | | | | |
| pH | 4.56c | 3.58a | 3.97b | 3.69ab | 5.71c | 3.49a | 4.38b | 4.10b | 0.117 | < 0.001 | < 0.001 | < 0.001 |
| Total SCFA[4] | 38.2b | 25.3a | 43.4b | 44.5b | 9.65a | 10.1a | 16.6b | 18.0b | 1.91 | < 0.001 | < 0.001 | 0.004 |
| Acetate | 32.2b | 25.0a | 26.9a | 28.3ab | 8.57 | 9.33 | 8.93 | 9.14 | 0.95 | 0.010 | < 0.001 | 0.001 |
| L-lactate | 1.85a | n.d.a5 | 15.4b | 15.8b | 0.167a | 0.08a | 6.73b | 8.13b | 0.887 | < 0.001 | < 0.001 | < 0.001 |
| Propionate | 2.39 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | 0.843 | 0.403 | 0.323 | 0.403 |
| Butyrate | 1.45 | n.d | 0.714 | n.d | 0.435 | 0.217 | 0.453 | 0.235 | 0.45 | 0.204 | 0.520 | 0.474 |
| Caproate | 0.339 | 0.315 | 0.388 | 0.424 | 0.159 | 0.141 | 0.16 | 0.166 | 0.067 | 0.769 | < 0.001 | 0.911 |
| Valerate | n.d | n.d | n.d | n.d | n.d | n.d | n.d | n.d | n.d | - | - | - |
| Iso-butyrate | n.d | n.d | n.d | n.d | 0.098 | 0.107 | 0.125 | 0.128 | 0.021 | 0.87 | < 0.001 | 0.87 |
| Iso-valerate | n.d | n.d | n.d | n.d | 0.22 | 0.192 | 0.222 | 0.226 | 0.014 | 0.635 | < 0.001 | 0.635 |
| $NH_3$ | 0.175b | 0.114a | 0.129a | 0.126a | 0.07c | 0.043a | 0.054b | 0.049ab | 0.007 | < 0.001 | < 0.001 | 0.066 |
| Mannitol | 255 | 226 | 237 | 229 | 196 | 220 | 235 | 224 | 10.1 | 0.755 | < 0.001 | 0.081 |
| Total soluble phlorotannins and antioxidant activity at 3 months | | | | | | | | | | | | |
| TSP (g $kg^{-1}$ DM) | 3.65c | 2.66a | 3.16b | 3.06b | 3.22b | 2.77a | 2.80a | 2.77a | 0.062 | < 0.001 | < 0.001 | < 0.001 |
| DPPH-$IC_{50}$[6] | 45.7b | 39.4ab | 33.7a | 29.5a | 84.8c | 34.4a | 54.5b | 46.3ab | 3.29 | < 0.001 | < 0.001 | < 0.001 |
| Total soluble phlorotannins and antioxidant activity at 12 months | | | | | | | | | | | | |
| TSP (g $kg^{-1}$ DM) | 1.99c | 0.79a | 1.80b | 1.81b | 1.94b | 1.08a | 1.34a | 1.34a | 0.063 | < 0.001 | < 0.001 | < 0.001 |
| DPPH-$IC_{50}$ | 50.3c | 57.2d | 16.1a | 37.6b | 68.0c | 32.9a | 44.5b | 50.8b | 2.16 | < 0.001 | < 0.001 | < 0.001 |

[1]CON: no additive = control; FA: 4 g of formic acid per silo bag; LABh: $5\times10^9$ CFU *Lactobacillus plantarum* per silo bag; LABm: $2.5\times10^9$ CFU *L. plantarum* and $2.5\times10^9$ CFU *Lactobacillus fermentum* per silo bag

[2]Dry matter = g $kg^{-1}$ fresh matter

[3]a,b,c—Mean values with different superscripts differed in PW or noPW treatment, respectively ($p<0.05$)

[4]Total SCFA = Ac + Llac + Pr + Bu + Cap + Val + IBu + Ival

[5]n.d.: not detected, entered in the analysis with the value zero

[6]DPPH-$IC_{50}$: the 50% inhibition of DPPH radical reduction (mg DM seaweed $mL^{-1}$ DPPH)

the total SCFA content was the lowest in FA treatment. In *Saccharina*-PW silages, the effects of additives were similar to the noPW silages, except for a similar content of total SCFA in CON and FA. The L-lactate content was significantly higher in LABh and LABm in both noPW- and PW-*Saccharina* compared to CON.

## TSP and DPPH radical scavenging capacity in 3- and 12-month silages

There was a strong reduction in the TSP content and the DPPH radical scavenging capacity of the silages over storage time (Tables 2 and 3). In *Alaria*, the average TSP content decreased from 27.9 g $kg^{-1}$ DM in the pre-ensiling biomass to $19.5\pm6.4$ g $kg^{-1}$ DM after 3-month storage and to $4.8\pm1.4$ g $kg^{-1}$ DM after 12-month storage ($p<0.001$). The DPPH-$IC_{50}$ value increased from 2.2 to $20.7\pm18.6$ mg DM $mL^{-1}$ after 3-month storage and to $39.3\pm23.3$ mg DM $mL^{-1}$ after 12-month storage ($p<0.001$). In *Saccharina*, the TSP content decreased from 7.4 g $kg^{-1}$ DM in the pre-ensiled biomass to $3.0\pm0.34$ g $kg^{-1}$ DM after 3-month storage, and to $1.5\pm0.4$ g $kg^{-1}$ DM after 12-month storage ($p<0.001$). The average DPPH-$IC_{50}$ increased from 39.3 mg DM $mL^{-1}$ in the pre-ensiling biomass to $46.0\pm18.3$ mg DM $mL^{-1}$ after 3-month storage and remained at $44.7\pm15.8$ mg DM $mL^{-1}$ after 12-month storage ($p=0.013$).

Prewilting negatively affected the TSP content the DPPH radical scavenging capacity (Tables 2 and 3). The average TSP content in noPW-*Alaria* was 2 times higher after 3-month storage (noPW: $25.5\pm1.9$ g $kg^{-1}$ DM, PW: $13.4\pm1.8$ g $kg^{-1}$ DM) and was 1.5 times higher after 12-month storage (noPW: $5.86\pm1.9$ g $kg^{-1}$ DM, PW: $3.7\pm1.8$ g $kg^{-1}$ DM). The average DPPH-$IC_{50}$ in PW-*Alaria* was 5 times higher after 3-month storage (noPW: $5.44\pm1.5$ mg DM $mL^{-1}$, PW: $36.1\pm14.8$ mg DM $mL^{-1}$) and was 1.5 times higher after 12-month storage (noPW: $24.9\pm12.2$ mg DM $mL^{-1}$, PW: $36.1\pm22.8$ mg DM $mL^{-1}$). The average TSP content in noPW-*Saccharina* was 8.5% higher after 3-month storage and was 15.3% higher after 12-month storage compared to PW-*Saccharina*. The average

DPPH-$IC_{50}$ in PW-*Saccharina* was 32.6% higher after 3-month storage and was 17.8% higher after 12-month storage compared to noPW-*Saccharina*.

The expected negative correlation between TSP content and DPPH-$IC_{50}$ values was observed only in the *Alaria* silages ($p < 0.05$), but not *Saccharina* silages. In noPW-*Alaria*, the highest TSP and lowest DPPH-$IC_{50}$ were in CON silages after 3- and 12-month storage. In PW-*Alaria*, there was no significant difference in the TSP content, and the DPPH-$IC_{50}$ was significantly lower in FA after 12-month storage. In *Saccharina*, the highest TSP content was found in both PW-CON and noPW-CON, and the DPPH-$IC_{50}$ was significantly lower in noPW-LAB and PW-FA after 3- and 12-month storage.

### Chemical composition, iodine, and heavy metal content in 3-month silages

The triplicates from each ensiling treatments in the original design were pooled into one sample for chemical composition analysis, and the results shown in Table 4 were the average of 2 samples, replicates resulting from the omitted factor of chopping time. The ash, C, and N contents in the silages were in the same range of those in the pre-ensiling biomass (Tables 1 and 4). The aNDF content was numerically higher in silages than in the pre-ensiling biomass in both seaweed species (Tables 1 and 4). Furthermore, the aNDF in PW-*Alaria* (23.6 ± 0.4% DM) was higher than noPW-*Alaria* (17.6 ± 0.7% DM). For iodine and heavy metal content, all 6 replicates from each ensiling treatments were pooled for analysis and presented in Table 4. The average iodine content was higher in *Alaria* (931.3 mg $kg^{-1}$ DM) than in *Saccharina* (628.8 mg $kg^{-1}$ DM). The Pb, Cd, and Hg contents were numerically higher in PW-*Alaria* (Pb: 0.4 mg $kg^{-1}$ DM, Cd: 1.7 mg $kg^{-1}$ DM, Hg: 0.006 mg $kg^{-1}$ DM), than in noPW-*Alaria* (Pb: 0.28 mg $kg^{-1}$ DM, Cd: 1.5 mg $kg^{-1}$ DM, Hg: < 0.005 mg $kg^{-1}$ DM). The Pb content was numerically higher in PW-*Saccharina* (0.35 mg $kg^{-1}$ DM) than in noPW-*Saccharina* (0.24 mg $kg^{-1}$ DM).

## Discussion

Silage-making is a complicated biological process where the growth of desirable bacteria (e.g. *Lactobacillus* spp.) compete with undesirable bacteria responsible for the biomass spoilage (e.g. Enterobacteriaceae family and *Clostridium* genus). The silage outcome is affected by many factors such as the moisture content, the chemical composition, and the epiphytic bacteria of the harvested biomass (McDonald et al. 1991). In this study, common silage practices of prewilting and addition of silage additives were used to overcome the

**Table 4** The chemical composition ($n = 2$), iodine ($n = 1$), and heavy metal content ($n = 1$) of *Alaria esculenta* and *Saccharina latissima* silages

| Ensiling treatments | | Chemical composition (% DM) | | | | | Heavy metal content (mg $kg^{-1}$ DM) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Prewilting | Additives[1] | Ash | C | N | aNDF | I | Pb | Cd | Hg | As | iAs |
| *Alaria esculenta* | | | | | | | | | | | |
| noPW | CON | 27.4 | 34.2 | 1.64 | 17.1 | 960 | 0.29 | 1.4 | < 0.005 | 39 | < 0.1 |
| | FA | 26.2 | 34.7 | 1.66 | 18.5 | 1100 | 0.28 | 1.6 | < 0.005 | 38 | < 0.1 |
| | LABh | 27.3 | 34.2 | 1.66 | 17.2 | 960 | 0.28 | 1.5 | < 0.005 | 37 | < 0.1 |
| | LABm | 28.1 | 33.7 | 1.66 | 17.4 | 910 | 0.27 | 1.4 | < 0.005 | 38 | < 0.1 |
| PW | CON | 27.9 | 34.8 | 1.91 | 23.7 | 860 | 0.41 | 1.8 | 0.005 | 34 | < 0.1 |
| | FA | 27.2 | 34.9 | 1.82 | 23.1 | 870 | 0.42 | 1.6 | 0.006 | 37 | < 0.1 |
| | LABh | 29.2 | 34.5 | 1.9 | 23.7 | 880 | 0.41 | 1.6 | 0.006 | 34 | < 0.1 |
| | LABm | 28.8 | 34.5 | 1.88 | 23.9 | 910 | 0.37 | 1.8 | 0.006 | 35 | < 0.1 |
| *Saccharina latissima* | | | | | | | | | | | |
| noPW | CON | 27.3 | 31.9 | 0.942 | 11.8 | 670 | 0.25 | 0.56 | 0.026 | 50 | 0.22 |
| | FA | 24.4 | 33.3 | 0.932 | 11.5 | 570 | 0.25 | 0.51 | 0.035 | 42 | 0.21 |
| | LABh | 25.0 | 33.3 | 0.973 | 12.0 | 600 | 0.24 | 0.49 | 0.028 | 35 | 0.59 |
| | LABm | 25.8 | 32.9 | 0.965 | 12.0 | 650 | 0.22 | 0.51 | 0.027 | 36 | 0.23 |
| PW | CON | 25.5 | 32.9 | 0.958 | 11.8 | 630 | 0.34 | 0.48 | 0.026 | 34 | 0.27 |
| | FA | 23.9 | 34.1 | 1.23 | 13.8 | 630 | 0.35 | 0.48 | 0.026 | 37 | 0.26 |
| | LABh | 25.5 | 33.1 | 0.988 | 13.6 | 640 | 0.35 | 0.50 | 0.032 | 36 | 0.26 |
| | LABm | 25.4 | 33.1 | 0.945 | 11.6 | 640 | 0.34 | 0.47 | 0.024 | 34 | 0.28 |

*aNDF* amylase neutral detergent fiber, *I* iodine content (mg $kg^{-1}$ DM), *iAS* inorganic As

[1]CON: no additive = control; FA: 4 g of formic acid per silo bag; LABh: $5 \times 10^9$ CFU *Lactobacillus plantarum* per silo bag; LABm: $2.5 \times 10^9$ CFU *L. plantarum* and $2.5 \times 10^9$ CFU *Lactobacillus fermentum* per silo bag

known difficulties in ensiling macroalgal biomass (Herrmann et al. 2015; Schiener et al. 2015; Cabrita et al. 2017). The silage pH, fermentation products, and mannitol content were measured to evaluate and better understand the silage quality and fermentation process in seaweed. The TSP and DPPH scavenging capacity were measured to assess the preservation of antioxidant activity in seaweed biomass after ensiling and long-term storage. The chemical composition was measured to estimate the level of fiber (aNDF), protein (N), carbohydrate, and ash content in the silages as essential parameters to evaluate for ruminant feed application. And finally, the iodine and heavy metal contents were measured for safety concerns in feed and food applications.

## Pre-ensiling biomass

The DM content of pre-ensiling *Alaria* and *Saccharina* in present study was low but within the previously reported range of 5 to 36% DM in macroalgae (Zhang & Thomsen 2019) and was lower than that of the wild biomass collected in a close region (Tayyab et al. 2016; Novoa-Garrido et al. 2020). The chemical composition was within the range of the reported seasonal variation (Table 1) (Schiener et al., 2015). Rinsing the seaweed biomass with water led to a lower ash content, as observed previously, thus rinsing is recommended in the seaweed silage making procedure (Novoa-Garrido et al. 2020).

## Silage pH, fermentation products, and mannitol content

There was no excessive production of propionate, butyrate, and $NH_3$ in any silage, indicating limited spoiling bacterial activity, and thus a well fermented silage (Tables 2 and 3). The total SCFA was higher in *Saccharina* silages than in *Alaria* silages, as expected from *Saccharina*'s higher C:N ratio. However, the total fermentation products were low in both seaweed silages (0.5–5% of DM) compared to common legume silages (0.8–11% DM) reported by Kung et al. (2018), indicating a lower fermentation activity when ensiling seaweeds. Unlike terrestrial crops silages, where lactate is the major fermentation product, acetate was the major component in our seaweed silages, in accordance with results reported in a previous study (Novoa-Garrido et al. 2020). High acetate and low lactate content in silages indicated limited lactic acid bacteria fermentation, and it can be explained by (1) the lack of epiphytic LAB in seaweed ($< 10^3$ CFU $g^{-1}$ fresh biomass) (Uchida et al. 2004; Herrmann et al. 2015), (2) the low fermentation temperature (15 °C) used in present study, and (3) the high moisture content of biomass (DM < 30%) which prolonged the fermentation period required for the silage pH to be sufficiently low to favor the growth of *Lactobacillus* spp.

Mannitol is the primary photosynthetic product in brown seaweeds and is nearly indigestible in monogastric animals with an unknown digestibility in ruminants. Meanwhile, mannitol has been widely applied in the food and pharmaceutical industries, and thus is an interesting component to preserve (Mišurcová 2011). The mannitol content in the pre-ensiling *Alaria* and *Saccharina* (before wash) was similar to a previous study (Stévant et al. 2017). After 90-day ensiling storage, an unchange mannitol content was observed in the noPW-*Alaria* in the present study and in other brown seaweed silages reported by Herrmann et al. (2015). To the authors' best knowledge, this is the first time the reduction of mannitol in PW-*Alaira* and the increase of mannitol in *Saccharina* silages is reported. These differences in the mannitol contents between seaweed species might be explained by the different level of sugar metabolites including mannitol and fructose in different brown seaweed species as shown in the metabolome profiling (Hamid et al. 2019). The profile of these fermentable sugars can affect the mannitol content in silages, due to the ability of some microorganisms to ferment fructose to mannitol (Groisillier et al. 2013) or mannitol to lactate (Plaisance & Hammer 1921) during silage fermentation. Further research on the fermentable carbohydrate composition of the seaweed and seaweed silages is needed to explain the change of mannitol content during ensiling.

The prewilting and additives treatment significantly affected the seaweed silage fermentation ($p < 0.01$). Reducing moisture content by prewilting is a normal practice in agriculture to facilitate the fermentation process (Borreani, et al, 2018), and it makes the handling and transportation of seaweed biomass easier. When the moisture content is reduced, the silage fermentation can reach the stable stage with less acidification because of the increased osmotic pressure (McDonald et al., 1991). In the present study, the DM content (g $kg^{-1}$ fresh matter) was 116.9 ± 7.2 in noPW-*Alaria*, 295.5 ± 11.9 in PW-*Alaria*, 93.5 ± 9.1 in noPW-*Saccharina*, and 277.6 ± 16.5 in PW-*Saccharina*. When using the earlier published equation for grass silages 'pH = 0.00359 DM + 3.44' (Haigh, 1987) to calculate the theoretically desirable pH, the desired pH would be about 3.8 for our noPW silages and about 4.5 for our PW silages. In noPW silages, the pH was above the desired 3.8 in CON- and LAB-*Alaria*, and in CON-*Saccharina*. In PW silages, the pH was close to the desired 4.5 in all silages but CON-*Saccharina*. A significant reduction of undesirable fermentation products (propionate, caproate, and $NH_3$) was also observed in the PW silages. However, the prewilting treatment in the present study led to an increase of protein and TSP degradation. Further, the acetate content was reduced from a normal range of 1–3% of DM in noPW silages to below 1% of DM in PW silages, which might result in a higher spoilage risk once opened, as acetate plays an important role in aerobic stability (Danner et al. 2003).

It has been reported that applying 0.4% (w/w) formic acid could significantly reduce the silage pH to below 4.0 in *Saccharina* (Novoa-Garrido et al. 2020), but, to our knowledge, this is the first time such results are reported in *Alaria*. Our results confirmed that the use of LAB additives had the benefits of facilitating lactate fermentation, regardless of using a one-strain culture or a two-strain culture, as suggested by Novoa-Garrido et al. (2020). In contrast to our findings, Herrmann et al. (2015) reported 50 g $kg^{-1}$ DM lactate content in 3-month *S. latissima* silages, and a LAB growth from less than $10^2$ CFU $g^{-1}$ fresh biomass to $10^8$ CFU $g^{-1}$ fresh biomass. Cabrita et al. (2017) also reported a high lactate content (200 g $kg^{-1}$ DM) in *S. latissima* silage after 9 weeks without the addition of LAB inoculant. The difference in the fermentation patterns can be partially explained by higher ensiling temperature (20 °C), which is more favorable for the growth of LAB, partially by the difference in biomass composition, as well as by different silage processing methods. It should also be considered that in the present study, we analyzed for L-lactate instead of the total lactate content. We based our approach in earlier findings showing that the average ratio of L- and D-lactate is close to 1:1 in whole crop and grass silages, making it possible to use 2 × L-lactate as an estimate for total lactate content. However, this estimation was found to be invalid in *Saccharina* silages (ratio of L-lactate and total lactate: 0.09) (Johansen et al. 2020) and might not be valid for other seaweed silages as well. To verify that the L-lactate measurement was not inhibited by the complex seaweed matrix, Johansen et al. (2020) spiked seaweed samples with a known amount of L-lactate and observed full recovery. Therefore, the L-lactate content in present study is valid but does not provide information of the total lactate content in silages.

The interactions of prewilting and additives treatments were significant on the DM content and fermentation products (Tables 2 and 3). The DM content in FA was significantly higher than in CON in noPW silages, but not in the PW silages in both *Alaria* and *Saccharina*. The same interaction was reported in *Palmaria palmata* silages using acids-based additive (Gallagher et al. 2021). Also using acids-based additive was reported to increase DM density in wet grass silages (Randby & Bakken 2021). However, this interaction was not seen in our previous publication under a similar setup in *Porphyra umbilicalis* and *S. latissima* silages (Novoa-Garrido et al. 2020). Both prewilting and FA additives restricted fermentation activity, indicated by a lower total SCFA and acetate content. However, a similar content in PW-CON and PW-FA indicated that the fermentation restriction did not intensify by combining both prewilting and FA treatments. Finally, the use of LAB inoculants facilitated lactate production in PW-*Alaria*, however, not in noPW-*Alaria*. This interaction was not observed in our *Saccharina* silages, and there is unfortunately a lack of publication for comparison.

## TSP and DPPH radical scavenging capacity in 3- and 12-month silages

Phlorotannins are oligomeric and polymeric derivatives of phloroglucinol (1,3,5-trihydroxybenzene) and are only found in brown macroalgae (Wang et al. 2009). Phlorotannins are valuable cellular compounds to preserve in brown seaweeds storage due to their antioxidant properties (Roleda et al. 2019; Gager et al. 2020). Based on the Folin-Ciocalteu assay, the TSP level in pre-ensiling *Alaria* was nearly 4 × higher than in *Saccharina* (Table 1), which is similar to previous studies where a range from 2.8 × to 5.0 × was reported (Stévant et al. 2017; Roleda et al. 2019).

There was a loss of 73–88% TSP across all treatments in the 12-month silages in both seaweed species (Tables 2 and 3). The TSP loss was much higher compared to conventional preservation methods as freezing (− 25 °C) and air-drying (20 °C, > 85% DM), where around 25% and 50% TSP loss was observed, respectively in brown seaweeds (Obluchinskaya and Daurtseva 2020). Thus, ensilage is less suitable for long-term storage when TSP is the targeted substance. However, ensilage seems promising for 3-month storage providing that the loss in TSP content remains at the level found in *Alaria* silages (3%), which is much better than the loss when freezing (11—16%) and drying (25—34%) reported by Obluchinskaya and Daurtseva (2020). Campbell et al. (2020) also reported an unchanged TSP content in *F. vesiculosus* and *S. latissima* silages after 3-month storage.

The progressive decline in the DPPH radical scavenging capacity of *Saccharina* and *Alaria* over the 12-month ensiling can be ascribed to the gradual degradation of TSP in the samples under the storage conditions. However, the TSP degradation does not necessarily reduce DPPH radical scavenging capacity in the case of the *Saccharina* silages with additives. This might be explained by a possibly alteration of the phlorotannins' chemical structure and/or an increase in the functional peptides in the samples due to LAB fermentation (Virtanen et al. 2007; Sun et al. 2009) and soaking in the acids. Unfortunately, it was not possible to compare our results with other studies on ensiled seaweeds as this is the first one conducting DPPH assay in such seaweed products. Further antioxidant activity assays and different analytical methods capable of detecting phlorotannins structures are required to confirm and further explain this enhanced antioxidant activity in seaweed silages due to the known limitation of DPPH assay (Foti 2015; Ford et al. 2019).

### Chemical composition, iodine, and heavy metal composition

The aNDF content, which measures the leftover fiber after dissolving protein, sugars, lipids, and other substance in neutral detergent, is routinely analyzed in ruminant feed ingredients as it affects the energy concentration in the feed. Compared to the aNDF content reported in whole plant corn and corn silages (> 400 g $kg^{-1}$ DM), the aNDF content in our seaweeds and seaweed silages were low, likely due to a different cell wall structure (Gheller et al. 2021). Compared to the pre-ensiling seaweed biomass, the aNDF content was higher in the seaweed silages as seen in previous study (Novoa-Garrido et al. 2020), indicated substance losses during ensiling process. Moreover, loss of small molecules during prewilting might led to the much higher aNDF content in the PW treatment in *Alaria*. It is worth noting that the higher content of both TSP and aNDF in *Alaria* has previously been reported to be unfavorable for rumen digestion (Campbell et al. 2020). Further studies are needed to investigate the protein and fiber digestibility of seaweeds in ruminant animals.

Seaweeds can accumulate heavy metals from its surrounding seawater and post a risk to human and animal health when being consumed. The European Union has established recommendations for the maximum levels of heavy metal contents in seaweed in food and feed products in the Official Journal of the European Union (OJEU L78/16, 2018). Following the recommendation, the present study measured the Pb, Cd, Hg, As, and inorganic As content in the silages (Table 4). The level of Hg and Pb in present study was low and is in accordance with previous records of *Saccharina* and *Alaria* collected in the Norwegian coasts (Biancarosa et al. 2018; Afonso et al. 2020). The total As content was generally high, but this could be mainly ascribed to the less toxic organic As since iAs was less than 5% of total, which is commonly observed in marine organisms. On the other hand, careful attention should be paid to the Cd content as it was found to exceed the maximum levels regulated by the EU recommendation (OJEU L78/16, 2018) in sample of all silages.

Brown seaweed is a natural source for dietary iodine. Iodine is an essential mineral for thyroid hormones synthesis, but both deficient and excessive iodine intake can disturb the body metabolism (Rohner et al. 2014). In the present study, the iodine content of *Saccharina* silages (630 mg $kg^{-1}$ DM) was relatively low compared to the concentrations reported in other investigations, where levels above 2000 mg $kg^{-1}$ DM and up to 4600 mg $kg^{-1}$ DM have been reported in both wild harvested and cultivated biomass from central and northern Norway, while a high iodine content was found in *Alaria* silages (930 mg $kg^{-1}$ DM) compared to other studies (Biancarosa et al. 2018; Roleda et al. 2018; Afonso et al. 2020). If our silages were subjected to whole food application, the safe consumption level will be limited to 2 g DM per week, according to the recommend iodine consumption for an adult person (Russel 2001).

The prewilting treatment led to numerically higher content of Pb, Cd, and Hg in *Alaria*, and higher content of Pb in *Saccharina*. However, the content of heavy metals and iodine were not expected to change during ensilage, as these elements are not supposed to disappear due to silage fermentation. Thus, the differences in concentration between treatments were likely due to the losses of chemical compounds in the effluents or during the prewilting process. Additionally, the concentration of heavy metals and iodine in the DM based unit are expected to be slightly higher than the pre-ensiling biomass due to the loss of carbon in $CO_2$ format during fermentation. Due to the lack of this data on pre-ensiling biomass, the exact change was not available.

## Conclusions

The results suggest that ensiling is a viable method for preserving *Alaria* and *Saccharina* biomass. The ensiling outcomes were significantly affected by prewilting and additive treatments ($p < 0.001$). In *Alaria*, prewilting led to a desirable silage pH of 4.5, a reduction of acetate, proprionate and $NH_3$, and higher production of L-lactate in the LAB treatments. In *Saccharina*, a reduction of acetate, L-lactate, caproate, and $NH_3$ was observed in PW treatment, and it led to insufficient silage acidification (pH = 5.7) when no additive was applied. Unfortunately, the prewilting process in the present study caused protein and TSP degradation, and a nymerically higher aNDF content indicated the losses of other small molecules. Future studies on alternative moisture reduction methods are therefore needed to minimize the loss. The silage pH dropped to below 3.8 when ensiled with FA in both noPW and PW silages. Adding FA also reduced the acetate and $NH_3$ content in noPW silages. Adding LAB inoculant enabled lactate production except in the noPW-*Alaria*. The antioxidant activity was preserved at a promising level (> 90%) in noPW-*Alaria* after 3-month storage; however, major loss of antioxidant activity was observed after 12-month storage in both seaweed silages. The prewilting and additive treatment appeared to negatively affect the TSP preservation, and the FA and LAB additive appeared to enhance the DPPH radical scanvenging capacity in *Saccharina* silages. Further studies on the carbyhydrate and phlorotannin composition in the silages are needed to explain these changes during ensiling.

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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Conflict of interest** The authors declare no competing interests.



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Applying cultivated seaweeds as feed ingredients is of great interest for sustainable animal production. This thesis tested different ensiling conditions for two of Norway's most cultivated seaweeds: *Saccharina latissima and Alaria esculenta*. Then, the CP and DM digestibility of seaweed silages in dairy cows were determined. Finally, the effect of *A. esculenta* silages on gut microbiota in monogastric animals was investigated using lab rats. The results suggest that formic acid and lactic acid bacteria improve silage fermentation, but oven-dry leads to nutrients degradation. *S. latissima* silages is with comparable nutrient composition and digestibility to other forage-like feedstuffs, but not *A. esculenta* as it showed no rumen degradable CP. Furthermore, dietary inclusion of ensiled *A. esculenta* in rats showed no clear indication of prebiotic effect. Therefore, future research for applying seaweeds as functional feed additives should focus on their bioactive extracts or on the other seaweed species.

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