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Department of Animal and Aquacultural Sciences
Liv Torunn Mydland

Isolation and Characterisation of Bioactive Components from *Saccharina latissima* and *Alaria esculenta* and their Effect on Proliferation and Migration of Intestinal Epithelial Cells (RTgutGC) *in vitro*

Ida Steine Oma
Molecular Biology
Faculty of Chemistry, Biotechnology and Food Science

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Abstract

The human population is projected to grow to 9.6 billion by 2050. This means increased demand for food, pharmaceuticals and feed for fish and farm animals. There is a high focus on meeting these demands by developing novel and sustainable feed ingredients and additives from new types of biomasses. Norway has a long coastline and good opportunities to cultivate brown macroalgae. The nutritional value of brown macroalgae is relatively low, but can be upgraded through novel processing methods. However, they are also a resource known to contain a wide variety of bioactive polysaccharides beneficial to health. These compounds are subject to species- and seasonal variations and it is important to obtain more knowledge about the chemical composition, optimise the methods for extraction and isolation, and to examine the bioactivity of such compounds. The aim of this thesis was to extract, isolate and characterise the bioactive components, laminarin and fucoidan, from two species of brown macroalgae, *Saccharina latissima* and *Alaria esculenta*. To achieve this, a solvent based acid extraction was applied, followed by a step-wise filtration of the extract using an ultrafiltration system to separate laminarin and fucoidan based on their molecular weight. The chemical composition in the native macroalgae and in the isolated fractions was analysed for dry matter, ash and elements, and the carbohydrates were analysed as the amount of monosaccharides released after two different methods of acid hydrolysis. The results indicated that the molecular weight of laminarin and fucoidan in the two species of macroalgae differed from one another. But, it was possible to isolate a laminarin and fucoidan fraction and test their bioactivity. Macroalgae is potentially an ingredient in feeding trials and is hypothesised to stimulate repair of the mucosal barrier. The bioactivity was tested in a wound healing assay using intestinal epithelial cells from rainbow trout. Laminarin stimulated cell migration and proliferation with increasing concentrations, whilst fucoidan seemed to prevent cell growth. These results give an indication of the immunostimulatory effects of these compounds and the potential use of laminarin in the promotion of intestinal cell migration and proliferation, or fucoidan to inhibit cell proliferation and growth e.g., as an anticancer reagent.

Sammendrag

Verdens befolkning er forventet å nå 9.6 milliarder innen 2050. Dette betyr økt etterspørsel etter mat, legemidler og fôr til fisk og husdyr. Det er et høyt fokus på å møte disse kravene ved å utvikle nye og bærekraftige fôringredienser og additiver fra nye typer biomasser. Norge har en lang kystlinje og gode muligheter til å dyrke brune makroalger. Næringsverdien i brune makroalger er relativt lav, men kan oppgraderes gjennom nye prosesseringsmetoder. I tillegg er de også kjent for å inneholde et bredt spekter av bioaktive polysakkarider med helsefremmende effekter. Disse komponentene er derimot gjenstand for store arts- og sesongvariasjoner, så det er viktig å framskaffe mer kunnskap om den kjemiske sammensetningen, optimalisere metoder for ekstraksjon og isolasjon, og å undersøke bioaktiviteten til disse komponentene. Formålet med denne masteroppgaven var å ekstrahere, isolere og karakterisere de to bioaktive komponentene, laminarin og fucoidan, fra to arter av brune makroalger, *Saccharina latissima* og *Alaria esculenta*. For å oppnå dette ble det anvendt en løsningsbasert syreekstraksjon etterfulgt av en trinnvis filtrering av ekstraktet ved å anvende et ultrafiltreringssystem for å separere laminarin og fucoidan basert på deres molekylvekt. Den kjemiske sammensetningen i selve makroalgene og i de isolerte fraksjonene ble analysert for tørrstoff, aske og elementer, og karbohydrater ble analysert som mengden av monosakkarider frigjort etter to forskjellige metoder for syrehydrolyse. Resultatene indikerte at molekylvekten av laminarin og fucoidan i de to artene av makroalger var forskjellig fra hverandre. Til tross for dette var det mulig å isolere en laminarin og fucoidan fraksjon og teste deres stimulerende effekt. Makroalgaer er en potensiell ingrediens i fôringsforsøk og er hypotisert til å kunne stimulere reparasjon av slimhinnen i tarmsystemet. Bioaktiviteten ble testet i et sårhelingsforsøk ved bruk av tarmepitelceller fra regnbueørret. Laminarin stimulerte til cellemigrasjon og proliferasjon med økende konsentrasjoner, mens fucoidan syntes å forhindre cellevekst. Disse resultatene gir en indikasjon på de immunstimulerende virkningene disse komponentene har og på den potensielle bruken av laminarin til å fremme migrasjon og proliferasjon av tarmceller, eller fucoidan til å hemme celleproliferasjon og vekst, for eksempel til bruk innen kreftbehandling.

List of Abbreviations

AE	*Alaria esculenta*
CP	Crude protein
DM	Dry matter
FAO	The Food and Agriculture Organization of the United Nations
HCl	Hydrochloric acid
HMWF	High molecular weight fucoidan
HPAEC	High-performance anion-exchange chromatography
HPLC	High-performance liquid chromatography
H_2SO_4	Sulfuric acid
IF	Isolated fraction
LMWF	Low molecular weight fucoidan
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MS	Mass spectrometry
MW	Molecular weight
MWCO	Molecular weight cut-off
PAD	Pulsed amperometric detection
PBS	Phosphate-buffered saline
RTgutGC	Epithelial gut cells from rainbow trout
SCFA	Short-chain fatty acids
SL	*Saccharina latissima*
SRS	Sugar recovery standard
TFA	Trifluoroacetic acid

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1. Introduction

1.1 Background

The human population is projected to grow to 9.6 billion by 2050, and the food production is expected to be increased by 70% (Skjermo et al. 2014). This means increased demand for food, materials, fuels, pharmaceuticals and feed for fish and farmed animals, etc. Moreover, there is a high focus on meeting these demands by developing novel, sustainable and environmentally friendly feed ingredients and additives from new types of biomasses. Norway has a long coastline and an old tradition of utilising the richness from the sea, including brown macroalgae. Macroalgae is a marine resource with many areas of utilisation and is known to contain a wide variety of vitamins, proteins, minerals and bioactive polysaccharides with health beneficial applications (El Gamal 2010; Gupta & Abu-Ghannam 2011). Macroalgae has long been used to feed livestock and industrially processed for thickening agents, such as alginate, agar and carrageenans (FAO 2016), but with the help of new technology it can serve as a high quality and sustainable feed resource and help increase the country's food security. In addition, utilisation of macroalgae has other environmental benefits. Norway has a low percentage of cultivable land area, consequently imported soy beans has been used as an important ingredient in compound feed. However, by exploring and utilising alternative feed resources, like the macroalgae, Norway can become more self-sufficient whilst at the same time reduce imports, emission and contribute to the replacement of fossil resources (Skjermo et al. 2014; Wang et al. 2016).

1.2 Macroalgae

Macroalgae – or seaweed as it is often referred to, can be divided into green algae (*Chlorophyta*), red algae (*Rhodophyta*) and brown algae (*Phaeophyta*) (El Gamal 2010). Large macroalgae, like the kelps, are one of the largest unexploited types of biomass and fastest growing plants in the world. Since macroalgae has proven to be rich in vitamins, minerals, polyphenols, lipids, proteins

and polysaccharides with bioactive properties they have been processed for the use as fertilisers, feed, food, pharmaceuticals and for the production of biofuels (Fleurence 1999; Holdt & Kraan 2011). Several countries in Asia already exploit seaweed for human consumption (Venugopal 2008). The growth conditions of macroalgae includes no need for fresh water, farmland or fertiliser, with only sunlight as an energy source whilst incorporating CO_2 into biomass (Skjermo et al. 2014). This, combined with the fact that macroalgae can produce large amounts of biomass in a short amount of time, makes them interesting for cultivation and attractive for industrial application.

According to the Food and Agriculture Organisation of the United Nations (FAO), the aquatic plant farming, and overwhelmingly seaweeds, is experiencing a rapid growth and expansion and is now practiced in over 50 countries (FAO 2016). Indonesia is a big contributor to this growth, while China remains the major producer. FAO also reports that in 2014, 28.5 million tonnes of seaweed and other algae were harvested for direct consumption or further processing. Skjermo et al. (2014) reported that in 2013 99.9% of the utilised seaweed in Asia comes from cultivated seaweed, whereas only 0.1% in Europe. However, this number is increasing according to the new trends and uses of the macroalgae. In Norway, several companies (Hortimare Norway, Ocean Forest, Seaweed Energy Solutions) started with commercial cultivation a few years ago, and achieving the first licenses are a major milestone. Through cultivation, the intended use of the biomass can be better controlled as harvest depth and time effects the amount of the different compounds in macroalgae (Sharma et al. 2018). If we are going to be able to meet the demand for food, feed and energy, that the increased population demand, we need to produce millions of tons of new biomass. The cultivation of macroalgae is an important step for upscaling the production of a renewable biomass.

1.3 Brown Macroalgae

Brown macroalgae is the dominant type of seaweed along the Norwegian coastline (Indergaard 2010), and these large kelps prefer the cold waters and growth conditions of the Norwegian coast (Skjermo et al. 2014). It is the dominance of a pigment called fucoxanthin that masks the other

pigments, such as Chlorophyll *a* and *c* and other xanthophylls, that gives the brown seaweed its colour (El Gamal 2010; Gupta & Abu-Ghannam 2011).

The nutritional value of brown macroalgae is relatively low (Øverland et al. in press), they have a high content of ash and water, and low protein levels. Compared with the protein content in green or red macroalgae (10-47% of the dry matter), the amount is only 3-15% of the dry matter in brown macroalgae (Fleurence 1999). However, they are also generally known to be rich in carbohydrates, minerals, vitamins and polyphenolic compounds (Adams et al. 2011; Holdt & Kraan 2011). For macroalgae to become an important feed for farm animals, it is important to upgrade the nutritional value. For this to happen, there is a need for novel processing strategies like the isolation of bioactive components and concentration of protein content through modern biorefinery processes, and thereby minimise the generation of waste streams. Most of the carbohydrates in macroalgae are polysaccharides. These function as a fiber source in the digestive tract of fish and monogastric animals, and hence do not have much nutritional contribution (Øverland et al. 2014). However, through the process of fermentation it is possible to produce protein rich yeast from seaweed hydrolysates that can be fed to farm animals (Ravanal et al. 2017). In addition, since the polysaccharides in macroalgae has shown to improve the immune system and gut health in several pig trials (Gardiner et al. 2008; Lynch et al. 2010), they can be used as a feed additive to improve both gut health and general health in farm animals.

The composition of brown seaweed is a subject of seasonal variation which makes it difficult for a generalisation. Harvest time, environment, maturity, season, currents and temperature are only some of the factors that can have an impact on the composition (Schiener et al. 2015). This also makes it difficult to prepare standardised products from brown macroalgae. However, Holdt and Kraan (2011) made a summary of scientific data from different studies concerning the biochemical composition of and seasonal variation in brown seaweed in Northwest Europe. It was reported that *Saccharina* sp. and *Laminaria* sp. contains a range of 10-27% dry matter (DM), 15-39% ash, 38-61% polysaccharides, proteins account for 3-21% whereas lipids only around 4,5% (percentages of DM). Moreover, the carbohydrates in the species *Saccharina latissima* and *Alaria esculenta* seems to accumulate during summer and autumn, whilst being utilised during the winter when the lowest levels appear (Adams et al. 2011; Schiener et al. 2015). This coincides with the content of ash, protein and moisture, which peaks during winter and spring. Apart from the environmental

factor, it was observed that cultivation depth and harvesting time could affect the chemical composition and biomass production in a study of *S. latissima* (Sharma et al. 2018). *S. latissima* and *A. esculenta* are among the fastest-growing (high biomass producing) European kelp species.

1.3.1 Saccharina latissima

Saccharina latissima, also known as sugar kelp ("sukkertare" in Norwegian) because of its high amount of the sugar alcohol mannitol, is a brown seaweed that belongs to the order of Laminariales and the family Laminariaceae (Manns et al. 2014). *S. latissima* is found growing along the shores of the northern hemisphere and widely along the Norwegian coast (Bekkby & Moy 2011). The seaweed has a big undivided blade and prefers the growth conditions in more sheltered areas (Lüning 1990). According to Schiener et al. (2015), the average content (percentages of DM) of polysaccharides in SL is 28.5% (±3.9) alginate, 8.2% (±5.3) laminarin and 18.6% (±4.7) mannitol.

1.3.2 Alaria esculenta

Alaria esculenta, or winged kelp ("butare" in Norwegian) as it is called, is found growing along the shores in the Atlantic Ocean where the temperature does not exceed 16°C. It is a brown seaweed from the family Alariaceae, also belonging to the Laminariales. The morphology can vary due to wave exposure, but is usually characterised by an upright stipe and a long blade along the midrib (Fredersdorf et al. 2009). *A. esculenta* grows in the upper sublittoral zone and can handle more wave-exposed areas than *S. latissima* (Lüning 1990). The average content of alginate is 37.4% (±4.0), whilst the amount of laminarin and mannitol is 11.1% (±7.2) and 12.1% (±3.5) (percentages of DM), respectively (Schiener et al. 2015).

1.4 Macroalgae as a Source of Bioactive Components

As mentioned, macroalgae contains several bioactive components that has proven to have several health beneficial functions. Alginate, fucoidan and laminarin are three polysaccharides produced by brown seaweed that has gained much attention for their biological activities. Laminarin and

mannitol are the main storage carbohydrates in brown macroalgae and the main structural components of the cell wall are alginate, fucoidan and cellulose (El Gamal 2010). Their bioactivity is reported to stimulate the immune system and to have antiviral, antibacterial, antitumor, immunomodulating, and anti-inflammatory properties (Gupta & Abu-Ghannam 2011; Holdt & Kraan 2011). These activities are thought to apply for both humans and animals and their effect is usually studied by evaluating effects of whole seaweed or isolated components in *in vitro* cell lines or in laboratory and farm animals. In general, their bioactivity is related to their molecular size and structure, type of linkages and sulfate content (Gupta & Abu-Ghannam 2011). However, there is still relatively little knowledge about the structure and size of these compounds, and their different bioactivities.

1.4.1 Alginate

Alginate, or alginic acid, are linear polysaccharides found in the cell walls of brown macroalgae made up of the two 1,4-linked monomers, α-L-guluronic acid and β-D-mannuronic acid, linked together in varying proportions. In the Laminariaceae, the levels of alginate varies between 17 to 45% of the DM with the highest amounts seen during winter and spring (Manns et al. 2014). As mentioned, Norway has a tradition of harvesting seaweed for the production of alginate. Alginate has a broad application in the food industry due to its gel forming properties in the presence of Ca^{2+}-ions and stabilising effects of several food products. It is used as a thickening-, stabilising- and gelling agent (Coultate 2009). Due to its gelling properties, alginate can easily be precipitated by adding $CaCl_2$ to a solution during an extraction process. Alginate can be quantified by HPAEC-PAD after hydrolysis to uronic acids, or total uronic acids can be measured spectrophotometrically or colourmetrically (Schiener et al. 2015).

1.4.2 Laminarin

Laminarin is a β-glucan and the main storage carbohydrate in brown seaweed, consisting of a linear β-(1,3) glucan backbone with some β-(1,6) branching (Adams et al. 2011). The degree of branching affects the solubility of laminarin. In general, highly branched polysaccharides are easily soluble in water, whereas unbranched are only soluble in hot water (Rioux et al. 2007).

There are two types of laminarin chains (G and M), where G chains ends with a glucose residue and M chains ends with a mannitol residue (Figure 1). Laminarin is a low molecular weight polysaccharide of approximately 2-10 kDa, with the size depending on the degree of polymerisation (Graiff et al. 2016; Gupta & Abu-Ghannam 2011; Hjelland et al. 2012).

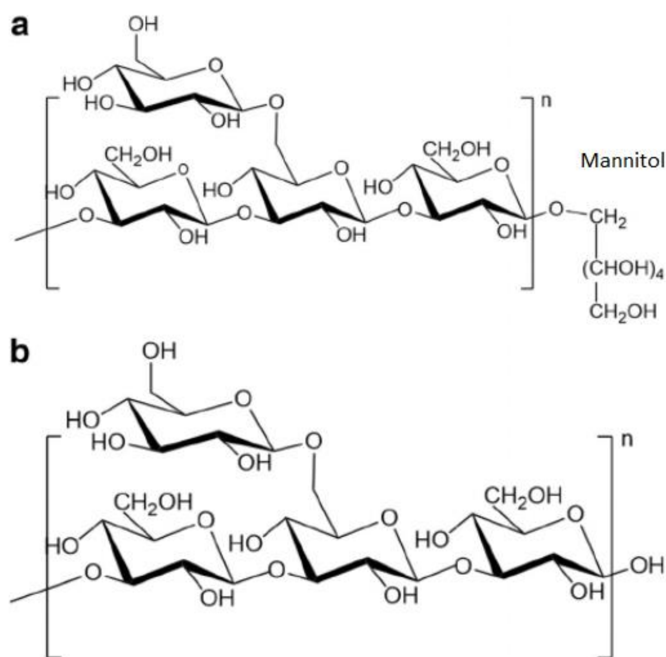

Figure 1. Structure of laminarin with M chain (a) and G chain (b) by Wang et al. (2016).

Laminarin is only found in brown macroalgae, mostly in the frond of the seaweed and is reported to reach up to 33% of the DM in *S. latissima,* with a peak during the summer and autumn (Holdt & Kraan 2011). The structure and biological activities of laminarin are thought to vary depending on age of seaweed, species and environmental properties, such as water temperature, salinity and current (Gupta & Abu-Ghannam 2011). Laminarin is one of the components in brown macroalgae that has proven to have health beneficial applications.

Different bioactivities have been identified for laminarins, such as antibacterial, antioxidant, immunomodulating and proliferative properties (Kadam et al. 2015; Zhang & Row 2014). These effects could be caused by a direct impact of the β-glucan on the immune cells or an indirect effect through their properties as dietary fibers. As a linear polysaccharide, laminarin is easily fermented by gut microbiota. This fermentation could affect the transit, stool output, intestinal mucosa and composition of the gut ecology (Devillé et al. 2004). Results show prebiotic effects of laminarin (Shang et al. 2017), where laminarin-fed rats showed an upregulation of gut beneficial bacteria (*Bifidobacterium* spp., *Lactobacillus* spp.) and an increased concentration of short-chain fatty acids (SCFA) in the cecum.

Several experiments have been carried out to evaluate the effect of laminarin and/or fucoidan on intestinal nutrient transporter gene expression, gut health and growth performance in weaned pigs. The weaning of pigs for commercial production is often done at an early stage, which can lead to an intestinal imbalance with inflammation, villous atrophy and reduction of the digestive capacity of the small-intestinal enterocytes (Heim et al. 2014). Laminarin and/or fucoidan derived from *Laminaria* spp. was included in the basal diet and the results showed that laminarin increased the expression of sodium-glucose linked transporters in the ileum and increased the average daily gain in the piglets, compared to the diet without laminarin. Moreover, piglets with a laminarin-supplemented diet showed a reduction in diarrhoea and fecal *Escherichia coli*. (Heim et al. 2014; McDonnell et al. 2010). However, the same effects of laminarin were not seen when combined with fucoidan in the diet. Also, a fucoidan-supplemented diet increased the number of *Lactobacilli* in the fecal bacteria population, but not when combined with laminarin.

Laminarin also have beneficial effects on wound healing. The wound healing process usually include an inflammatory phase, a proliferative phase with cell migration and a remodeling phase (Nguyen et al. 2009). Choi et al. (2013) showed that laminarin isolated from *Euglena gracilis,* promoted wound healing in immortalised human corneal epithelial cells, and that migration rates enhanced with increasing concentrations of laminarin. Some of the suggested effects of laminarin in this process are stimulation of tissue granulation, recruitment of macrophages to the wound site and re-epithelialization (Du et al. 2014).

Laminarin could also reduce acute inflammatory responses. Neyrinck et al. (2007) tested the hypothesis that laminarin, extracted from brown macroalgae, could modulate a response to a

systemic inflammation in rats. The results showed a different immune response in the rats fed laminarin compared to the control rats that suggested that laminarins immunomodulatory properties play a role in the tissue protective effects with lowered recruitment of inflammatory cells inside the liver tissue and lowered secretion of inflammatory mediators. Immunostimulatory effects have also been demonstrated in RAW 264.7 mouse macrophages (Lee et al. 2012). Here, laminarin significantly increased the release of several inflammatory mediators, such as cytokines, and showed signs that laminarin strengthened immune reactions through transcription factor pathways.

Laminarin has also been used in studies for the prevention or treatment of fish diseases. In one study, Atlantic cod was injected with fluorescence-labelled laminarin and when examined with fluorescence microscope, an accumulation of laminarin was observed in heart, spleen and kidney, and in immunologically relevant cells, such as macrophages (Dalmo et al. 1996). Also, head kidney macrophages from Atlantic salmon stimulated with laminarin isolated from *Laminaria hyperborea* showed an increase in organelles and spreading compared to the control cells (Dalmo & Seljelid 1995).

1.4.3 Fucoidan

Fucoidans from brown macroalgae are sulfated polysaccharides with a backbone composed primarily of α-(1,3)-L-fucopyranose residues, but can have altering α-(1,3)- and α-(1,4)-linked residues. Sulfate (SO_4^{2-}) groups appear usually at both C-2 and C-4 or at one of these alone (Figure 2). Some structures of fucoidans are also reported to contain small amounts of glucose, galactose or xylose, but it is unknown if these represent contaminations or actual substitutions on the fucoidan molecule (Ale & Meyer 2013). Fucoidan is widely found in the cell walls in brown macroalgae and have a high molecular weight that usually varies in the range of 100 kDa to 1600 kDa (Gupta & Abu-Ghannam 2011). However, low molecular fucoidan (~20 kDa) have been isolated from brown macroalgae, and one species can contain several types of fucoidan (Haroun-Bouhedja et al. 2000; Ponce et al. 2003). According to Manns et al. (2014), the amount of fucoidan found in Laminariaceae is reported to vary between 3-10% (w/w) depending on the season with highest levels reported during winter and spring, when the storage carbohydrates are at the lowest. Fucoidans are mainly found in brown macroalgae, and its structure differ among the species.

Actually, the structure varies so much between the species that it has been proposed that it is hard to confirm a consistent basic structure (Ale & Meyer 2013; Hjelland et al. 2012).

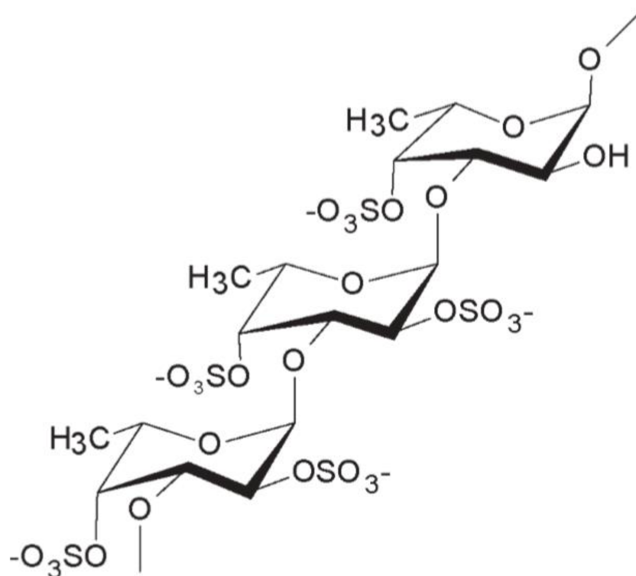

Figure 2. Modified structure of fucoidan isolated from *Saccharina latissima* (Ale & Meyer 2013).

As with laminarin, fucoidan is also reported to have a wide range of bioactive properties, such as anticancer, anti-inflammatory, anticoagulant and antiproliferative properties. The stimulatory effects of fucoidan depends on the species it is isolated from, molecular weight and position of and amount of the sulfate groups (Ale & Meyer 2013; Gupta & Abu-Ghannam 2011; Zhang & Row 2014).

Fucoidan can stimulate the immune system by its ability to modify properties on the cell surface or act as an immunomodulator directly on macrophages, T-lymphocytes, B-cells, natural killer (NK) cells and induce production of interleukin 1 (IL-1) and interferon-γ (INF- γ), *in vitro*. Though less clear, fucoidan is also demonstrated to produce antitumor effects (Chevolot et al. 1999; Holdt & Kraan 2011). In several studies examining the role of fucoidan in the inflammatory processes associated with ischemia and collagen-induced arthritis in mice and *in vitro* macrophage cell lines, results indicated that low molecular weight fucoidan (LMWF) showed more potent bioactivity

than high molecular weight fucoidan (HMWF). LMWF are usually isolated from algae or hydrolysed from HMWF (Hwang et al. 2016; Luyt et al. 2003; Park et al. 2010). Both types of fucoidans showed an effect, but it was indicated that HMWF enhanced arthritis by increasing the activation of macrophages, while LMWF reduced arthritis through the suppression of specific cytokine-mediated immune reactions. In a study by Tanoue et al. (2008), fucoidan was added to an *in vitro* co-culture system with intestinal epithelial Caco-2 and macrophage RAW264.7 cells. For patients with inflammatory bowel diseases (IBD), their intestinal epithelial cells and macrophages secrete excessively amounts of pro-inflammatory cytokines like interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α), and this dysregulation plays a part in the pathogenesis of IBD. The results showed that cells treated with fucoidan showed a down-regulation of IL-8 mRNA expression and a decreased TNF-α production.

Pereira et al. (1999) studied the anticoagulant properties of fucoidans from brown macroalgae. Their results indicated that the structural differences not only determined the anticoagulant potency, but also the mechanisms by which they carried out their activity. Fucoidan seemed to directly inhibit thrombin, and a single difference in one sulfate group per tetrasaccharide repeating unit altered the activity notably. In platelet aggregation assays, fucoidan with a high sulfate content (>20%) have shown greater anticoagulant activity in LMWF than fucoidan with a low sulfate content (<20%) (Dürig et al. 1997; Haroun-Bouhedja et al. 2000). In the same study by Haroun-Bouhedja et al. (2000), the results showed that the highly sulfated fucoidans also exerted greater antiproliferative effect of CCL39 cells. Interestingly, it was also indicated that fucoidans with anticoagulant and antiproliferative activities had different structures, where desulfated fucoidans that had lost their anticoagulant effect were still able to inhibit cell growth.

Several studies have been performed on the effect of fucoidan on cell migration and proliferation *in vitro*. Kim et al. (2015) found in a migration assay of osteoblast cells that fucoidan treated cells slightly decreased migration compared to the control cells. In addition, the cells shrunk and showed decreased spreading and adhesion. Similarly, Giraux et al. (1998) did a study on the effect of fucoidan, isolated from *Ascophyllum nodosum,* on cell proliferation and migration in human umbilical vein endothelial cells in culture. Fucoidan stimulated cell growth in the presence of fibroblast growth factor-1 whilst inhibited proliferation induced by fibroblast growth factor-2. Similarly, in the presence of another sulfated polysaccharide (heparin), the cell migration was also

inhibited. These results indicate that fucoidan can be used as a tool in the investigation of cellular mechanisms regulating cell migration and proliferation. The same mechanisms could also be the cause of the anticancer effect shown by fucoidan (Yang et al. 2008), where fucoidan from a Japanese brown kelp has shown growth inhibition in a cancer cell line.

Because of the complex chemical structure of fucoidan, it cannot be fermented by gut microbiota. Still it has shown prebiotic-like effects and could increase the abundance of benign microbes in the gut, like *Lactobacillus spp*. and SCFA-producers, whilst decreasing the number of opportunistic pathogens (Shang et al. 2017). These compositional changes in the gut could lead to indirect health promoting effects for the host and could potentially be used as a treatment of intestinal dysbiosis. Fucoidan degrading enzymes may be a way of identifying various immunostimulatory effects. Both fucoidanases, cutting the fucoidan backbone, and sulfatases may be valuable tools in addressing which structural elements are causing biological effects.

It is important to emphasise that many of the publications on the bioactive effects of laminarin and fucoidan do not mention where their substrates are isolated from. One certainty is that the huge variety of bioactivity these compounds cause, highly depends on their structure, size, branching and sulfate groups, and this varies between the different species of macroalgae. This makes it difficult for a generalisation of their function and press the importance of more detailed research on species and structure specific function.

1.5 Extraction Methods

There are several methods for extracting fucoidan and laminarin from macroalgae and no standard method exists. This is also a reason for there being no official approval for the use of these or any other of the macroalgae's bioactive components for commercial applications (Garcia-Vaquero et al. 2017). Different extraction methods exist depending on the compound you want to isolate. The extraction method can cause changes in the structural and compositional traits of the polysaccharides, because treatment with different solvents, acids, pH and temperature can alter the molecular weight or the degree of sulfation. This again can lead to changes in their bioactivity (Ale & Meyer 2013).

Before extraction, the macroalgae is usually dried and then milled to get a high surface-to-volume ratio. To date – the traditional extraction methods have been solvent based, where the polysaccharides are extracted from the macroalgae and ends up in the solvent. The most common solvents are water, ethanol and slightly acidic solutions as HCl with a low molarity (Ale et al. 2012; Garcia-Vaquero et al. 2017). The incubation also varies from room temperature to 120°C and from one to several hours. It is also not uncommon to pretreat the macroalgae for the removal of unwanted compounds (e.g. lipids, proteins) prior to extraction.

Over the recent years, more efficient extraction technologies like ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and enzyme-assisted extraction (EAE) have been developed. These techniques proposes improvements in time, cost and yield and have all been used to isolate laminarin and fucoidan from macroalgae (Garcia-Vaquero et al. 2017). UAE transforms sound waves to mechanical energy that disrupts the cell walls, whereas MAE uses a thermal based approach for the same purpose. These extraction methods can easily be used in upscaled industrial application, and they have a lower solvent consumption and a high level of automation. EAE is also a promising alternative as it has a high specificity and efficiency, but the costs in industrial applications will be high (Michalak & Chojnacka 2014).

For the bioactive components in macroalgae to become of commercial value, it is important with standardised gentle extraction methods that both preserves the biological properties of the components as well as being fitted for industrial scale production (Ale & Meyer 2013). This includes lower temperature, lower acid levels and shortened extraction times.

1.6 Methods for Analysis of Polysaccharides from Macroalgae

The complexity of polysaccharide structures in macroalgae has been a source of analytical challenge. Carbohydrates have different monosaccharide compositions including amino, acidic and neutral sugars as well as complex linkage and branching patterns (Zhang et al. 2012) and substitutions of sulfates (Ale & Meyer 2013). There are several ways of measuring the polysaccharides from macroalgae. Composition of the different monosaccharides, types and amounts of substitutions, and the molecular weight is relevant for the structural understanding of

macroalgae. The structure of fucoidan and laminarin is often analysed by methylation analysis, desulfation and NMR spectroscopy (Bilan et al. 2002; Ponce et al. 2003), where the latter can provide structural details such as degree of polymerisation, positions of branching or sulfations and linkages (Jiao et al. 2011). The molecular weight of the polysaccharides on the other hand is commonly determined by methods such as high-performance size exclusion chromatography and gel permeation chromatography (Kadam et al. 2015). The molecular size of the polysaccharides can also be determined by the use of an ultrafiltration system with different molecular weight cut-off membranes, though more imprecise as charges on the polymers may affect the cut-off.

The composition of different carbohydrates in macroalgae are usually analysed after an extraction process and then by measuring the amount of monosaccharides released after an acid hydrolysis (Manns et al. 2014). As with the extraction, there are several different methods of hydrolysing the polysaccharides to monosaccharides. In fact, it is difficult to have one universal method since the optimal type of acid hydrolysis depends on the biomass and your compound of interest. In a study done by Manns et al. (2014), several methods for the quantitative determination of the carbohydrate composition in brown seaweeds were compared. A two-step sulfuric acid (H_2SO_4) treatment of 72% (w/w) H_2SO_4 and then a dilution to 4% (w/w) H_2SO_4 released most monosaccharides, only beaten by enzymes. This method resembles one recommended by the US National Renewable Laboratory (NREL) (Sluiter et al. 2008b) widely used for analysis of lignocellulosic biomass. A disadvantage with H_2SO_4 hydrolysis is the inability to completely hydrolyse the β-(1,4) linkages in alginate (De Ruiter et al. 1992). Manns et al. (2014) suggested that treatment with trifluoroacetic acid (TFA) is favoured for biomass rich in uronic acids. This was also the conclusion made by De Ruiter et al. (1992) when comparing different methods for the hydrolysis of uronic acid containing polysaccharides. However, TFA alone was not sufficient for complete hydrolysis, but combined with methanolysis it was superior.

Several chromatographic methods have been developed to analyse monosaccharide content with high precision and sensitivity. Some of these methods use high-performance liquid chromatography (HPLC) or high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). Chromatography is based on the principle that the sample molecules with regards to differences in their physiochemical properties (charge, size, solubility with solvents) can be separated (Bird 1989). In column chromatography, the separation

occurs when the mobile phase mixed with the sample is passed through a column coated with a stationary phase (e.g. silica, polymers, etc.). HPLC uses pumps to apply pressure to the liquid mobile phase to pass it through the column. Each component in the sample interacts slightly different to the stationary phase, leading to a separation of the compounds (Transgenomic 2007) and usually detected by HPLC refractive index detector, charged aerosol detection, evaporative light scattering, fluorescence detection, or pulsed amperometric detection as described above.

HPAEC-PAD can be used to analyse monosaccharides and oligosaccharides without analyte derivatisation. For carbohydrates to be separated by anion-exchange chromatography, they need to be ionised at high pH since they are not anionic at neutral pH. Hence, the mobile phase usually is around pH 12 and greater and it is important to have a stationary phase that tolerates high pH. PAD directly detect the anionic carbohydrates that are oxidised on the surface of a gold electrode and the current measured is proportional to the concentration (Rohrer et al. 2013). Methods for analysing neutral sugars are well known, but the analysis of acidic sugars, such as the uronic acids, is less known. Zhang et al. (2012) developed a method to profile all of these on HPAEC-PAD, which used 15 mM sodium hydroxide to separate neutral and amino sugars and adding a linear sodium acetate gradient to accelerate the elution and separation of acidic sugars.

1.7 Aim of the Thesis

The aim of this thesis is to extract and isolate laminarin and fucoidan from two species of brown macroalgae common along the Norwegian coast, *Saccharina latissima* and *Alaria esculenta*. To achieve this, a solvent based acid extraction will be applied on both fresh and dried macroalgae, followed by a filtration of the extract through an ultrafiltration system to separate laminarin and fucoidan based on their molecular weight. The chemical composition in the native macroalgae and the isolated fractions will be analysed by proximate and elemental analysis and the carbohydrates will be analysed as the amount of monosaccharides released after two different methods of acid hydrolysis. Finally, the bioactivity of the isolated components will be tested in a wound healing assay in intestinal epithelial cells from rainbow trout.

2 Material and Methods

2.1 Material Collection

Two species of cultivated macroalgae, *Saccharina latissimia* (SL) and *Alaria esculenta* (AE), were provided by Seaweed Energy Solutions in Trondheim. The macroalgae was harvested in the end of May 2016 and frozen the same day. Extraction was performed on both fresh and dried form of the macroalgae.

2.2 Proximate and Elemental Analysis

The water and ash content in native SL and AE were measured as proposed by Sluiter et al. (2008a). Moisture and dry matter was calculated after drying the biomass in an oven (Thermo Fischer Scientific, USA) at 105°C overnight. Ash was determined by using a muffle furnace (Carbolite, UK) set to 550°C for 2 h, as the percentage of remaining residue after the 105°C oven dry weight of the sample. The amount of carbon, hydrogen, nitrogen and sulfur (CHNS) were analysed in the native macroalgae samples and the freeze dried isolated fractions. This was done using a CHNS element analyser (Elementar, Germany). Crude protein (CP) was calculated using a nitrogen-to-protein conversion factor. According to Lourenço et al. (2002), the traditional factor of 6.25 is unsuitable for macroalgae because of their high content of "non-protein-nitrogen" and proposes a new average factor of 4.92, which was used in this experiment. Angell et al. (2016) also states that the traditional conversion factor of 6.25 over-estimates the protein content by 43% and proposes a conversion factor of 5.

2.3 Extraction

For the extraction of the dried macroalgae the seaweed was first dried in an oven at 50°C before milling in a cutter mill with sieve size 1 mm (Retsch SM 2000, Germany). The dried and milled

seaweed was mixed with 0.03 M hydrochloric acid (HCl) (Merck, Germany) in the ratio of 1 kg to 20 L, incubated in an oven at 70°C for 1 hour and shaken every 15 minutes (Keihani 2016). After incubation, the solution was passed through a nylon filter to remove the largest particles before it was mixed 1:1 with 1 M calcium chloride ($CaCl_2$) (VWR, USA) and stored in 4°C overnight for the precipitation of alginate. The next day the extract was filtered again through a finer nylon filter (pore size 80 µm) to remove the precipitated alginate.

The same extraction method was applied to the fresh macroalgae with some adjustments regarding milling and concentration of HCl. Frozen macroalgae was grinded through a meat grinder with a disc size 3 mm (MADO Optimo, Germany) and mixed to a homogenous mass, before the dry matter in the samples were analysed. Depending on the moisture in the samples, the volume and concentration of HCl was adjusted to result in 1 kg dry matter per 20 L 0.03 M HCl. The HCl and macroalgae samples were preheated to obtain the desired temperature during incubation. The HCl was preheated to 70°C, but to avoid causing degradation in the macroalgae compounds, the samples were heated to maximum 40°C before mixing with the HCl solution. Because of the high amount of water in the fresh macroalgae itself, the temperature dropped when mixed with the solvent and reached only a temperature of 60°C. The time of incubation was thus prolonged to 1.5 h for SL and 3.5 h for AE, to increase the yield of polysaccharides.

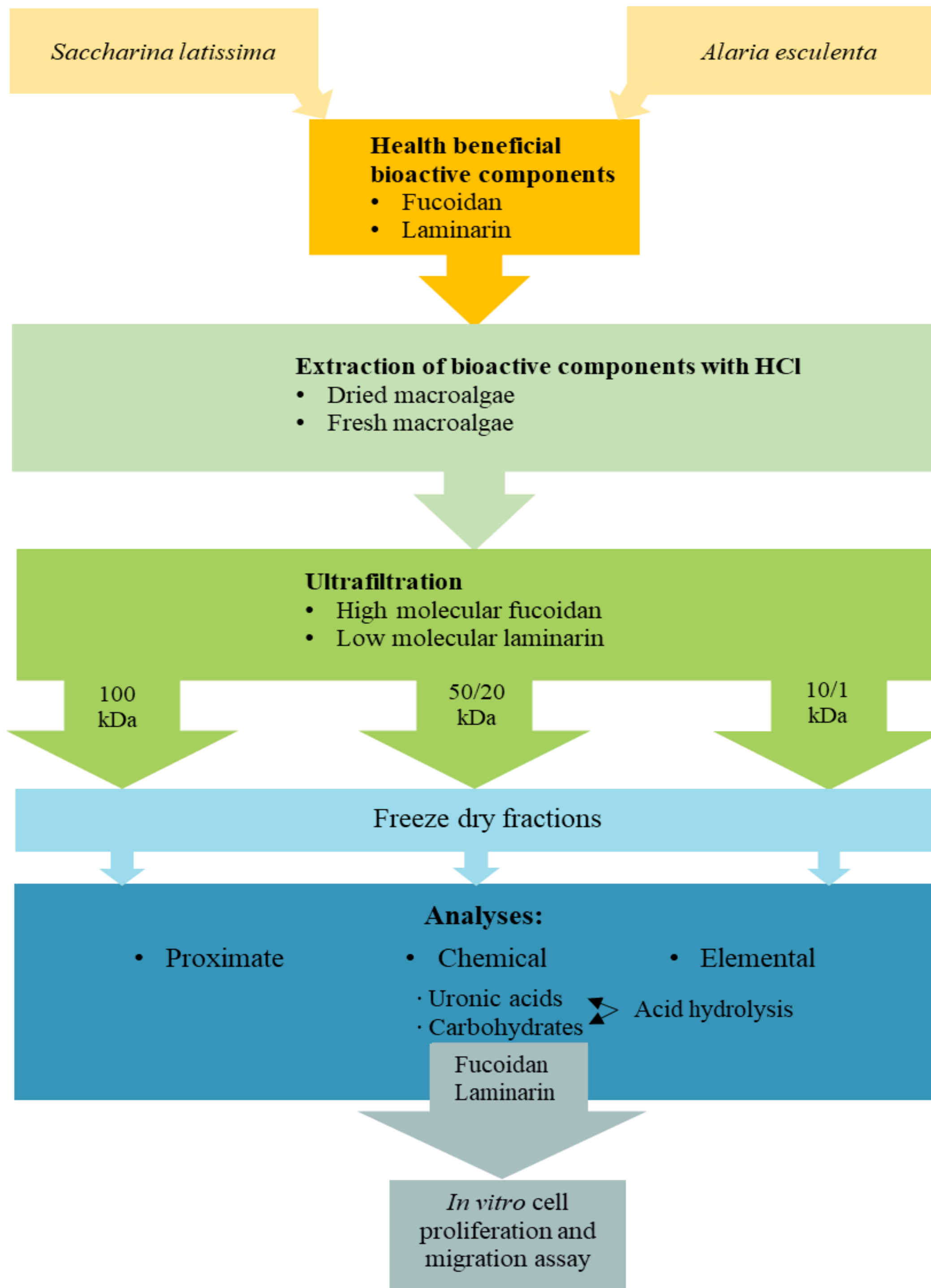

Figure 3. Flowchart of the experiments in this thesis. Bioactive components from *Saccharina latissima* and *Alaria esculenta* were extracted from dried and fresh form of the macroalgae. The extract was filtered through an ultrafiltration system to separate components based on molecular weight. The fractions were freeze dried prior to compositional analyses, and the carbohydrates were hydrolysed with sulfuric and trifluoroacetic acid. Lastly, the bioactivity of isolated laminarin and fucoidan was tested in an *in vitro* cell proliferation and migration assay.

2.4 Ultrafiltration

After extraction, the filtrate was passed through an ultrafiltration system (GEA, Germany) to separate the polysaccharides. For this, spiral membranes (Alfa Laval, Sweden) with different molecular weight cut-offs (MWCO) were used. Based on the literature and work done by Keihani (2016) the MWCO-membranes were chosen. For the extract from the dried macroalgae MWCO-membranes with sizes of 100 kDa, 20 kDa and 1 kDa were used. After the extraction from dried macroalgae, 100 kDa, 50 kDa and 10 kDa were chosen for the extract from the fresh macroalgae. The spiral membranes were made of either polysulphone or polyethersulphone cast on polyester support material, except for the 1 kDa, which was made of composite fluoro polymer cast on polypropylene. The retentate from each round of filtration was collected and freeze dried for analysis, whilst the permeate (filtrate) was collected and filtered again on a lower spiral membrane size. The extract circulated in the ultrafiltration system until the conductivity was below ~400 µS (~ the same conductivity as the water used for filtration) before collection of the retentate, to enhance the purity of the fraction.

2.5 Chemical Analyses

2.5.1 Acid Hydrolysis

For the quantitative determination of the carbohydrate composition in the samples, two different methods of acid hydrolysis were applied. The analyses were performed in triplicates on dried and ground SL and AE, freeze-dried fractions from ultrafiltration and sugar recovery standards (SRS) (glucose, xylose, mannitol, fucose, mannuronic acid and guluronic acid). The standards and most important chemicals are listed in Appendix E. The first method was a modified 2-step sulfuric acid hydrolysis of the NREL method (Sluiter et al. 2008b), starting with a strong acid swelling by adding 72% (w/w) H_2SO_4 (Merck Emsure, Belgium) to the biomass (100 mg dry material per mL) in pressure tubes, and then an incubation in water bath set to 30°C for 1 hour. For the second step, the acid was diluted to 4% (w/w) H_2SO_4 by adding deionised water before the samples were placed in an autoclave at 121°C for 40 minutes (Manns et al. 2014). After hydrolysis the samples were

filtered through centrifugal filters 10K (VWR, USA). For the second method, the biomass was weighed in screw-cap vials and exposed to 2 M TFA (Sigma-Aldrich, Germany) (10 mg dry material per mL). The vials were tightly sealed and heated in an oven (Thermo Fischer Scientific, USA) at 121℃ for 2 hours (Manns et al. 2014). The hydrolysed samples were placed under nitrogen flow to evaporate the acid, before being re-dissolved in deionised water and filtered through centrifugal filters, 10K (VWR, USA).

2.5.2 Carbohydrate Analysis

The neutral monomeric sugars (glucose, fucose, xylose) and mannitol were analysed on a Dionex UltiMate 3000 HPLC instrument (Thermo Fischer Scientific, USA) using a 300×7.8 mm Rezex ROA-Organic Acid H^+ (8%) analytical column with a stationary phase consisting of sulfonated styrene-divinylbenzene (Phenomenex 2017) equipped with a cation-H cartridge guard column. The sulfate groups in the stationary phase are negatively charged, allowing and separating neutral and partially charges molecules that are eluted according to their pKa (Transgenomic 2007). The system was operated with a 5 mM H_2SO_4 mobile phase, 8 µL injection volume and with a 0.6 mL/min flow rate at 65℃. As standards for the calibration curve, glucose, fucose, xylose and mannitol in the concentration of 0.0125, 0.025, 0.05, 0.1, 0.5, 1.25 and 2.5 g/L were used.

The uronic acids (guluronic and mannuronic) were detected using Dionex ICS-3000 HPAEC-PAD with a Dionex CarboPac PA1 column (Thermo Fischer Scientific, USA) using two eluents: A: 0.1 M sodium hydroxide (Honeywell, Germany) and B: 0.1 M sodium hydroxide with 1 M sodium acetate (Sigma-Aldrich, Germany). The eluents were made fresh prior to every sample sequence and sonicated to avoid formation of CO_2. The stationary phase in the column is composed of non-/micro-porous polystyrene-divinylbenzene beads (10 µm) agglomerated with smaller polystyrene-divinylbenzene beads (<1 µm) that works as a strong anion-exchanger and can tolerate high pH (Rohrer et al. 2013). Several gradients and different pH in the samples were tested for the best separation of guluronic and mannuronic acid. The best gradient in this experiment is described in the following. The method had an initial condition of 100% A and the separation of the uronic acids were performed by a linear gradient of 0-14% B from 0 to 2 minutes, followed by an exponential gradient of 14-100% B from 2 to 14 minutes, then 100% B for 5 minutes before 10 minutes reconditioning with 100% A. The flow rate was set to 0.25 mL/min and 5 µL injection

volume. The samples were injected after acid hydrolysis with a pH of ~2. D-Mannuronic acid and L-Guluronic acid in the concentrations of 6.25, 12.5, 25, 50, 100 mg/L were used as calibration curve standards. For the identification and quantification of the neutral sugars and uronic acids, the Dionex Chromeleon version 7.2.3.7553 (Thermo Fischer Scientific, USA) was used.

2.6 Evaluation of the Hydrolysis Methods and Results

2.6.1 Examining of the Hydrolysis

To assess if there had been a complete hydrolysis of the polysaccharides to monosaccharides after the acid hydrolysis, it was examined if the method used to analyse the samples on the HPAEC-PAD could detect oligosaccharides. Samples of alginate enzymatically hydrolysed with alginate lyase containing oligomers of different sizes, was run on the same method as the experiment samples on the HPAEC-PAD.

2.6.2 Purification of Isolated Fractions

The results from the carbohydrate analysis (Figure 6 and 7) showed that the "fucoidan" fraction (100 kDa) from SL contained a high amount of glucose. Some purification methods are discussed in 4.4, and one mentioned is based on enzymatically cleavage of unwanted polysaccharides in a fraction followed by a new filtration for the removal of these. A small experiment was conducted in an attempt to enzymatically cleave the β-(1,3) glucan of laminarin. Fucoidanases are not commercially available. In addition, the AE 100 kDa fraction was included in this experiment because of its high glucose level, to get an indication if this could derive from laminarin. The samples were analysed using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS).

The substrates were dissolved in a sodium acetate buffer (50 mM, pH 5.0). A 5 mg/mL stock solution was made of SL 100 kDa and AE 100 kDa, whilst a 1 mg/mL solution of laminarin from *Laminaria digitata* (Sigma-Aldrich, Germany) was used as a positive control. 1 µL of 10 mg/mL

β-(1→3)-D-Glucanase from *Helix pomatia* (Sigma-Aldrich, Germany) was added to 500 µL of substrate and the reaction ran over nigh at 37°C on a ThermoMixer (500 rpm, Eppendorf).

The samples were diluted 1:10 and 1 µL was added in a 2 µL droplet of matrix (2,5-dihydroxybenzoic acid in 30% acetonitrile) on an MTP 384 ground steel target plate TF (Bruker Daltonics, Germany). The samples were dried under a stream of air and analysed with a Ultraflextreme MALDI-TOF/MS instrument (Bruker Daltonics, Germany) that provides the capability of mapping molecules in a sample. The instrument was controlled by the software FlexControl (Bruker Daltonics, Germany) (Version 3.4), the acquisition range was 300 to 4000 *m/z* and the data was collected from 200 laser shots. The spectra were analysed with the FlexAnalysis software (Bruker Daltonics, Germany) (Version 3.4).

2.7 Cell Migration and Proliferation Assay

2.7.1 Cell Culture

The effect of isolated fucoidan and laminarin from SL and AE on cell migration and proliferation were tested using an intestinal epithelial cell line from rainbow trout (*Oncorhynchus mykiss*) (RTgutGC). This cell line was established in Niels C. Bols' laboratory at the University of Waterloo, Canada (Kawano et al. 2011). The cells were grown in 75-cm^2 flasks (Thermo Fisher Scientific, Denmark) in Leibovitz's L-15 medium (Thermo Fisher Scientific, USA) supplemented with 5% fetal bovine serum (Sigma-Aldrich, Germany) (FBS) and 1% gentamicin (Thermo Fisher Scientific, USA) (L15/FBS). The cells were maintained at 18°C and passaged every week in a ratio of 1:2, detached using trypsin (Biowest, France).

2.7.2 Wound Healing Assay

Fucoidan isolated in the 100 kDa fraction from SL and laminarin in the 10 kDa fraction from AE (see results 3.4), both extracted from fresh macroalgae, were tested for effects on cell migration and proliferation (wound healing) in RTgutGC cells. 35 mm µ-dishes (Ibidi, Germany) with a 2-

well culture-insert was used. The culture-insert created two small wells which was separated by a plastic divider to generate a gap between the cells. The cells were counted using an automated cell counter (TC20, Bio Rad, USA) and approximately 10,000 cells in L15/FBS were seeded into each well and incubated until becoming fully confluent. The freeze dried isolated fucoidan from SL and laminarin from AE were re-dissolved in phosphate-buffered saline (PBS) and added to the cells at 10, 100 and 500 µg/mL, respectively. Fucoidan (≥95%) from *Fucus vesiculosus* (Sigma-Aldrich, Germany) and laminarin from *Laminaria digitata* (Sigma-Aldrich, Germany) were used as positive controls and added at the concentration of 500 µg/mL. PBS was added as a negative control. The experiment was performed in duplicates. When the cells were confluent, the plastic divider was removed to create a 500 µm gap between the cells, imitating a wound. Immediately after the removal, the substrates were added to the µ-dish in medium and phase contrast microscopy (Axio, Zeiss, Germany) pictures were taken from day 0 until 7. A gap area in the pictures were analysed using ImageJ (http://imagej.nih.gov/ij/docs/index.html) and the level of cell migration was estimated by measuring the migration rate compared to the original wound in the same sample at day 0.

2.7.3 Statistical Analysis

A two-way ANOVA ($p<0.05$) was applied to examine if time and dose of laminarin and fucoidan significantly influenced the cell migration. The results are expressed as the mean from two experiments. Further on, Tukey's multiple comparison test ($p<0.05$) was used to determine which means differed from the rest amongst a set of means.

3. Results

3.1 Chemical Composition of Native Macroalgae

The chemical composition of the native *S. latissima* and *A. esculenta* with regards to monosaccharide content and elemental analysis (CHNS) are presented in Table 1 and 2, respectively. The dry matter in the *S. latissima* in this experiment was 7.7%, while *A. esculenta* had a dry matter content of 11.1%. The following percentages are expressed on a DM basis, unless otherwise stated. The ash content was 33.5% and 25.5% in SL and AE, respectively. Moreover, the total carbohydrate content (laminarin, fucoidan, xylose, mannitol and alginate) accounted for 68.0% in *S. latissima*, distributed in 13.1% laminarin (estimated by glucose), 2.2% fucoidan (estimated by fucose), 3.6% xylose, 19.0% mannitol and 30.1% alginate (estimated by uronic acids). Results are given from which hydrolysis method that gave the highest yield. By the use of a nitrogen-to-protein factor of 4.92 (Table 2), *S. latissima* contained 8.9% crude protein. There is a generally low nitrogen content, and hence a low protein content in all the samples. *A. esculenta* had a total carbohydrate content of 66.2%, where laminarin accounted for 12.0%, fucoidan 2.4%, xylose 3.7%, mannitol 13.4% and alginate for 34.7%. From the elemental analysis (Table 2) AE has the highest carbon content, but the lower carbon to nitrogen factor indicates a higher protein content than in SL, which also is indicated by the crude protein content of 11.4%.

Table 1. Carbohydrate composition of native *Saccharina latissima* (SL) and *Alaria esculenta* (AE) after hydrolysis with sulfuric acid and trifluoroacetic acid (TFA).

Monosaccharide content (g/kg DM)	Glucose[1]	Fucose[1]	Xylose[1]	Mannitol[1]	Uronic acids[2]
Samples			Sulfuric acid		
SL	131	21	36	164	189
AE	120	17	35	112	180
			TFA		
SL	67	22	35	190	301
AE	81	24	37	134	347

[1]Determined by HPLC. [2]Determined by HPAEC-PAD.

Table 2. Content of carbon (C), nitrogen (N), hydrogen (H), sulfur (S), crude protein (CP) and the ratios C to N (C:N) and C to S (C:S) in *Saccharina latissima* (SL) and *Alaria esculenta* (AE) and fractions isolated from fresh macroalgae.

Samples	C	N	H	S	CP[1]	C:N	C:S
			g/kg DM in native macroalgae				
SL	312	18	49	12	89	17.2	25.1
AE	365	23	51	13	114	15.8	28.0
			g/kg isolated fraction				
SL 100 kDa	257	9	59	63	46	27.2	4.1
SL 50 kDa	63	6	26	60	31	9.9	1.0
SL 10 kDa	163	6	37	78	30	26.5	2.1
AE 100 kDa	300	5	55	22	23	64.4	13.5
AE 50 kDa	60	6	24	46	29	10.4	1.3
AE 10 kDa	140	6	29	54	29	23.9	2.6

[1] Calculated by a protein conversion factor of 4.92

3.2 Extraction

Results from the chemical analyses performed on the fractions from the dried macroalgae extraction are not presented because it is believed that the extraction was incomplete. The extraction was not performed with a preheated solution, resulting in a too low temperature during incubation. This is indicated from the quantity of dry product from each filter after ultrafiltration, which was in the range of 0.005 – 1.8 g/L retentate, compared to 0.285 – 2.4 g/L retentate from fresh macroalgae extraction. The amount of initial macroalgae dry matter was approximately the same for extraction of dried and fresh macroalgae. A fraction of the permeate after ultrafiltration on the 1 kDa membrane of the dried macroalgae (SL and AE) was freeze dried and analysed for carbohydrates on HPLC and HPAEC-PAD in case some components had gone through, but the permeate did not contain any detectable amounts of sugars (data not shown). The same was also shown by CHNS analysis, where SL and AE permeates after the 1 kDa membrane only contained 1.7% and 1.6% carbon, respectively.

During the extraction we wanted to precipitate alginate, which cause a potential impurity in the fractions aimed to isolate here. With the extraction on both dried and fresh macroalgae there was no visible precipitation of alginate after the extract was incubated with $CaCl_2$. However, results from the HPAEC-PAD analyses of the sulfuric acid hydrolysates of the retentates (solid-fractions) removed by the two first nylon filters showed that a large proportion of the alginate had in fact been removed (Appendix A and B). The retentates from the first filter consisted of 28 and 29% uronic acids, for SL and AE, respectively. Whereas the retentates from the filtration with 80 µm nylon filter after incubation with $CaCl_2$ consisted of 8 and 18% uronic acids for SL and AE, respectively. Thus, the amount of alginate in all the fractions from ultrafiltration is low (Figure 6).

3.3 Carbohydrate Analyses

The carbohydrate composition of the native *S. latissima* and *A. esculenta,* expressed as yield of monomeric sugars, mannitol (sugar alcohol), and uronic acids (sugar acid) after two different hydrolysis methods are shown in Table 1. In general, the hydrolysis with sulfuric acid results in a higher release of glucose than hydrolysis with TFA (Table 1 and Figure 4), whereas samples hydrolysed with TFA results in much higher yield of the uronic acids. The amount of fucose, xylose and mannitol are quite similar from the two different methods of hydrolysis. Samples with oligomers of different length (enzymatically hydrolysed with alginate lyase) of uronic acid were analysed using the same method as the samples on the HPAEC-PAD to check if the method detected oligomers, which it did (Figure 5). However, no oligosaccharides were detected in the acid hydrolysed samples from this experiment, thus it can be assumed that the polysaccharides have been completely hydrolysed.

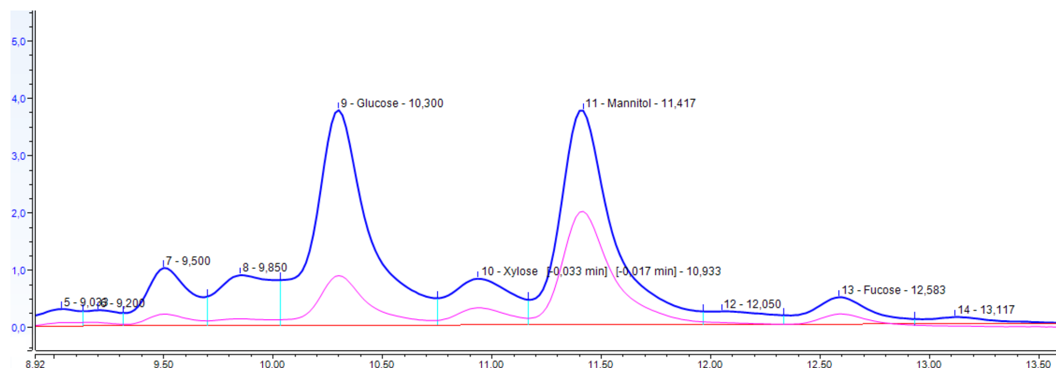

Figure 4. Representative chromatogram of the same sample of native *Saccharina latissima* hydrolysed with sulfuric acid (blue) and trifluoroacetic acid (pink). The figure shows overlaying chromatograms after analysis of glucose, xylose, mannitol and fucose on HPLC. The x-axis display retention time (minutes) and the y-axis show measuring range of refractive index µRIU.

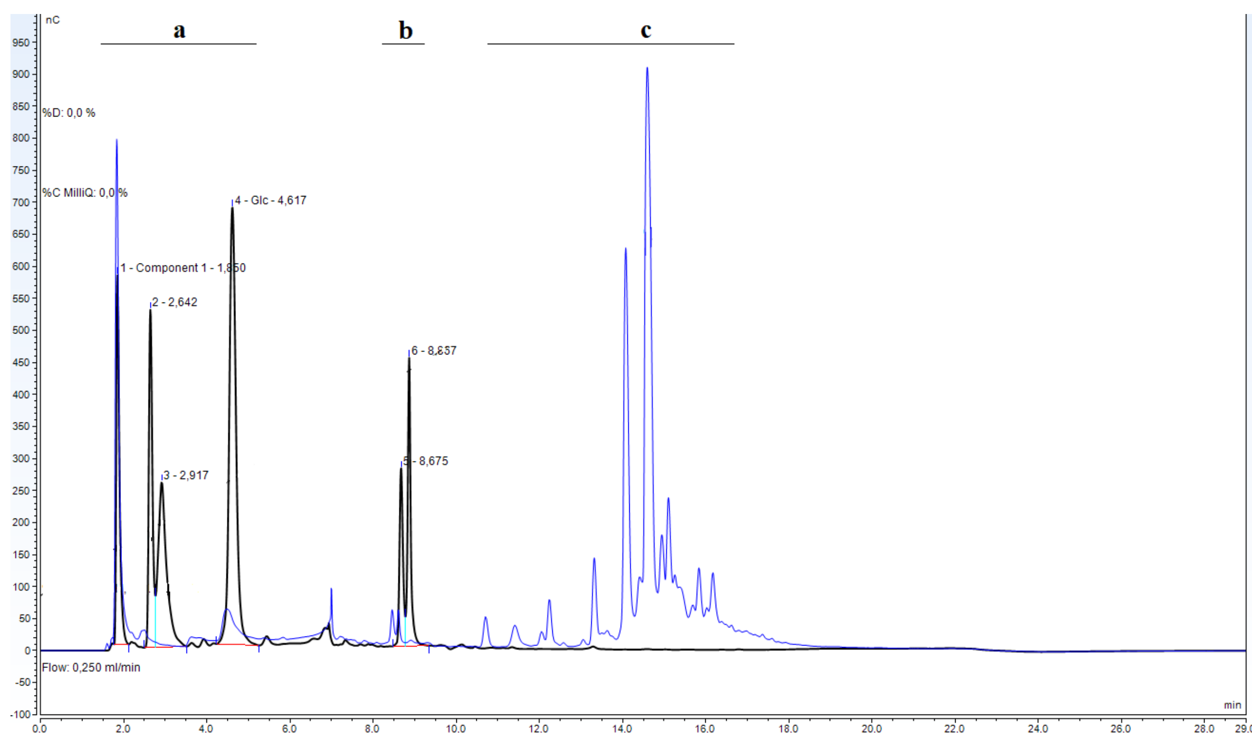

Figure 5. Overlaying chromatograms of native *Alaria esculenta* (black) hydrolysed with sulfuric acid, and a sample with a range of different uronic acid oligomers enzymatically produced (blue). The figure shows monomeric neutral sugars (a), monomeric guluronic and mannuronic acid (b) and oligomers of uronic acid (c) detected by HPAEC-PAD.

3.4 Isolation of Laminarin and Fucoidan

The profiles of different carbohydrate components in the isolated fractions from fresh macroalgae, and after acid hydrolysis with sulfuric acid and TFA are presented in Figure 6 and 7, respectively. As mentioned, the results from the chemical analyses performed on the fractions from the dried macroalgae extraction are not presented. However, the results indicated that there were some fucoidan and alginate left in the 20 kDa fraction, so for the ultrafiltration of fresh macroalgae extracts, the 50 kDa MWCO-membrane was introduced to improve the purity of the low MW laminarin fraction (10 kDa).

The 100 kDa fractions targeted fucoidan. This fraction from SL contains a high amount of fucose, but also of glucose and xylose. The same fraction from AE has a very high amount of glucose, while fucose levels are low. In addition, AE 100 kDa has the lowest level of sulfur measured from CHNS analysis (Table 2). Except from this, all the fractions have relatively high levels of sulfur, which can indicate the presence of sulfated polysaccharides such as fucoidan. Proteins also contain sulfur (from cysteine and methionine), but the low nitrogen content in all the fractions indicate low levels of protein. The 10 kDa fraction aimed to isolate laminarin, and they do have high levels of glucose. AE seems to have a very pure laminarin fraction compared to SL, which contains a lot of fucose. The AE 10 kDa fraction contains 21.2-30.5% glucose, and in the range of approximately ~1-2% (Figures 6, 7 and Table 4) of the respective other carbohydrates whilst 2.9% crude protein (Table 2). Despite the low fucose content, it has a high amount of sulfur. In SL, there are high fucose levels in both the 100 kDa and 10 kDa fractions. The 10 kDa fraction has a higher amount of sulfur and a lower carbon to sulfur ratio. This can indicate that SL contains fucoidans with different molecular weight and degree of sulfation. In the native macroalgae samples, the glucose results were higher after sulfuric acid hydrolysis than with TFA. In the isolated fractions however, TFA seems to result in a higher glucose yield.

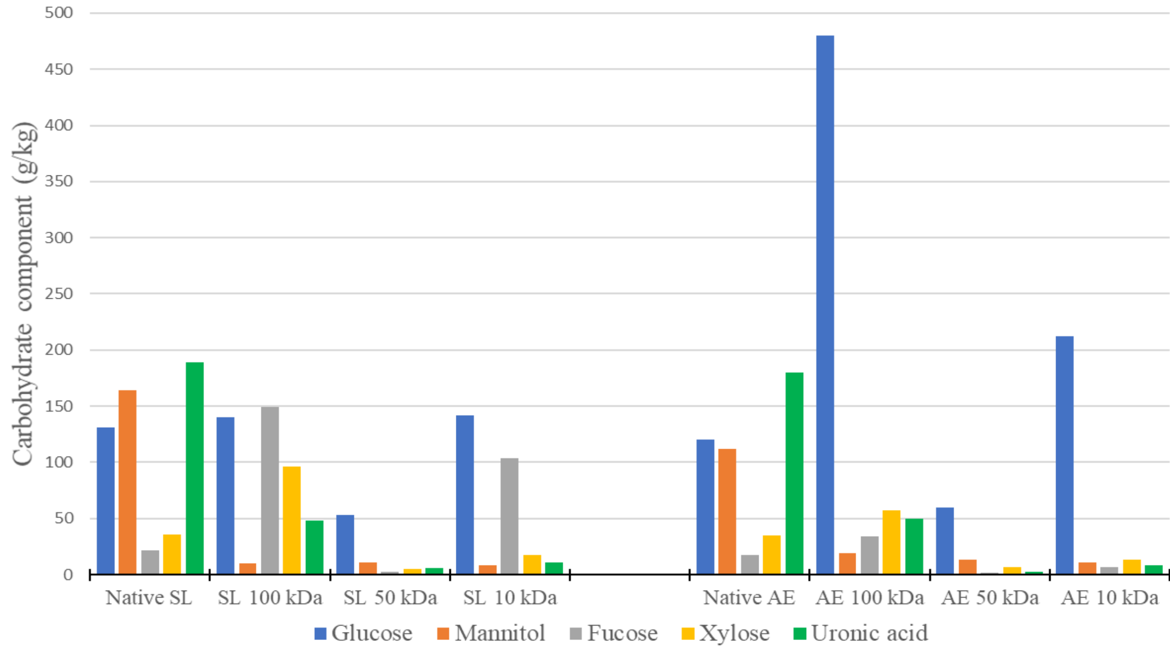

Figure 6. The average amount of glucose, mannitol, fucose, xylose and uronic acid found in native *Saccharina latissima* (SL) and *Alaria esculenta* (AE) (g/kg DM) and in the fractions (g/kg isolated fraction). The monosaccharides are analysed after extraction of fresh macroalgae and hydrolysis with sulfuric acid (H_2SO_4).

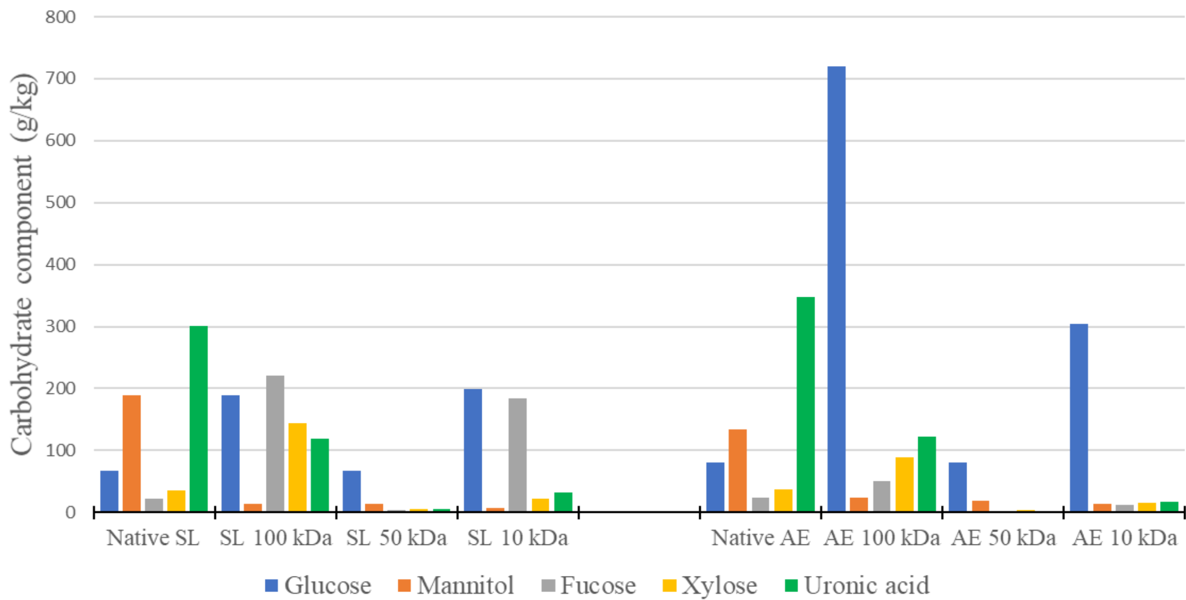

Figure 7. The average amount of glucose, mannitol, fucose, xylose and uronic acid found in native *Saccharina latissima* (SL) and *Alaria esculenta* (AE) (g/kg DM) and in the fractions (g/kg isolated fraction). The monosaccharides are analysed after extraction of fresh macroalgae and hydrolysis with trifluoroacetic acid (TFA).

3.5 Cell Migration and Proliferation Assay

The results from the wound healing experiment are presented from day 0 until day 6 when the wounds were closed in most samples or in the negative control. Pictures of the wound healing are presented in Figure 8 and 9, and results from the statistical analysis are displayed in Figure 10. The statistical analysis showed that there were significant differences in migration rates in the same day between samples treated with laminarin or fucoidan and the negative control. There were no significant differences between the different concentrations from the same day (Appendix F and G). Cell migration and proliferation seems to be stimulated in the cells treated with laminarin compared to the untreated cells in the negative control. This applies both for the isolated laminarin (10 kDa AE fraction) and the laminarin from Sigma-Aldrich (positive control) (Figure 8). In the first days a rapid growth was observed in the cells treated with the lower concentrations of laminarin, and a significant difference ($p<0.001$) (Figure 10) was seen between the gap closure area in the wounds treated with 100 µg/mL laminarin compared to negative control at day 4. At day 6 there was a significant difference ($p<0.05$) in the migration rate in cells treated with 100 and 500 µg/mL isolated laminarin and the positive control, compared to the negative control the same day.

In contrast to the stimulatory effect of laminarin on cell migration and proliferation, fucoidan seems to prevent growth. The wound is closed after 6 days in the negative control (Figure 9), but in neither of the fucoidan treated cells (isolated 100 kDa SL fraction and fucoidan from Sigma-Aldrich). Already at day 4 there is a significant difference ($p<0.01$) in the growth between the negative control and fucoidan treated cells in all concentrations (Figure 10). From the pictures it seems that there is a higher cell migration rate in the cells treated with low concentration (10 µg/mL), compared to the two high concentrations of fucoidan, but there were no significant differences.

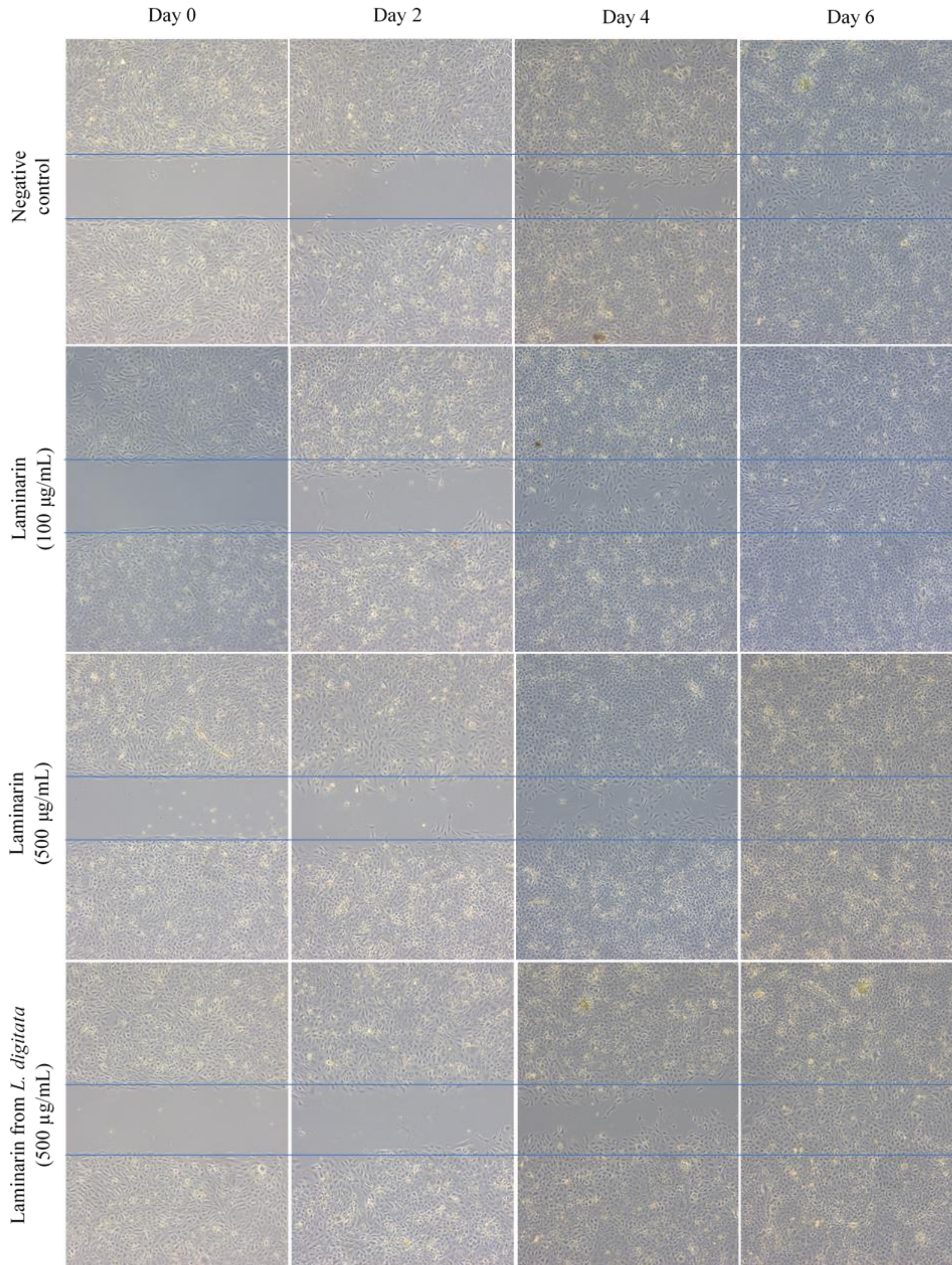

Figure 8. Pictures of RTgutGC cells treated with different concentrations (100 and 500 μg/mL) of isolated laminarin from *Alaria esculenta* and laminarin provided by Sigma-Aldrich isolated from *Laminaria digitata*. The pictures illustrate wound healing at day 0, and 2, 4 and 6 days after treatment.

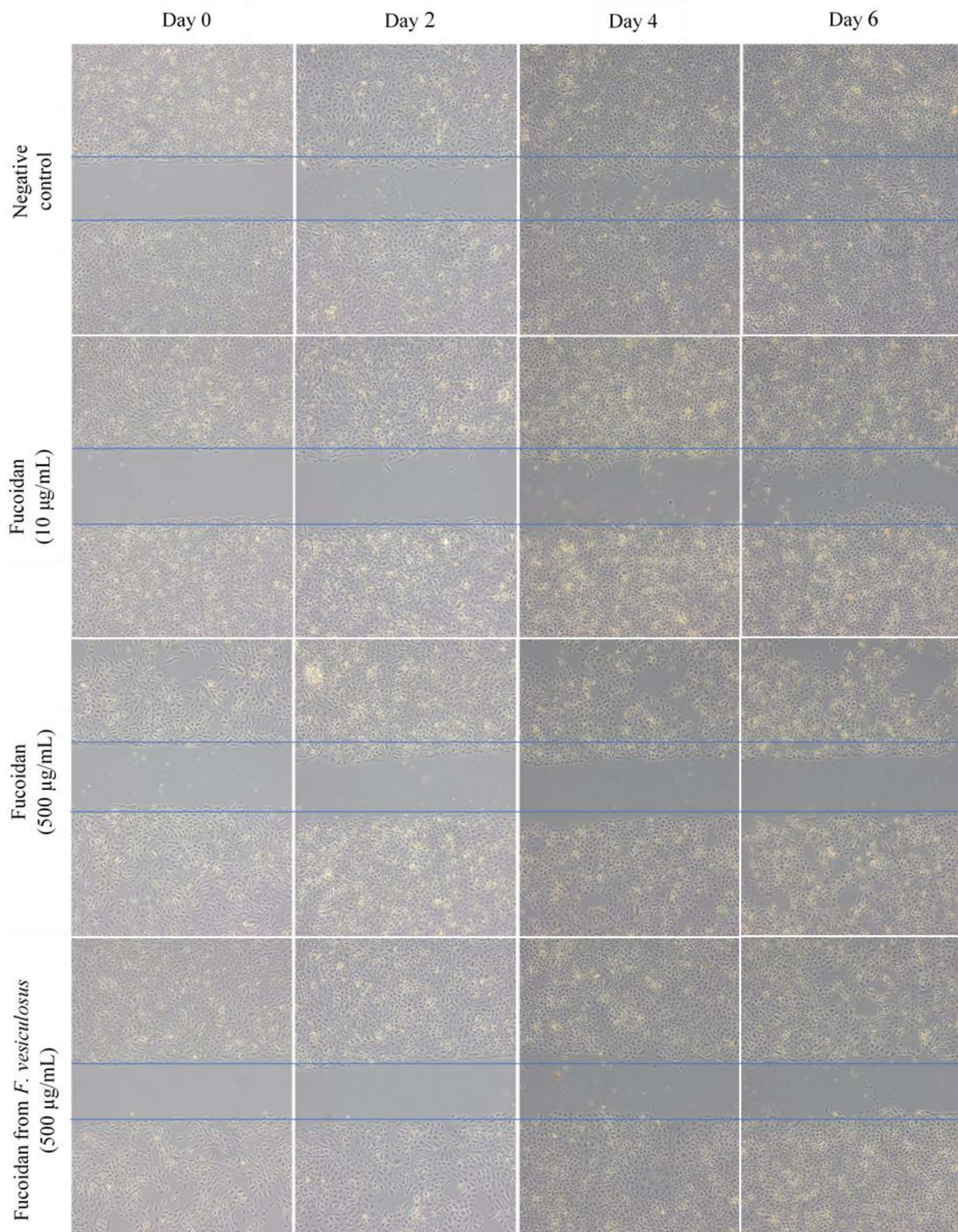

Figure 9. Pictures of RTgutGC cells treated with different concentrations (10 and 500 µg/mL) of isolated fucoidan from *Saccharina latissima* and fucoidan from Sigma-Aldrich isolated from *Fucus vesiculosus*. The pictures illustrate wound healing at day 0, and 2, 4 and 6 days after treatment.

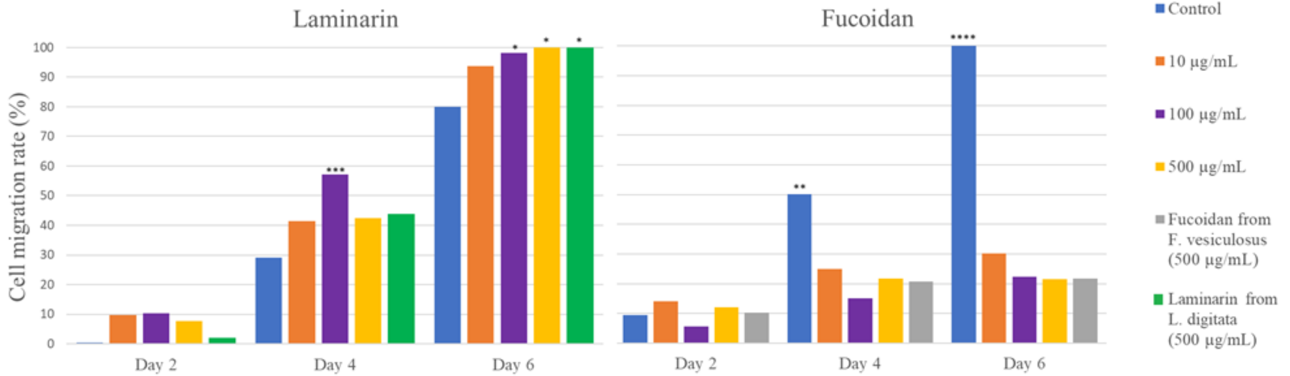

Figure 10. Cell migration rate (%) calculated as percentage of wound closure rate in each sample compared to original wound at day 0. The percentages are expressed as the mean from two experiments and samples statistically significant ($p<0.05$) from the negative control in each day is marked with a "*" (*$p<0.05$ **$p<0.01$ ***$p<0.001$ ****$p<0.0001$). In the fucoidan treated cells the negative control is marked with a "*" since all the samples at day 4 and 6 was significantly different from the negative control.

3.6 Enzymatically Treated Isolated Fractions

From the MALDI-TOF/MS (Figure 11) it was detected a series of molecular ions in the SL and AE 100 kDa fractions that corresponds to the masses of reducing hexose-based oligomers (162 *m/z*). These were detected in samples treated with β-(1→3)-Glucanase and not in the untreated samples. This indicates that the fractions contain laminarin.

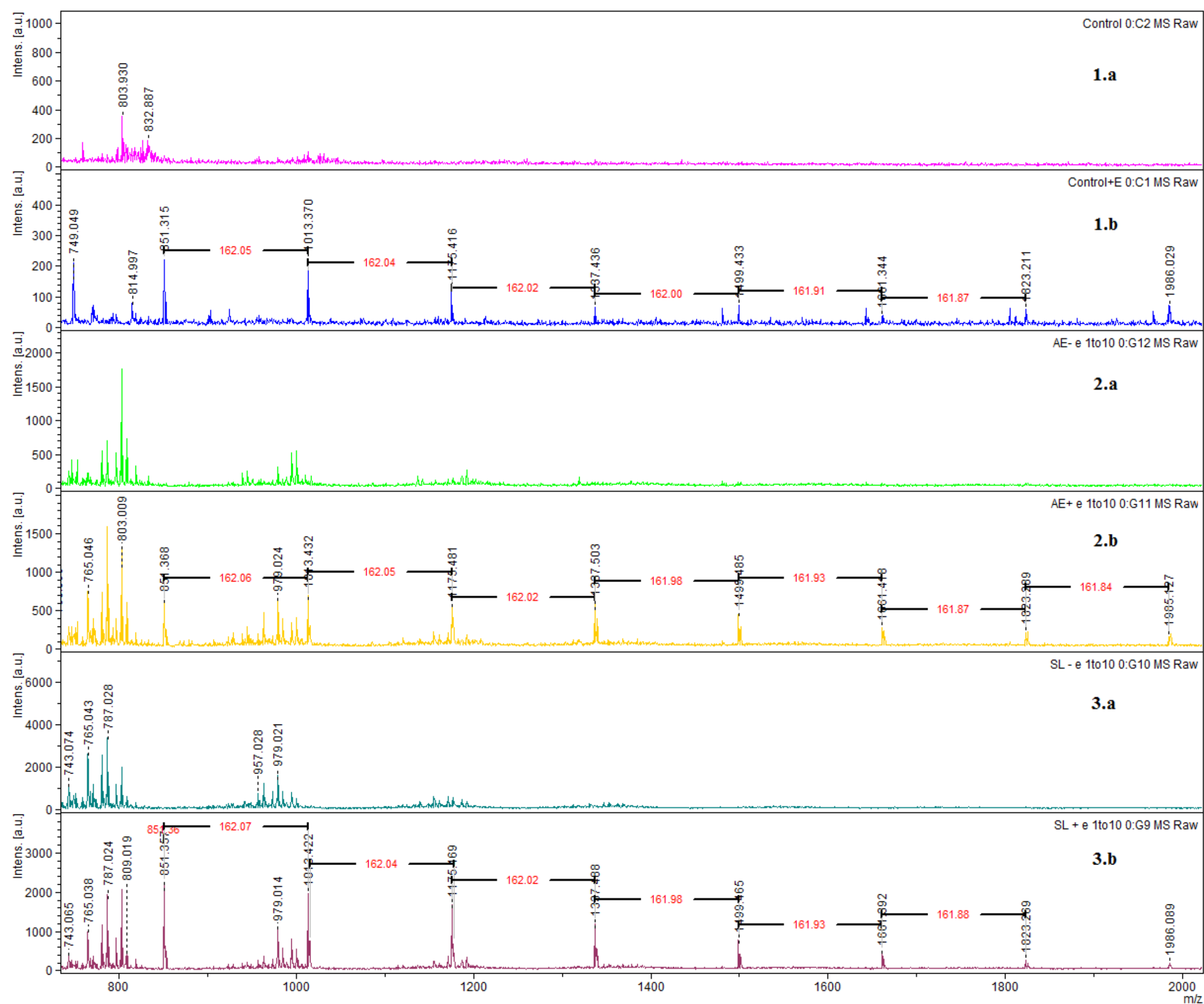

Figure 11. MALDI-TOF/MS analysis showing spectra of samples with products of hexose-based oligomers. The figure shows the mass spectra of the positive control (1 a), and the fractions SL 100 kDa (2 a), AE 100 kDa (3 a), with corresponding samples treated with enzyme (b).

4. Discussion

The increasing human population means increased demand for food, feed for fish and farmed animals, pharmaceuticals, etc., thus there is a high focus on utilising new types of biomass for this purpose. There is a growing interest in the bioactive components in macroalgae and their potential for beneficial impact on health. The aim of this thesis was to extract and isolate laminarin and fucoidan from two species of brown macroalgae, *Saccharina latissima* and *Alaria esculenta,* and to test their effect on cell migration and proliferation using intestinal epithelial cells from rainbow trout.

4.1 Chemical Composition of Native Macroalgae

The macroalgae in this experiment has a high moisture content (89-92% of the wet weight), but was harvested in late spring when moisture content in brown seaweeds are reported to be at a high level. Furthermore, these cultivated plants are young. The amount of total carbohydrates found in the macroalgae in this experiment are a bit higher than reported by Schiener et al. (2015) from the same species, but they did not include fucose or xylose in their calculations. SL has a higher mannitol content than AE, but this species is known for its high content of the sugar alcohol. AE is higher in protein indicated by the higher crude protein level and slightly lower carbon to nitrogen ratio. Otherwise, the results from the chemical composition of the native macroalgae show a similar composition, and fit the average composition of brown macroalgae reported in the literature (Holdt & Kraan 2011; Schiener et al. 2015). Through the cultivation of macroalgae, the optimal growth conditions (time, depth) for harvesting your compound of interest can be adjusted, as seen with the highest amount of sugars found in *S. latissima* at 3 meters depth (Sharma et al. 2018).

4.2 Extraction

The first round of extraction was performed on the dried macroalgae (data not presented). The results discussed are from extraction from fresh macroalgae, if not otherwise stated. Based on the

low yield in each fraction after freeze drying, it is believed that the initial extraction of dried macroalgae was incomplete. This is thought to have to do with the temperature during the extraction and the fact that it probably did not reach 70°C. It is expected that the yields could be higher if the extraction reached a higher temperature, shown by comparing the results from extraction of dried and fresh macroalgae. The incubation time of extract from fresh macroalgae was elongated to obtain the desired temperature. However, according to literature, too long incubation can negatively alter the composition and quality of specific components, so the incubation ceased before reaching 70°C. According to Ale and Meyer (2013); Holdt and Kraan (2011), solvent based extraction is common, but harsh conditions like temperature and pH causes degradation and can alter their biological properties, such as cleaving sulfate groups from fucoidans. However, results from the elemental analysis (Table 2) indicate attached sulfate groups, based on high levels of sulfur in all the fractions. Temperature and incubation time clearly play an important role in the extraction of laminarin and fucoidan, since the yield after the freeze drying of the fractions from fresh macroalgae was much higher than from the dried. Ale et al. (2012) observed that it was rather the acid concentration that negatively affected the yield and polysaccharide composition after extraction, whilst temperature and time exerted positive effects.

During the extraction, there was no visible precipitation of alginate after adding $CaCl_2$. A portion of the extract was taken out and added higher concentration of $CaCl_2$ (2 M and 3 M) and incubated in the fridge over night, but no visible precipitation was observed. However, from the results (Appendix A and B) it is shown that a considerable proportion of alginate was removed during the first rounds of filtration during the extraction and could be the reason for not seeing any precipitation. Still, there is some alginate left in the 100 kDa fractions from both species of macroalgae, but the amount of alginate decreases in the lower fractions. Thus, it could be possible to purify these fractions by the use of alginate lyase.

There are many difficulties in performing extraction from macroalgae in large volumes. To extract from fresh algae, it can be hard to control and adjust the temperature. Drying the macroalgae requires a large oven, the water from the macroalgae must be drained and the seaweed needs to be turned regularly to avoid the growth of moulds. In this experiment, we dried the macroalgae at 50°C, to avoid moulds, and to reduce the risk of changing the structure of the desired components. On the other hand, when the macroalgae is dry, it is a more stable product, easily milled and easier

to control the amount and concentration of acid to add and also the temperature during incubation. Compared, extracting fresh seaweed is a less costly approach, it does not suffer the risk of getting mould during drying or the possibility of altering the components when exposed to direct heat. However, as 25-37% of the water (of the macroalgae in this experiment) in the extraction comes from the macroalgae itself, it is more difficult to control the final concentration and temperature of the incubation, especially when dealing with large volumes. But, drying macroalgae is costly and time consuming, which you will avoid by extracting from fresh macroalgae.

4.3 Carbohydrate Analyses

Ideally, the HPAEC-PAD could be used to analyse both the neutral monosaccharides and the acidic ones, as done by Zhang et al. (2012). However, it was decided to analyse the neutral sugars with HPLC because it was a well-known and faster method. There was experienced some instability with the analysis with HPAEC-PAD concerning the running, separation and detection. The problems were suspected to be correlated with pH in the samples, which was around pH 1-2, since the mobile phase has a high pH. The instability was also experienced more frequently in the TFA hydrolysed samples. With the samples from the dried macroalgae extraction several gradients for the separation of guluronic and mannuronic acid were tested, where the best condition was chosen (described in 2.5.2). The pH in the samples were also adjusted by diluting the samples in different concentrations of NaOH, without any success. The best results from the HPAEC-PAD came from the undiluted acidic samples, as the amount injected into the mobile phase is small. These experiences were later used for the samples from the fresh macroalgae extraction.

Both hydrolysis methods (TFA hydrolysis and 2-step sulfuric acid hydrolysis) applied are fairly simple methods, but both have limitations concerning standardisation if the number of samples are high. Ideally, one would need equipment (autoclave, nitrogen gas outflow/freezedrier) to process all the samples at the same time. This was found limiting in this experiment and both methods had to be performed several times to get all the samples hydrolysed. The 2-step sulfuric acid hydrolysis method is widely used for the quantitative determination of the carbohydrate composition in macroalgae (Sluiter et al. 2008b). In a comparative study, Manns et al. (2014) concluded that this hydrolysis method released most glucose, only beaten by enzymes. The results from this

experiment (Table 1) also showed that hydrolysis with sulfuric acid resulted in a higher yield of glucose than hydrolysis with TFA in the native macroalgae samples. These studies are based on hydrolysis of native macroalgae and the same effect may not apply for isolated fractions, as shown in Figures 6 and 7 where it seems as TFA yields the highest glucose results.

The reason for including hydrolysis with TFA in this experiment was because it has resulted in higher yields of uronic acids compared to other methods. Some literature (De Ruiter et al. 1992; Manns et al. 2014) argues that the 2-step sulfuric acid hydrolysis has a disadvantage in biomass rich in uronic acids, where it has an inability to completely hydrolyse the β-(1,4) linkages in alginate. Despite lower uronic acid results, it was indicated that there had been a complete hydrolysis of the polysaccharides after sulfuric acid hydrolysis when analysed on HPAEC-PAD (Figure 5), so it might be an adequate method. Still, hydrolysis with TFA resulted in a higher yield of uronic acids than with sulfuric acid hydrolysis (Table 1). Background noise from the sample matrix could also be a possible reason for differences in the results. However, these results suggest that a combination of the two methods of hydrolysis, sulfuric acid for analysis of neutral sugars whereas TFA for uronic acid, will give the best results.

4.4 Isolation of Laminarin and Fucoidan

The objective for using the ultrafiltration system was to isolate fractions of fucoidan and laminarin based on their molecular weight. The membranes were chosen based on an experiment performed on another type of brown macroalgae, *Laminaria hyperborea* (LH) (Keihani 2016), where the highest content of fucoidan was found in the 100 kDa fraction and laminarin in the 10 kDa. In the present experiment, we did not manage to isolate pure laminarin and fucoidan fractions devoid of the other major polysaccharides, except for the 10 kDa "laminarin" fraction from AE (Figure 6 and 7). This fraction seems to be a quite pure laminarin fraction, with 21.2-30.5% glucose. In comparison, Keihani (2016) found 36.7% glucose in the laminarin from *Laminaria digitata* control from Sigma-Aldrich. However, the 10 kDa fraction from LH consisted of 83.4% glucose. There is also a high sulfur content in the 10 kDa fraction from AE, whilst fucose levels are low. This could indicate that the fucoidan present has a high degree of sulfate groups, or the sulfur could originate

from other sources such as other polysaccharides or proteins. However, the low nitrogen content in all of the fractions gives an indication of low amounts of protein.

SL has a high fucose and glucose content in both the 100 kDa and 10 kDa fractions. This could possibly be explained by a too harsh extraction method that could have degraded the fucoidan and cleaved off its sulfate groups, but a high amount of sulfate in all of the fractions (Table2) indicate attached sulfate groups. Also, an incomplete ultrafiltration on the 100 kDa membrane could have left some low molecular laminarin behind. However, the retentate was not harvested until the conductivity was below 400 µS on any of the membranes, which is considered to be a clean fraction and theoretically containing components larger than the given MW of the membrane. In comparison, the water used for the filtration has approximately the same conductivity. This give rise to the question if the macroalgae has components with different molecular weights. Especially fucoidans have been reported to appear in multiple sizes in the same macroalgae, and the components MW vary between species (Haroun-Bouhedja et al. 2000; Ponce et al. 2003). The highest amount of fucose is in the 100 kDa fraction, but the elemental analysis show that the highest amount of sulfur measured is in the 10 kDa fraction from SL. In addition, the carbon to sulfur ratio indicates that the fucose in the 10 kDa fraction has a higher degree of sulfation. A different degree of sulfation also means a difference in charge, which possibly could lead to a repulsive interaction with the coating on the ultrafiltration membrane. Still, this can indicate the presence of two types of fucoidans in SL, one high MW fucoidan and one low MW richer in sulfate groups.

Surprisingly, very high levels of glucose were found in the AE 100 kDa fraction. The MW of laminarin is reported to usually be around 2-10 kDa (Graiff et al. 2016; Hjelland et al. 2012). The other main source of glucose except for laminarin in brown macroalgae is cellulose, but is found to a lesser extent than laminarin and is not highly soluble (Holdt & Kraan 2011). It would be interesting to find out if this was a high molecular laminarin. The fraction was enzymatically treated with a β-(1→3)-Glucanase intended to cleave laminarin. It was indicated that the fraction contained laminarin since hexose-based oligomers did appear in the sample after treatment with enzyme. However, more analyses on structure and further investigation to characterise the different fractions would have been interesting. Despite low levels of fucose, only 3.4% hydrolysed with sulfuric acid, the 100 kDa fraction contains the highest amount of fucose of the

fractions isolated from AE. Either the presumed molecular weight of fucoidan in AE and hence the choice of membrane was incorrect, or fucoidan could have been lost in the filters prior to ultrafiltration. Again, the membranes were chosen based on the work on a different macroalgae species, and these results display the structural variations in macroalgae, with regards to different MW of the same component between species, and also within the same species.

It would be ideal to perform a small-scale filtration with multiple MWCO-membranes to get an indication of the composition and MW of the different polysaccharides in the macroalgae prior to the upscaled ultrafiltration experiment. Also, there are ways of purifying the fractions of unwanted components. In the 100 kDa SL fraction, it could have been possible to purify the freeze dried fraction by precipitating fucoidan with ethanol (Keihani 2016), and collecting the pellet as fucoidan. The high molecular laminarin or cellulose in the same fraction could also be enzymatically cleaved, and a new round of filtration of the fraction on a 100 kDa MWCO-membrane could have purified the fucoidan. Because of time limitations, neither were performed in this experiment.

4.5 Cell Migration and Proliferation Assay

Cell migration was significantly enhanced after treatment with laminarin in the higher concentrations. Enhanced migration rates with increasing concentration of laminarin (from 50 to 200 μg/mL) was also seen in a study by Choi et al. (2013). The two laminarin treatments at 500 μg/mL (laminarin isolated from *A. esculenta* in the present thesis and laminarin from *Laminaria digitata* purchased from Sigma-Aldrich) resulted in the same level of stimulation on cell growth and migration. However, the opposite effect was observed in all assays containing fucoidan. At the last day (day 6), the migration of 10 μg/mL fucoidan-treated cells were only 30% of the control, whereas the higher concentrations inhibited the migration of the cells even more. Similar results have been observed in cell migration and proliferation in osteoblasts and human endothelial cells (Giraux et al. 1998; Kim et al. 2015), which could also cause the anticancer effect shown for fucoidan (Yang et al. 2008), where it inhibited growth of cancer cells. Similar to Kim et al. (2015), it also looked like the cells showed a loss of adhesion (Appendix D) in wells added fucoidan in concentrations 100 and 500 μg/mL. Laminarin did not have this effect.

The cell migration assay is an easy way of indicating the bioactivity of laminarin and fucoidan. Ideally, it would have been interesting to test several of the isolated fractions. From the results, it was indicated that there could be two types of fucoidans in SL, one high molecular and one more sulfated low molecular. It would be interesting to test both, but because of limited amounts of wound healing wells, only a few of the fractions were included. The SL 100 kDa was chosen based on its high fucoidan content and that the molecular weight coincided with the literature and expectations.

Several studies (Heim et al. 2014; McDonnell et al. 2010) have observed that when laminarin and fucoidan are combined they lose their bioactivity. It would also be interesting to test a combination of the laminarin and fucoidan, either from Sigma-Aldrich or isolated here, to see if this affected laminarins proliferative effect. The SL 100 kDa fraction tested for the effect of fucoidan was not pure as it contained considerable amounts of glucose (possibly laminarin), xylose and alginate (Figure 6 and 7). However, that did not seem to inhibit fucoidans antiproliferative effect. It would also be good to attempt to purify the fractions and see if that would have made a difference on the stimulatory effect. Enzyme treatments would be a subject to future analyses, where it would have been helpful with fucoidan-active enzymes, like fucoidanases and sulfatases, to get a better structural understanding of the extraction effects.

5. Further Work

Macroalgae are characteristic by that their chemical composition varies depending on species and environmental factors. The most important biochemical components are known, but their varying structures and functions are more uncertain, thus, more detailed research in this field is needed. For macroalgae to become of commercial interest, a pipeline for the production of several different products is needed; i.e., more research to map the nutritional value for farm animals, and knowledge on how to up concentrate nutrients through fermentation and biorefinery processes is needed. Also, more research on how cultivation, environment and species differences can affect the composition of macroalgae, will facilitate choosing the optimal time of harvest for increased content of the compounds of interest (e.g., high protein level or higher level of specific bioactive components). Documentation of positive health effects is crucial to increase the value of specific macroalgae-derived products, together with more detailed research on the mode of action for their bioactivity. At the same time, it is important to continue the search for standardised, well-defined methods with gentle extraction techniques with lowered acid levels and temperature that preserves the structure and bioactivity of the components.

6. Conclusion

The chemical composition of the native macroalgae was within the range of what has been reported in the literature. Solvent based extraction from fresh macroalgae may save time and cost compared to drying the macroalgae, but it is more difficult to control the temperature during the incubation, which was proven to be an important factor in the extraction. The results indicated that the presumed molecular weight of laminarin and fucoidan in the two species of macroalgae differed from one another, resulting in some fractions not devoid of other polysaccharides. This showed the importance of performing a small scale filtration first to get an indication of the composition of the species prior to a full scale ultrafiltration. This experiment also showed that a combination of hydrolysis with sulfuric acid and trifluoroacetic acid is best for carbohydrate analysis, as sulfuric acid results in a higher release of glucose than TFA, whereas TFA hydrolysis results in higher yield of the uronic acids. However, it was managed to isolate a laminarin and fucoidan fraction and test their bioactivity. The results showed that laminarin stimulated cell migration and proliferation in intestinal epithelial cells from rainbow trout, whilst fucoidan prevented growth. These results give an indication of the immunostimulatory effects of these compounds and the potential use in the promotion or inhibition of cell migration and proliferation.

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Appendix

Appendix A: Carbohydrate Composition of Native *Saccharina latissima* and Isolated Fractions after Acid Hydrolysis with H_2SO_4 and TFA

Table 3. Carbohydrate composition after hydrolysis with sulfuric acid and trifluoroacetic acid (TFA) of native *Saccharina latissima* (SL) (g/kg DM) and fractions (g/kg isolated fraction) from extraction of fresh macroalgae.

Monosaccharide content (g/kg)	Glucose[1]	Fucose[1]	Xylose[1]	Mannitol[1]	Uronic acid[2]
Samples			Sulfuric acid		
SL	131	21	36	164	189
1. filter extraction SL	138	31	46	68	280
2. filter extraction SL	26	18	20	15	81
SL 100 kDa	140	149	96	10	48
SL 50 kDa	53	3	5	11	6
SL 10 kDa	142	104	18	8	10
			TFA		
SL	67	22	35	190	301
1. filter extraction SL	37	28	49	84	639
2. filter extraction SL	11	27	24	19	25
SL 100 kDa	190	221	144	14	118
SL 50 kDa	67	4	5	14	5
SL 10 kDa	199	183	23	7	33

[1]Determined by HPLC. [2]Determined by HPAEC-PAD

Appendix B: Carbohydrate Composition of Native *Alaria esculenta* and Isolated Fractions after Acid Hydrolysis with H_2SO_4 and TFA

Table 4. Carbohydrate composition after hydrolysis with sulfuric acid and trifluoroacetic acid (TFA) of native *Alaria esculenta* (AE) (g/kg DM) and fractions (g/kg isolated fraction) from extraction of fresh macroalgae.

Monosaccharide content (g/kg)	Glucose[1]	Fucose[1]	Xylose[1]	Mannitol[1]	Uronic acid[2]
Samples			Sulfuric acid		
AE	120	17	35	112	180
1. filter extraction AE	104	22	38	48	292
2. filter extraction AE	41	13	23	20	184
AE 100 kDa	480	34	57	19	50
AE 50 kDa	60	2	7	13	2
AE 10 kDa	212	7	13	11	8
			TFA		
AE	81	24	37	134	347
1. filter extraction AE	28	33	38	49	255
2. filter extraction AE	22	19	24	23	62
AE 100 kDa	719	51	90	24	121
AE 50 kDa	80	0	3	18	2
AE 10 kDa	305	12	16	13	17

[1]Determined by HPLC. [2]Determined by HPAEC-PAD

Appendix C: Dry Matter, Moisture and Ash Measured in Dried Macroalgae

Table 5. The table shows the dry matter, moisture and ash measured in the dried macroalgae *Saccharina latissima* (SL) and *Alaria esculenta* (AE).

	SL	AE
Dry matter	94.5%	93.6%
Moisture	5.5%	6.4%
Ash	33.5%	25.5%

Appendix D: Loss of Cell Adhesion in Fucoidan Treated Wound Healing Assay

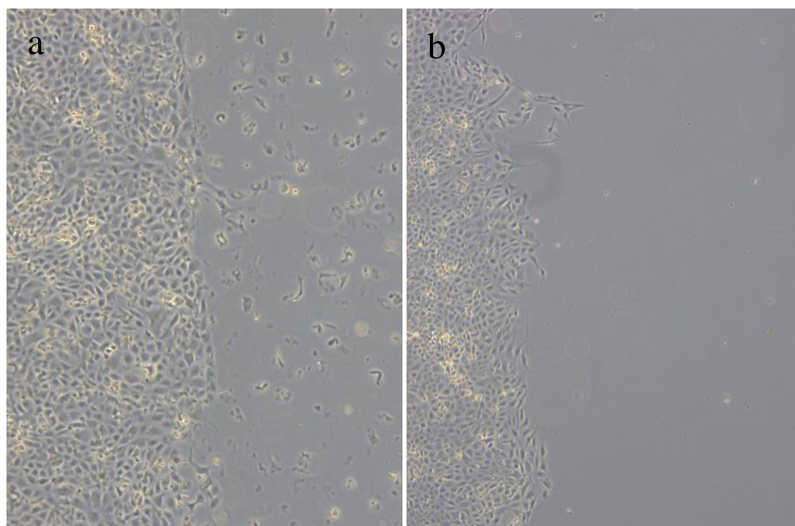


Figure 12. The pictures show cells with loss of adhesion in sample treated with 100 µg/mL isolated fucoidan (a). The picture is compared to cells treated with 100 µg/mL isolated laminarin (b). Pictures are taken with 5x phase contrast microscopy.

Appendix E: Table of Standards and Most Important Chemicals

Table 6. The table shows a list of standards and the most important chemicals in this experiment.

What	From
Purified Calcium chloride ($CaCl_2$) 22328.364 Batch: 15K270002	VWR Chemicals, USA
Hydrochloric acid 32% for analysis Batch: 1.00319.2500	Merck Emsure, Germany
Crystallized Citric acid monohydrate 20278.460 Batch: 15F150001	VWR Chemicals, USA
Sulfuric acid 98% for analysis Cas: 7664-93-9	Merck Emsure, Belgium
Trifluoroacetic acid (TFA) ≥99% Lot: STBF4960V	Sigma-Aldrich, Germany
Sodium acetate, anhydrous ≥99% Lot: BCBW1746	Sigma-Aldrich, Germany
Sodium hydroxide solution 50% Lot: H123A	Honeywell, Germany
Leibovitz's L-15 medium Cat: 21083027	Thermo Fisher Scientific, USA
5% fetal bovine serum (FBS) Cat: F7524	Sigma-Aldrich, Germany
1% gentamicin Cat. 15710049	Thermo Fisher Scientific, USA
Trypsin Cat: L0910-100	Biowest, France
β-(1→3)-D-Glucanase from *Helix pomatia* ≥0.2 U/mg Cas: 9044-93-3	Sigma-Aldrich, Germany
Standards	
L-Guluronic acid sodium salt Batch: MG615761603	Carbosynth, UK
D-Mannuronic acid sodium salt Batch: MM615751701	Carbosynth, UK
D-(+)-Glucose anhydrous Lot: 16D124104	VWR Chemicals, USA
D-(+)-Fucose ≥98% F8150 Lot: BCBM3647V	Sigma-Aldrich, Germany
D-(+)-Xylose ≥99% X3877 Lot: SLBN4994V	Sigma-Aldrich, Germany
D-Mannitol ≥98% M4125 Lot: 069K0057	Sigma-Aldrich, Germany
Fucoidan (≥95%) from *Fucus vesiculosus* F8190 Lot: SLBT5471	Sigma-Aldrich, Germany
Laminarin from *Laminaria digitata* L9634 Lot: SLBM6723	Sigma-Aldrich, Germany

Appendix F: Tukey's Multiple Comparisons Test from Samples Treated with Fucoidan

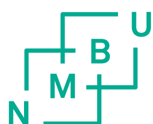
Table 7. Tukey's Multiple Comparisons Test (alpha 0.05) performed on the migration rate values measured from cells added different concentrations of fucoidan.

Tukey's Multiple Comparisons Test	Mean Diff.	95,00% CI of Diff.	Significant?	Summary	Adjusted P Value
Day 0					
Fucoidan 0 vs. 10	0	-16,6 to 16,6	No	ns	>0,9999
Fucoidan 0 vs. 100	0	-16,6 to 16,6	No	ns	>0,9999
Fucoidan 0 vs. 500	0	-16,6 to 16,6	No	ns	>0,9999
Fucoidan 0 vs. Fucus vesiculosus	0	-16,6 to 16,6	No	ns	>0,9999
10 vs. 100	0	-16,6 to 16,6	No	ns	>0,9999
10 vs. 500	0	-16,6 to 16,6	No	ns	>0,9999
10 vs. Fucus vesiculosus	0	-16,6 to 16,6	No	ns	>0,9999
100 vs. 500	0	-16,6 to 16,6	No	ns	>0,9999
100 vs. Fucus vesiculosus	0	-16,6 to 16,6	No	ns	>0,9999
500 vs. Fucus vesiculosus	0	-16,6 to 16,6	No	ns	>0,9999
Day 2					
Fucoidan 0 vs. 10	-4,75	-21,35 to 11,85	No	ns	0,9093
Fucoidan 0 vs. 100	3,8	-12,8 to 20,4	No	ns	0,9574
Fucoidan 0 vs. 500	-2,6	-19,2 to 14	No	ns	0,9893
Fucoidan 0 vs. Fucus vesiculosus	-0,8	-17,4 to 15,8	No	ns	0,9999
10 vs. 100	8,55	-8,049 to 25,15	No	ns	0,5492
10 vs. 500	2,15	-14,45 to 18,75	No	ns	0,9948
10 vs. Fucus vesiculosus	3,95	-12,65 to 20,55	No	ns	0,9513
100 vs. 500	-6,4	-23 to 10,2	No	ns	0,7765
100 vs. Fucus vesiculosus	-4,6	-21,2 to 12	No	ns	0,9184
500 vs. Fucus vesiculosus	1,8	-14,8 to 18,4	No	ns	0,9974
Day 4					
Fucoidan 0 vs. 10	25,1	8,501 to 41,7	Yes	**	0,0017
Fucoidan 0 vs. 100	34,9	18,3 to 51,5	Yes	****	<0,0001
Fucoidan 0 vs. 500	28,3	11,7 to 44,9	Yes	***	0,0005
Fucoidan 0 vs. Fucus vesiculosus	29,35	12,75 to 45,95	Yes	***	0,0003
10 vs. 100	9,8	-6,799 to 26,4	No	ns	0,4188
10 vs. 500	3,2	-13,4 to 19,8	No	ns	0,977
10 vs. Fucus vesiculosus	4,25	-12,35 to 20,85	No	ns	0,9374
100 vs. 500	-6,6	-23,2 to 9,999	No	ns	0,757
100 vs. Fucus vesiculosus	-5,55	-22,15 to 11,05	No	ns	0,852
500 vs. Fucus vesiculosus	1,05	-15,55 to 17,65	No	ns	0,9997
Day 6					
Fucoidan 0 vs. 10	69,8	53,2 to 86,4	Yes	****	<0,0001
Fucoidan 0 vs. 100	77,65	61,05 to 94,25	Yes	****	<0,0001
Fucoidan 0 vs. 500	78,45	61,85 to 95,05	Yes	****	<0,0001
Fucoidan 0 vs. Fucus vesiculosus	78,25	61,65 to 94,85	Yes	****	<0,0001
10 vs. 100	7,85	-8,749 to 24,45	No	ns	0,6255
10 vs. 500	8,65	-7,949 to 25,25	No	ns	0,5384
10 vs. Fucus vesiculosus	8,45	-8,149 to 25,05	No	ns	0,5601
100 vs. 500	0,8	-15,8 to 17,4	No	ns	0,9999
100 vs. Fucus vesiculosus	0,6	-16 to 17,2	No	ns	>0,9999
500 vs. Fucus vesiculosus	-0,2	-16,8 to 16,4	No	ns	>0,9999

Appendix G: Tukey's Multiple Comparisons Test from Samples Treated with Laminarin

Table 8. Tukey's Multiple Comparisons Test (alpha 0.05) performed on the migration rate values measured from cells added different concentrations of laminarin.

Tukey's Multiple Comparisons Test	Mean Diff.	95,00% CI of Diff.	Significant?	Summary	Adjusted P Value
Day 0					
Laminarin 0 vs. 10	0	-16,68 to 16,68	No	ns	>0,9999
Laminarin 0 vs. 100	0	-16,68 to 16,68	No	ns	>0,9999
Laminarin 0 vs. 500	0	-16,68 to 16,68	No	ns	>0,9999
Laminarin 0 vs. Laminaria digitata	0	-16,68 to 16,68	No	ns	>0,9999
10 vs. 100	0	-16,68 to 16,68	No	ns	>0,9999
10 vs. 500	0	-16,68 to 16,68	No	ns	>0,9999
10 vs. Laminaria digitata	0	-16,68 to 16,68	No	ns	>0,9999
100 vs. 500	0	-16,68 to 16,68	No	ns	>0,9999
100 vs. Laminaria digitata	0	-16,68 to 16,68	No	ns	>0,9999
500 vs. Laminaria digitata	0	-16,68 to 16,68	No	ns	>0,9999
Day 2					
Laminarin 0 vs. 10	-9,384	-26,06 to 7,294	No	ns	0,4654
Laminarin 0 vs. 100	-9,802	-26,48 to 6,876	No	ns	0,4233
Laminarin 0 vs. 500	-7,233	-23,91 to 9,445	No	ns	0,6956
Laminarin 0 vs. Laminaria digitata	-1,673	-18,35 to 15,01	No	ns	0,9981
10 vs. 100	-0,4179	-17,1 to 16,26	No	ns	>0,9999
10 vs. 500	2,151	-14,53 to 18,83	No	ns	0,9949
10 vs. Laminaria digitata	7,711	-8,967 to 24,39	No	ns	0,6446
100 vs. 500	2,569	-14,11 to 19,25	No	ns	0,99
100 vs. Laminaria digitata	8,129	-8,549 to 24,81	No	ns	0,5993
500 vs. Laminaria digitata	5,56	-11,12 to 22,24	No	ns	0,8534
Day 4					
Laminarin 0 vs. 10	-12,31	-28,98 to 4,373	No	ns	0,2172
Laminarin 0 vs. 100	-28,13	-44,81 to -11,45	Yes	***	0,0005
Laminarin 0 vs. 500	-13,21	-29,89 to 3,465	No	ns	0,1645
Laminarin 0 vs. Laminaria digitata	-14,81	-31,48 to 1,872	No	ns	0,0973
10 vs. 100	-15,82	-32,5 to 0,8533	No	ns	0,0681
10 vs. 500	-0,9076	-17,59 to 15,77	No	ns	0,9998
10 vs. Laminaria digitata	-2,501	-19,18 to 14,18	No	ns	0,991
100 vs. 500	14,92	-1,761 to 31,6	No	ns	0,0936
100 vs. Laminaria digitata	13,32	-3,354 to 30	No	ns	0,1588
500 vs. Laminaria digitata	-1,593	-18,27 to 15,09	No	ns	0,9984
Day 6					
Laminarin 0 vs. 10	-13,78	-30,46 to 2,899	No	ns	0,1372
Laminarin 0 vs. 100	-18,07	-34,75 to -1,393	Yes	*	0,0297
Laminarin 0 vs. 500	-20,02	-36,7 to -3,346	Yes	*	0,0139
Laminarin 0 vs. Laminaria digitata	-20,02	-36,7 to -3,346	Yes	*	0,0139
10 vs. 100	-4,292	-20,97 to 12,39	No	ns	0,9363
10 vs. 500	-6,245	-22,92 to 10,43	No	ns	0,794
10 vs. Laminaria digitata	-6,245	-22,92 to 10,43	No	ns	0,794
100 vs. 500	-1,953	-18,63 to 14,72	No	ns	0,9965
100 vs. Laminaria digitata	-1,953	-18,63 to 14,72	No	ns	0,9965
500 vs. Laminaria digitata	0	-16,68 to 16,68	No	ns	>0,9999



Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway