Norwegian University of Life Sciences

Master's Thesis 2022 60 ECTS

Faculty of Biosciences
Norwegian University of Life Sciences (NMBU)

Seaweeds Pretreatment Methods for Long Term Storage; Effect on Nutrients, Bioactive Compounds and General Sensory Characteristics.

Kant Owusu Fokuo
Master of Science – Feed Manufacturing Technology

Acknowledgements

I would like to thank my main supervisor Dr. Liv Torunn Mydland for her guidance, patience, and support and who besides reading and reviewing this thesis, explored all possible means to make my efforts most productive. I would also like to thank my supervisor Prof. Margareth Øverland for giving me the opportunity and resources to work in such a fascinating field. My sincere gratitude also goes to my co-supervisors Dr. Nanna Rhein-Knudsen for her guidance and support and to Dr. Ruth Tamara Montero Meza for her guidance, inspiration, and assistance.

Furthermore, I would like to thank Jon Øvrum Hansen and Leszek Michalak for the opportunity and for introducing me to macroalgae research. I also thank Prof. Svein Jarle Horne for the resources and laboratory space during the compositional analysis and to Elise, Ashwath, Dominic, and Diego for their assistance. Finally, I would like to acknowledge the entire team at Foods of Norway (FoN) – Sergio, Veronica and Christine for their invaluable assistance and support during the fucoidans bioactivity analysis.

Abstract

Global human population is projected to increase by more than 34 % to over 9 billion people by 2050. As food, feed, fuels, and pharmaceutical sources are crucial to support this population increase, researchers are putting efforts in to finding novel sustainable ingredients and means to produce these valuable resources. Currently, there is high focus on macroalgae as an alternative sustainable ingredient due to its positive contribution to ecosystem preservation, particularly in reducing greenhouse gases and in bioremediation. Norway has an extensive coastline and an already established competence in marine raw materials processing and related technologies and thus have great prospects in macroalgae cultivation and value addition. The large-scale utilization of macroalgae, particularly in the food and pharmaceutical industries, will require continuous supply of the macroalgae biomass. However, harvesting is currently done seasonally (usually between the months of April to June) in Norway. Therefore, it is imperative to develop suitable low cost and low energy methodology for preserving the harvested biomass for long periods, while maintaining an intact chemical composition. The aim of this thesis was to investigate the storage stability of brown macroalgae – *Saccharina latissima*, preserved for a long period (approx. 2.5 months) and to determine the effects of different pretreatment methods such as ensiling with different additives, microbes, and chemical stabilizers, on the general sensory properties and chemical composition. Another aim was to determine the bioactivity of fucoidans – an important biomolecule found in brown macroalgae. To do this, the macroalgae samples were subjected to 20 different treatments and stored vacuum packed for approx. 2.5 months. Afterwards the samples were physically analysed to check for colour change, smell, and fungal growth. Compositional analyses were conducted to assess any differences in compositions due to the different pretreatments. And subsequently, extraction of fucoidans and bioactivity analysis in head kidney leukocytes of Atlantic salmon was conducted. The results indicated that higher volumes of the additives were generally more effective in preserving the samples than lower volumes. Furthermore, although freezing is an effective preservation method, it is not feasible for larger volumes of macroalgae biomass, due to high energy and space requirements and high associated cost. Instead, preservation using formic acid 85 % was the most effective method based on the amount of fucose (major constituent of fucoidans) and purity of the

fucoidan-rich seaweeds extracts. The other effective pretreatments were ensiling with Sil-All® 4x4, no additive (storing at room temperature), GrasAAT® Plus and GrasAAT® Lacto. Finally, the bioactivity of fucoidan-rich seaweeds extracts from *S. latissima* was confirmed based on its ability to induce an upregulation of the *tnfa* gene in head kidney leukocytes from Atlantic salmon.

Sammendrag

Den globale menneskelige befolkningen anslås å øke med mer enn 34 % til over 9 milliarder mennesker innen 2050. Ettersom mat, fôr, drivstoff og farmasøytiske kilder er avgjørende for å støtte denne befolkningsøkningen, satser forskere på å finne nye bærekraftige ingredienser og midler å produsere disse verdifulle ressursene. For tiden er det høyt fokus på makroalger som en alternativ bærekraftig ingrediens på grunn av dets positive bidrag til bevaring av økosystemer, spesielt for å redusere klimagasser og i bioremediering. Norge har en omfattende kystlinje og en allerede etablert kompetanse innen marin råvareforedling og relaterte teknologier og har dermed store utsikter innen makroalgedyrking og verdiøkning. Storskala utnyttelse av makroalger, spesielt i næringsmiddel- og farmasøytisk industri, vil kreve kontinuerlig tilførsel av makroalgenes biomasse. Innhøstingen foregår imidlertid i dag sesongmessig (vanligvis mellom april og juni måned) i Norge. Derfor er det viktig å utvikle passende lavkostnads- og lavenergimetodikk for å bevare den høstede biomassen i lengre perioder, samtidig som den opprettholder en intakt kjemisk sammensetning. Målet med denne oppgaven var å undersøke lagringsstabiliteten til brune makroalger – *Saccharina latissima*, konservert over en lang periode (ca. 2,5 måneder) og å bestemme effekten av ulike forbehandlingsmetoder som ensilering med ulike tilsetningsstoffer, mikrober, og kjemiske stabilisatorer, om generelle sensoriske egenskaper og kjemisk sammensetning. Et annet mål var å bestemme bioaktiviteten til fucoidans – et viktig biomolekyl som finnes i brune makroalger. For å gjøre dette ble makroalgeprøvene utsatt for 20 ulike behandlinger og lagret (vakuumpakket) i ca. 2,5 måneder. Etterpå ble prøvene fysisk analysert for å se etter fargeendring, lukt og soppvekst. Sammensetningsanalyser ble utført for å vurdere eventuelle forskjeller i sammensetninger på grunn av de forskjellige forbehandlingene. Og deretter ble det utført ekstraksjon av fucoidans og bioaktivitetsanalyse i hodenyreleukocytter fra atlantisk laks. Resultatene indikerte at høyere volumer av tilsetningsstoffene generelt var mer effektive for å bevare prøvene enn lavere volumer. Videre, selv om frysing er en effektiv konserveringsmetode, er det ikke mulig for større volumer av makroalgebiomasse, på grunn av høye energi- og plassbehov og høye tilknyttede kostnader. I stedet var konservering med maursyre 85 % den mest effektive metoden basert på mengden fucose (hovedbestanddelen av fucoidans) og renheten til det fucoidanrike tangekstraktet. De andre effektive

forbehandlingene var ensilering med Sil-All® 4x4, uten tilsetning (oppbevaring ved romtemperatur), GrasAAT® Plus og GrasAAT® Lacto. Til slutt ble bioaktiviteten til fucoidan-rik tangekstrakt fra *S. latissima* bekreftet basert på dens evne til å indusere en oppregulering av *tnfa*-genet i hodenyreleukocytter fra atlantisk laks.

List of Abbreviations

A.G.D	Amoebic gill disease
CP	Crude protein
DM	Dry matter
DS	Degree of sulfation
FCSPs	Fucose containing sulfated polysaccharides
Gulu A (G)	α-L-guluronic acid
H.K	Head kidney
HMW	High molecular weight
HPAEC	High performance anion exchange chromatography
LAB	Lactic acid bacteria
LMW	Low molecular weight
Man A (M)	ß-D-mannuronic acid
RT	Room temperature
S.L	*Saccharina latissima*
tnfa	Tumor necrosis factor alpha

List of Tables

List of Figures

Table of Contents

1.0 INTRODUCTION

1.1 Background

Global human population is expected to increase drastically by more than 34 %, corresponding to over 9 billion people, in the next half century. As food sources are crucial to support this population increase, food production must also increase by about 70 % to meet the demands of the population (Wise, 2013). This makes it imperative to find sustainable means of producing food and feed, fuels, and medicine. Traditional means of agriculture have a deteriorating effect on the environment because of the negative impacts on biodiversity, erosion, and desertification (Cai et al., 2021). Currently, there is high focus on macroalgae, because of their potential as an alternative ingredient source for producing functional food and feed. Additionally, macroalgae positively contribute to the ecosystem preservation especially by reducing greenhouse gases and in bioremediation (Tanaka et al., 2020, Cai et al., 2021). The large-scale utilization of macroalgae as food and feed ingredient or through a biorefinery process requires a continuous and dependable supply of the macroalgae biomass throughout the year, although cultivation and harvesting in Norway is currently done seasonally, usually between the months of April to June. Therefore, preservation and long-term storage of the biomass, as well as maintaining an intact nutritional and bioactive composition is particularly important. The two commonly used methods for macroalgae preservation – drying and freezing – tend to be very costly, especially to preserve large volumes of the biomass. Hence a more efficient method is needed (Øverland et al., 2019).

According to a publication by Black (1955), lactic acid fermentation could be an effective preservation method for macroalgae due to the production of lactate and other organic acids which lower the pH and prevent the growth of spoilage microbes. However, this is challenging because naturally occurring acidification of macroalgae is improbable, due to the inadequate amount of rapidly fermentable carbohydrates and lactic acid bacteria (LAB), and also the high buffering capacity of the macroalgae. Other literature have reported that, the inclusion of a starter culture of LAB, a process called ensiling, and the activities of cellulase in hydrolyzing polysaccharides such as cellulose and laminarin found in macroalgae, result in fermentation leading to preservation of the biomass (Uchida et al., 2013). Thus,

the ensiling process, involves the biochemical change of carbohydrates into lactic acid, acetic acid and other organic acids, leading to a lower pH which prevents growth of spoilage microbes and favoring preservation of macroalgae biomass (Maneein et al., 2018). Ensiling is also effective particularly for wet biomasses and requires lower energy input than other storage methods like freezing and drying (Herrmann et al., 2015). Therefore, by exploring and further investigating a potentially effective and sustainable preservation method like ensiling, it will facilitate the large scale utilization of macroalgae as an alternative sustainable ingredient for feed, fuel, cosmetics, fertilizers and pharmaceutical products, ensuring an all year round supply of chemically intact macroalgae biomass (Herrmann et al., 2015, Miyashita et al., 2013).

1.2 Macroalgae

Macroalgae or seaweeds refer to the many different species of aquatic plants and algae that are photosynthetic in nature, and are also able to absorb essential nutrients from their surrounding environment using their thalli (de Castro et al., 2017). They are broadly classified into three main taxonomic classes namely (Cai et al., 2021):

i. Rhodophyta or red seaweeds (over 7200 species)
ii. Chlorophyta or green seaweeds (more than 1800 species)
iii. Phaeophyta or brown seaweeds (around 2000 species)

Presently, out of the over 220 species of seaweeds of commercial interest, only less than 10 % are actively cultivated, studied, and intensely utilized. The North Atlantic Ocean in Norway, has a wide array of cultivated kelp (large brown algae) species – mainly sugar kelp *Saccharina latissima* (S.L) and winged kelp *Alaria esculenta*; while the Eastern Pacific Ocean is dominated with kelp species *Macrocystis*, *Undaria pinnatifida*, S.L and *A. esculenta* (Kim et al., 2017). Seaweeds are more commonly used in Asia for human consumption than in western societies. The initial use of seaweeds in Europe was for extracting high-value hydrocolloids such as alginate, agar, and carrageenans, used as gelling agents in food and biotechnological processes (Kim et al., 2017, Rhein-Knudsen et al., 2015).

Currently, seaweeds are used more commonly as vegetable or nutraceuticals in Europe and America. In fact, the kelp aquaculture industry in Europe has become one of the fastest growing industries (Kim et al., 2017). Nearly all cultivated or farmed macroalgae are grown in Asia – predominantly in China. Whereas wild macroalgae are predominantly harvested in China, Chile and Norway (Cai et al., 2021).

In Europe, Norway leads in total macroalgae production (farmed and wild) and has a very large reserve and potential for increase production (Cai et al., 2021). This is due to the wide and extensive Norwegian coastline, which covers approx. 102,936 km (2nd largest overall coastline after Canada) – highly suitable for aquaculture (Thorsnæs, 2022). And also an already established competence in marine raw materials processing and related technology (Stévant, 2019). Increasing cultivation of macroalgae is essential, not only to expand the production of this renewable resource but also to selectively control the chemical composition, biomass yield and other preferable features in the macroalgae. For instance, Forbord et al. (2020) investigated the effects of cultivation depth, season, light exposure, temperature and area of cultivation of macroalgae (S.L) in regards to biomass yield, length of frond, biofouling and chemical composition. The research concluded that biomass growth was optimal at 1 m to 2 m depth (than at depths higher that 7 m), biofouling was minimal at areas influenced by fresh water and a variance in chemical composition due to latitude and season of cultivation were observed. Thus, preferable features or compositions of macroalgae can be attained through selective cultivation of species and control of cultivation conditions.

1.3 Nutrients in macroalgae

Macroalgae are known for the polysaccharides, minerals, and vitamins they contain, as well as other bioactive compounds (like fucoidan in Phaeophytes) beyond basic nutrition (Holdt et al., 2011, Stévant, 2019). The moisture content of seaweeds is very high ranging between 72 % to 94 %, while the dry matter (DM) content is very low (less than 15%). Macroalgae have small amounts of lipids and very high mineral content of about 10 to 20 times that of land plants (Stévant, 2019). Some authors have also reported a carbohydrate concentration of 26 % to 74 % of DM, crude protein concentration of 5 % to

17 % of DM, lipid concentration of less than 1 % of DM, ash concentration of 6 % to 45 % of DM and 75 % to 96 % moisture content (Ahmad et al., 2016). Macroalgae have high amounts of non-protein nitrogen (N), and hence the level of protein may be overestimated. To correct this, a nitrogen-to-protein factor of 5 has been proposed instead of 6.25 for many feed ingredients (Angell et al., 2016, Stévant, 2019). Furthermore, other studies have reported that the carbohydrate composition of seaweeds is about 50 % of DM, made up mainly of polysaccharides with a low quantity of disaccharides and monosaccharides (Rioux et al., 2015). Polysaccharides are polymers of monosaccharides joined by glycosidic bond (Holdt et al., 2011). The polysaccharides of brown seaweeds include laminarin, alginate and fucoidans which have the potential to perform immune-modulatory, nutritional and antioxidant functions (Ahmad, 2015, Holdt et al., 2011). The polysaccharides in red seaweeds are agar and carrageenan while green seaweeds contain ulvans (Shao et al., 2022) The polysaccharides can be easily hydrolysed under acidic conditions to produce monosaccharides (Manns et al., 2014). Hence acid hydrolysis and compositional analysis provide an insight into the concentrations of neutral sugars like glucose and fucose present in the seaweeds.

1.4 Brown macroalgae

Brown macroalgae are a very common type of macroalgae found along the coast of Norway and they are known for their large size and high biomass yield (Øverland et al., 2019). They belong to the phylum Phaeophyta and contain the xanthophyll called fucoxanthin, that masks the green coloured chlorophyll pigments leading to their brown colour and hence name (Morrissey et al., 2001). The moisture content of Phaeophytes or brown seaweeds ranges between 61 % to 94 % while ash content is between 15 % to 39 % of DM, protein content around 3 % to 15 % of DM and polysaccharides content ranging between 38 % to 61 % of DM for species in genera *Saccharina* and *Laminaria* (Holdt et al., 2011). Other authors have also reported that the polysaccharide content accounted for more than 50 % of the total DM and can reach up to 70 % in some species (Hakim et al., 2020). Therefore, brown seaweeds have high moisture content with moderate levels of ash and carbohydrate but very low protein content.

Furthermore, the low amounts of protein and metabolizable energy but high amount of minerals reported in brown macroalgae prevents the possibility of being used to replace fishmeal or other main protein sources in animal feed production (Øverland et al., 2019). However, brown macroalgae can serve as a valuable source of essential bioactive compounds.

The cell wall of macroalgae is made up of cellulose and hemicellulose that fortify the structure of the thallus. The main polysaccharides present in brown macroalgae are fucoidan, laminarin, cellulose and alginate (Deniaud-Bouët et al., 2014). These polysaccharides can be hydrolysed to neutral monosaccharides such as fucose, glucose, galactose, mannose, xylose, and mannitol as well as uronic acids. Fucose is the main constituent of fucoidans while glucose is the main constituent of laminarin and cellulose. Mannuronic acid (Man A) and guluronic acid (Gulu A) are the main constituents of alginate with S.L having Man A, Gul u A and glucuronic acid (Glu A) concentrations of around 36.1 % of DM, 9.3 % of DM and 1.9 % of DM respectively, depending on season and place of harvest (Nguyen et al., 2020).

Brown seaweeds are the most cultivated seaweeds in Norway, mainly due to their large size and high biomass production (Stévant, 2019, James et al., 2020). The levels of carbohydrates in brown seaweeds found in cold regions in the northern hemisphere (e.g., Europe) changes throughout the year. For instance, the level of matrix polysaccharides such as alginate and fucoidan are highest at early days of spring season where the storage materials mannitol and laminarin are lowest. The opposite happens in the fall season when matrix polysaccharides are lowest compared to high levels of reserve materials – mannitol and laminarin (Manns et al., 2014). Additionally, the carbohydrate structures and composition of brown seaweeds vary with species, age of the algae population, season and geographical location (Ahmad, 2015, Holdt et al., 2011). Consequently, it is difficult to generalize on the concentrations of biochemical components of brown seaweeds due to the seasonal changes, species and environmental conditions (Ahmad, 2015, Holdt et al., 2011). Finally, instead of using whole brown seaweeds as feed of low nutritional value – for fish and monogastric organisms – a better utilization option will be to extract valuable bioactive compounds like fucoidans to potentially modulate the immune system, improve growth rate and development of organisms (Øverland et al., 2019)

1.4.1 *Saccharina latissima*

Saccharina latissima (S.L) is a well-known brown macroalgae species found along the coastline of Norway. At present, there is a fast rising industry around its cultivation and high interest to utilize its bioactive compounds as a supplement in food and feed, pharmaceutical products, biofuels and other value-added products (Sharma et al., 2018). S.L belongs to the class Phaeophyceae, family Laminariaceae and genus *saccharina*. S.L is also called sugar kelp because of the dried whitish, sweet tasting powder that forms on the fronds (White et al., 2007). It usually grows in the sublittoral zone in a depth range less than 30 m but preferably around 3 m to obtain high levels of sugars and around 8 m depth to obtain high levels of amino acid, and minerals (White et al., 2007, Sharma et al., 2018). S.L are characterized by a long undivided frond, up to about 4 m long and undergo the fastest growth rate from winter to spring at a rate of approximately 1.1 m per day or higher. However growth rate for this brown macroalgae specie declines in June and can completely halt in late summer due to nitrate reduction in summer (White et al., 2007). **Table 1** shows the content of monosaccharides, uronic acids, total sugars, total phenolic content, and ash content of sugar kelp S.L cultivated at different depths (3 m and 8 m) and harvested in different months (May, June, August). It can be observed from **Table 1** that the chemical composition of S.L is affected by depth of cultivation and season of harvest. For example, the chemical composition of S.L cultivated at a depth of 3 m and harvested in May is different from the composition of S.L cultivated at 8 m depth and harvested in August (**Table 1**). In addition, the highest total sugar and uronic acids levels were observed at a depth of cultivation of 3 m while highest minerals level were observed at 8 m for the 3 months of study (**Table 1**). The highest glucose and fucose levels were measured in May and June (at the beginning of spring), at a depth of cultivation of 3 m except an anomaly where there was higher fucose level at 8 m depth of cultivation in June (Sharma et al., 2018).

Table 1: Amount of glucose, mannitol, xylose, fucose, uronic acids, ash, total phenolic, and total sugars (in g/ kg DM) present in S.L at different seasons (months) of harvesting and depth of cultivation (Sharma et al., 2018).

Chemical constituents	May 3m	May 8m	June 3m	June 8m	August 3m	August 8m
Glucose	140.8 ± 2.0	120.1 ± 2.2	173.4 ± 5.5	112.4 ± 3.0	66.8 ± 1.3	37.7 ± 0.5
Mannitol	140.7 ± 2.7	115.4 ± 2.5	158.4 ± 5.2	96.5 ± 1.2	68.9 ± 0.2	20.5 ± 0.5
Uronic acids	170.7 ± 3.1	170.6 ± 4.4	148.5 ± 2.3	127.9 ± 3.3	64.7 ± 5.4	63.7 ± 4.3
Xylose	41.6 ± 1.5	42.4 ± 1.4	31.9 ± 2.4	41.2 ± 1.5	18.9 ± 0.3	18.4 ± 0.4
Fucose	28.5 ± 0.2	25.5 ± 0.8	22.3 ± 2.7	27.9 ± 10.6	13.9 ± 0.2	13.1 ± 0.3
Total sugars	522.3 ± 9.5	474 ± 11.3	534.5 ± 18.1	405.9 ± 10.6	233.2 ± 7.4	153.4 ± 6.0
Total phenolic content	0.9 ± 0.04	1.1 ± 0.04	1.0 ± 0.1	0.7 ± 0.01	1.3 ± 0.01	1.4 ± 0.01
Ash	272.8 ± 5.6	275.9 ± 2.8	252.7 ± 10.0	327.4 ± 4.9	366.6 ± 4.4	411.5 ± 9.6

1.5 Bioactive components of brown macroalgae

1.5.1 Cell wall structure

The cell wall of Phaeophytes is a layered matrix of composite polysaccharides fundamentally made up of alginate, fucoidans, laminarin, hemicellulose, proteins, polyphenol, and other compounds (Deniaud-Bouët et al., 2014, Maneein et al., 2018) . Thus, Phaeophytes are made up of cell wall system of assembled and inter-connecting polysaccharides. Fucoidan, alginate, cellulose and hemicellulose provide structural support while laminarin is a storage polysaccharide (Deniaud-Bouët et al., 2017). The fucoidans (and other Fucose-containing sulfated polysaccharides (FCSPs)) interlock with the ß-glucan structural frame composed of alginates and polyphenols that are joined together. Other constituents that support this structure are proteins, and ions such as calcium ion. The cross-linking between the phenols and alginates, together with the interlocking between alginate and calcium ions, function to ensure the

rigidity of the cell wall structure (Deniaud-Bouët et al., 2017, Maneein et al., 2018) as shown in **Figure 1.**

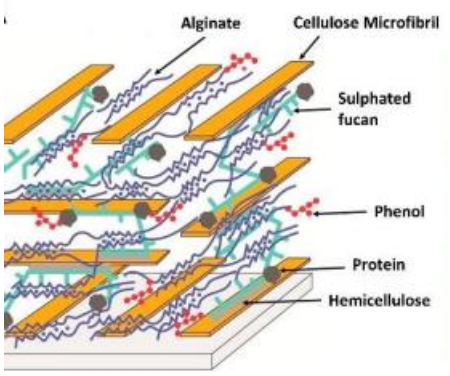


Figure 1: Cell wall model of brown macroalgae (Maneein et al., 2018).

Alginate and fucoidans (and FCSPs) constitute a major part of the cell wall polymer. The FCSPs interlock with alginate and other components like cellulose while phenols form bridging with alginate. These interlockings of cell wall components make their extraction complicated (Deniaud-Bouët et al., 2017). In consequence, breaking down the cell is required to release and easily extract these components. The gelling abilities and physicochemical properties of alginate reduce efficiency of extraction of other water-soluble polysaccharides like fucoidan (Deniaud-Bouët et al., 2014, Deniaud-Bouët et al., 2017). Conversely, laminarin is situated in the vacuoles present in the cells hence breaking down the cell wall does not directly lead to higher laminarin yield (Kadam et al., 2015).

1.5.2 Fucoidans

FCSPs refer to a group of chemically different sulfated polysaccharides that are rich in fucose and extracted from brown macroalgae (Ale et al., 2011, Li et al., 2008). Among the various types of FCSPs, fucoidans or fucans (formerly called fucoidin) are the most studied; however, there are other types which include sulfated galactofucans extracted from *Sargassum sp.* with a variation in structure to fucoidans. Fucoidans are made up of a backbone of (1,3)- linked α-L fucopyranosyl molecules or alternating (1,3) and (1,4) linked α-L fucopyranosyl molecules and a variety of substitutions. The

substitution on the fucopyranosyl molecules may be a sulfate molecule or fucoside side chain at the carbon 2 or 4, in addition to a minor substitution of monosaccharides or uronic acids (Ale et al., 2011, Ale et al., 2013). According to Li et al. (2008) fucoidans perform many immune functions such as anticoagulation and anti-tumor functions. Other immune functions performed by fucoidans include anti-inflammatory, anti-arteriosclerosis, antioxidant, aging and Alzheimer's prevention, anti-cancer, anti-proliferation, inhibition of tumor cells growth, directly killing tumor cells through Th1 and NK cell responses, anti-viral, prebiotic and antibacterial functions against *Escherichia coli* (Holdt et al., 2011). These functions underscore why fucoidans have gained great attention in recent years.

Regarding the chemical composition of fucoidans, **Table 2** shows the amount of neutral monosaccharides extracted from two species of brown macroalgae *Fucus evanescens* and S.L using different methods – enzymatic and chemical methods. It can be observed from **Table 2** that the neutral monosaccharides present in fucoidans are fucose (main constituent), glucose, galactose, and xylose with minimal quantities of mannose, rhamnose and mannitol. Also, there is minimal amount of Glu A, but relatively higher Man A concentration, likely due to alginate contamination during the fucoidans extraction (Deniaud-Bouët et al., 2014). The amount of glucose after the enzymatic method versus the chemical method in those 2 species of brown macroalgae is different. This is because, most of the glucose in the macroalgae originates from cellulose and laminarin, however the Cellic®CTec2 enzyme used in the enzymatic method, degrade these polysaccharides, explaining the lower glucose levels observed in the enzyme assisted method (Nguyen et al., 2020). Furthermore, the presence of uronic acids in the compositional analysis of fucoidans shown in **Table 2**, presupposes alginate contamination. The uronic acids concentrations are higher in the enzymatic method than the chemical method. Therefore, enzymatic extraction techniques must ensure optimum working conditions and amount of the enzyme – such as alginate lyase – used, or incorporate a chemical like calcium chloride, to selectively precipitate the inter-connecting bonds between alginate and fucoidans, thus extracting less contaminated fucoidans. This difference in alginate contamination observed between the two extraction techniques is reinforced by the observed lower fucose content in the enzymatic method (which has higher amount of alginate) compared to the chemical method.

According to Deniaud-Bouët et al. (2014) fucoidans and alginate are part of the matrix structure of brown seaweeds and thus are found any time in the macroalgae. However, their exact concentrations vary depending on the season and extraction method used. Concentration ranging between 3 % to 10 % (w/w) have been reported for fucoidans with the highest levels recorded in spring (Manns et al., 2014).

Table 2: Chemical composition of fucoidans extracted from *Fucus evanescens* and S.L using enzymatic and chemical methods (Nguyen et al., 2020).

Content	Monomer	*Fucus evanescens*		*S. L*	
		Enzymatic Method	**Chemical Method**	**Enzymatic Method**	**Chemical Method**
Neutral monosaccharides (%mol)	Mannitol	0.2 ± 0.0	0.43 ± 0.0	2.1 ± 0.2	2.2 ± 0.3
	Fucose	24.8 ± 2.9	60.9 ± 0.9	12.6 ± 0.4	31.2 ± 4.2
	Rhamnose	0.2 ± 0.1	0.9 ± 0.2	0.2 ± 0.0	0.2 ± 0.0
	Galactose	0.9 ± 0.1	5.4 ± 0.1	2.3 ± 0.1	2.9 ± 2.4
	Glucose	0.7 ± 0.1	6.2 ± 0.1	1.6 ± 0.0	57.7 ± 3.1
	Xylose	0.8 ± 0.1	5.8 ± 0.1	0.8 ± 0.0	3.0 ± 0.0
	Mannose	0.4 ± 0.0	2.6 ± 0.1	0.9 ± 0.0	0.9 ± 0.1
Uronic acid (%mol)	GuluA	12.6 ± 1.8	0.9 ± 0.1	18.6 ± 0.9	0.2 ± 0.1
	GluA	1.0 ± 0.2	3.9 ± 0.1	1.3 ± 0.2	0.7 ± 0.1
	ManA	58.4 ± 2.6	13.1 ± 0.4	59.6 ± 1.9	1.0 ± 0.2

Regarding the structure of fucoidans, Ale et al. (2013) reported variations in the chemical structure from different species of brown macroalgae. These structural variations reinforce their natural biodiversity, different chemical substitutions, and compositions. Thus, no single structure has been proposed for all fucoidans. However in spite of the variations, the primary chemical composition that encompasses most fucoidans is made up of the α(1,3)-linked α-L fucopyranosyl backbone or alternating α(1,3) and α(1,4) linked α-L fucopyranosyl backbone structures with various degrees of sulfate substitution (Ale et al., 2013, Nguyen et al., 2020). **Figure 2** shows the proposed structure of fucoidans found in S.L, which is composed predominantly of α (1,3)- linked α-L fucopyranose residues with various degrees of sulfation.

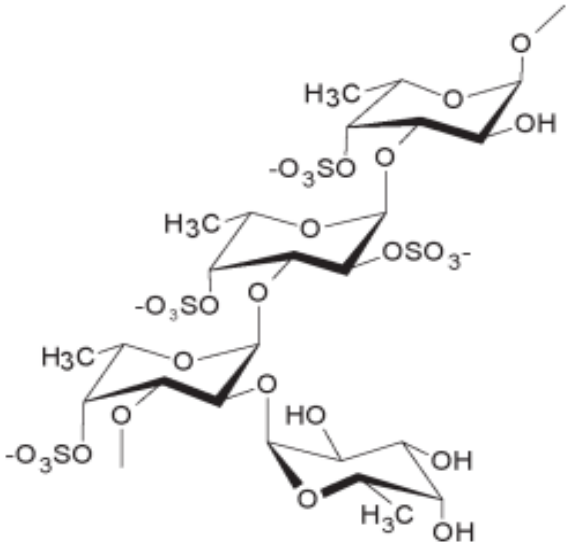


Figure 2: Chemical structure of fucoidans extracted from S.L (Ale et al., 2013).

According to research, the bioactivity of fucoidans is directly connected to their constituent monosaccharides, structure, and molecular mass (Li et al., 2008) Accordingly, it is very important that fucoidans extraction techniques focus on isolating the intact biomolecule without destroying its structure or contaminating it with other compounds. The mechanism of many previously widely used extraction methods such as using acids, organic solvents or hot water relied on their ability to dissolve the polysaccharides in the cell wall of the macroalgae. However, these methods presented disadvantages as they could be toxic, are less efficient, tedious and could destroy the structure of the fucoidans which could affect the bioactivity. Thus to remedy these disadvantages, novel cutting edge extraction techniques such as microwaves, ultrasound and enzyme assisted extraction techniques have been developed which have high propensity in maintaining the fine structure and hence bioactivity of extracted fucoidans (Flórez-Fernández et al., 2017, Yuan et al., 2015, Alboofetileh et al., 2019).

The enzyme-assisted technique operates by getting rid of polysaccharides (that are not fucoidans) in the cell wall matrix of the macroalgae and completely breaking down laminarin. The challenge of this technique is that, some concoction of enzymes do not selectively target alginate which is present in large quantities in the cell wall matrix of the macroalgae (Alboofetileh et al., 2019). Also, alginate forms a cross-linkage with fucoidans and cellulose, thus it is extremely essential that these linkages are broken down in a non-destructive way to the fucoidans while selectively removing the alginate cross-linkage

(Deniaud-Bouët et al., 2014). A combination of Cellic®CTec2 enzyme to depolymerise laminarin and other polysaccharides and alginate lyase to degrade the alginate or calcium chloride to precipitate alginate is being used in recent extraction protocols (Nguyen et al., 2020)

1.5.3 Alginate

Alginate, which is the salt form of alginic acid, is the most abundant polysaccharide component located in the cell wall and intercellular matrix of brown macroalgae (Li et al., 2021). The main source of commercially available alginate is obtained from brown macroalgae such as *Laminaria digitata* and *Macrocystis pyrifera* (Rhein-Knudsen et al., 2017). Alginate is a linear polysaccharide composed of ß-D-mannuronic acid (M or Man A) and α-L-guluronic acid (G or Gulu A). Thus the polymers may be bound and arranged in three types of blocks with different proportions of homopolymeric MM, GG or heteropolymeric MG blocks depending on the location of cultivation, season of harvest and species of the brown macroalgae (Rhein-Knudsen et al., 2017, Li et al., 2021). **Figure 3** shows the structure of alginate with alternating GG, MM, and MG blocks.

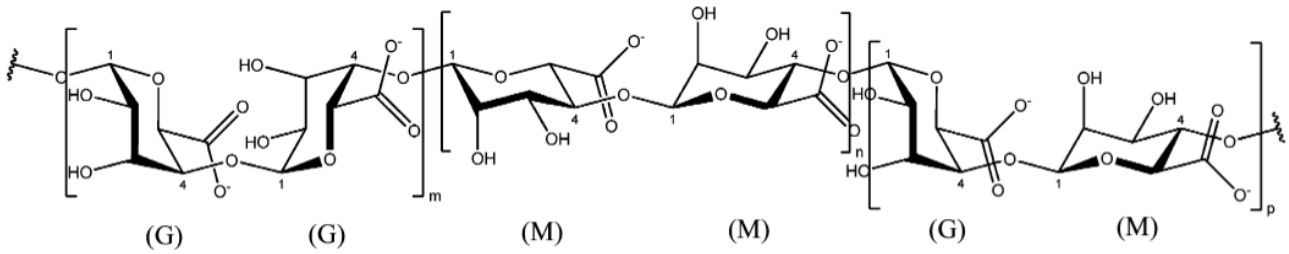


Figure 3: Structure of alginate showing alternating GG, MM and MG blocks (Agulhon et al., 2012).

Alginate forms complexes with divalent ions such as calcium ions. Calcium ions form ionic complexes with aligned alginate chains to produce a 'stacked structure' or gel network formation called the egg-box model. The ratio of M and G blocks, the arrangement of the copolymers in the uronic acid chain, and degree of polymerisation all affect the rheological features of the alginate (Rhein-Knudsen et al., 2017). For instance, alginate with high proportions of G residues form rigid and delicate gel structure while a high proportion of M residues produce better gelling properties.

Manns et al. (2014) reported that the concentration of alginate and fucoidans in brown macroalgae may vary with season but are always present, since they are constitutive of the cell wall matrix. Alginate proportions ranging between 17 % and 45 % of DM have been measured, with highest levels recorded in spring and winter when there were lowest levels of the storage polysaccharides. Alginate composition in brown macroalgae can be measured by hydrolysing the alginate with sulfuric acid and quantifying the uronic acid concentrations using high performance anion exchange chromatography (HPAEC-PAD) or by quantifying total uronic acids spectrophotometrically (Manns et al., 2014, Rhein-Knudsen et al., 2017). Alginate is used in several food and feed, cosmetics, and medicinal applications as a thickening, stabilizing and gelling agent.

1.5.4 Laminarin

Laminarin is a principal storage carbohydrate of brown macroalgae and it is made up of a backbone of ß-1,3-bonded glucopyranoses with some having branched ß-1,6 glucose residues (Manns et al., 2014). D-mannitol joined to laminarin at the reducing end is called M-chain whereas G-chain is when there is no mannitol at the reducing end as shown in **Figure 4** (Li et al., 2021, Kadam et al., 2015). For instance, a laminarin chain is predominantly made up of 25 units that could have either D-mannitol or glucose reserve unit at the reducing end, thus an M-chain or G-chain respectively. These substitutions have effect on the bioactivity as Moroney et al. (2015) reported, with the antioxidant properties of laminarin affected by variations in structure, monosaccharide composition and degree of substitution (Moroney et al., 2015). Also, there is variation in concentrations of laminarin in most brown macroalgae due to seasonal and species differences with concentration levels ranging between 0 – 35 % of DM for S.L and other phaeophytes; with the highest levels recorded around autumn when the reserve compound are highest (Manns et al., 2014, Holdt et al., 2011, Kadam et al., 2015). Laminarin has a low molecular weight of around 5 kDa and are hydrolysed by laminarinases to produce oligosaccharides and glucose. The endolytic laminarinase enzymes degrade the bonds between adjacent glucose units to produce

oligosaccharides while the exolytic enzymes cleave off the glucose found on the non-reducing end releasing it (Li et al., 2021).

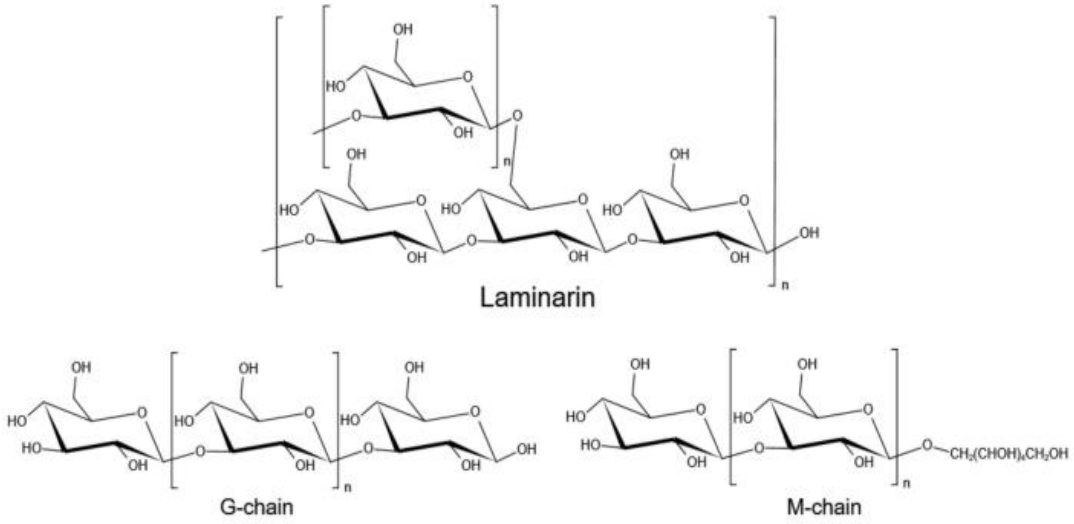


Figure 4: Chemical structure of laminarin (Li et al., 2021).

Recently, laminarin just like fucoidans, have generated a lot of attention due to their bioactive properties. Being a ß-glucan, laminarins are reported to stimulate macrophages which results in stimulation of the immune system, eliciting anti-tumor growth responses, enhancing wound repair and improving resistance against infections (Kadam et al., 2015). They are also easily fermented by microbes in the gastrointestinal tract of many organisms, evidencing a great potential in the pharmaceutical industry and for producing functional food (Li et al., 2021).

1.6 Preservation techniques for macroalgae

Freshly harvested macroalgae have a very high moisture content (between 70–90%) and contain many sensitive molecules, which make it deteriorate quicker, restricting its post-harvest processing prospects, particularly in industrial and pharmaceutical uses (Albers et al., 2021, de Castro et al., 2017, Holdt et al., 2011). Therefore, it is crucial to find an appropriate preservation technique that can be used to store and maintain their quality from time of harvest till eventual use. Industrial use of seaweeds, such as in a biorefinery to produce different products requires an understanding of the possible changes in

chemical composition, molecular structure and physical characteristics likely to occur due to preservation (Albers et al., 2021). The following are some methods used in preserving macroalgae:

1.6.1 Dehydration.

This method encompasses several common preservation methods of seaweeds. Commonly, freshly harvested seaweeds from the sea are dehydrated by sun-drying, oven-drying or, less commonly, freeze-drying for consumption or long-term storage before industrial processing (Albers et al., 2021).

1.6.2 Sun-drying.

Because of simplicity and economic reasons, sun drying is widely used as a preservation method for seaweeds used for producing agar, carrageenan and alginate (Albers et al., 2021). The major setback associated with sun-drying is its dependence on the weather conditions and the duration of availability of the sun within the day. Hence, it is the most difficult method when it comes to control of drying rate.

1.6.3 Heating.

This is another common method for stabilization of macroalgae as the speed of drying is quicker but needs high amounts of energy. Research shows that heat drying may affect the nutritional level of the seaweeds (Albers et al., 2021). For instance, heat drying at room temperature (RT) between 25 ℃ to 30 ℃ decreases phenolic content, flavonoids, and vitamin C in seaweeds. Furthermore, using higher temperatures between 60 ℃ to 70 ℃, negatively affects the nutritional value compared to fresh seaweeds (Albers et al., 2021).

1.6.4 Freezing.

This method is an excellent and regularly used preservation method, that slows down or prevents chemical reactions and growth of microbes by the low temperature and conversion of water into ice. The particulate ice molecules that are formed can cause severe destruction to the frozen material, especially cell walls of seaweeds. In addition, the patches of unfrozen liquid containing salts and other substances may become highly concentrated and increase the rate of chemical reaction leading to

spoilage particularly upon cooling or thawing (Albers et al., 2021). It has been reported that freezing and freeze-drying maintain polysaccharides with higher molecular weights than salting or hot-air drying (Albers et al., 2021).

1.6.5 Ensiling as a storage technique for macroalgae

There is a lot of attention currently on ensiling as a preservation method because it serves as a low energy preservation option and can be used to store large amounts of macroalgae biomass. Ensiling or anaerobic fermentation is a widely used method for preserving silage based on reduction in the pH (usually to a pH less than 4) of the sample and elimination of oxygen to inhibit growth of microbes that cause spoilage such as fungi, clostridia etc. (Albers et al., 2021) The decrease in pH can be attained by addition of additives, such as specific bacteria, enzymes, or acids. LAB is a microbe commonly used in ensiling, and it functions to ferment the water soluble carbohydrates (WSC) or fermentable sugars to produce lactic acid, acetic acid and other organic acids (Elferink et al., 2000). Ensiling also involves exclusion of air and this is achieved by vacuum packing in some cases. The processes involved in ensiling can be grouped into four stages. The first stage, also called the aerobic stage involves the depletion of atmospheric oxygen between sample particles due to respiration of the cells and tissues of the biomass, and due to the presence of aerobic microbes like yeasts and enterobacteria (Elferink et al., 2000). This stage lasts for a short period of time till the oxygen is depleted and then follows the second stage, also called fermentation stage. Lactic acid fermentation refers to the chemical breakdown of carbon molecules like glucose, other monosaccharides and disaccharides, into smaller molecules like lactic, acetic, or other organic acids as shown in **Figure 5 and 6** (Monteiro et al., 2021, Maneein et al., 2018). **Figure 5** shows the breakdown of polysaccharides into their composite monosaccharides during fermentation while **Figure 6** shows the chemical representation of lactic acid fermentation during ensiling.

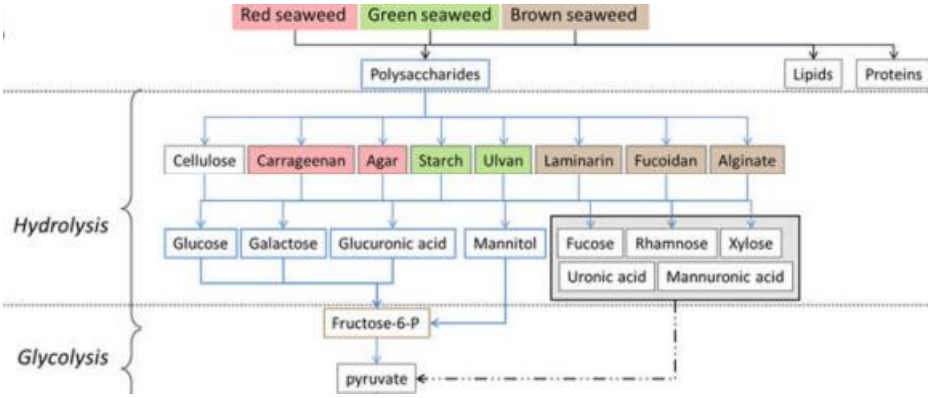


Figure 5: Breakdown of polysaccharides in macroalgae during fermentation (Maneein et al., 2018)

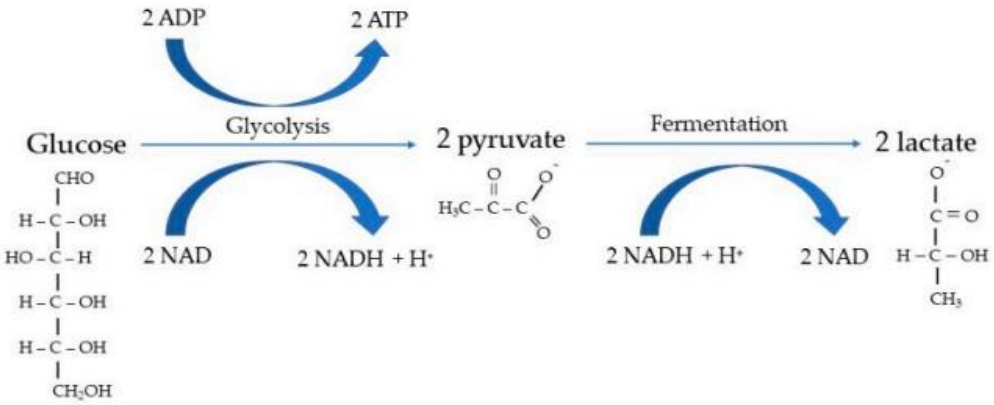


Figure 6: Lactic acid fermentation which occurs during ensiling (Monteiro et al., 2021).

During the second stage, if LAB is included in the ensiling treatment, they begin to grow and multiply in number and become the dominant population leading to high lactic and organic acids production and a lower pH – between 3.8 to 5.0 (Elferink et al., 2000). The third stage or stable stage is where microbes die off due to the exclusion of oxygen and low pH, except acid tolerant microbes like clostridia and facultative bacilli that form spores. Carbohydrase and proteases that are acid tolerant also remain active at minimal level. The final stage or aerobic spoilage stage begins when the packaging for the ensiled material, bag or container is compromised. For instance, by insects, rodents or when it is purposefully opened. Spoilage at this stage is divided into 2 phases and it occurs due to breakdown of the preserving organic acids by yeast and acetic acid bacteria present (Elferink et al., 2000). This causes pH increase (first phase) and leads to the next phase of spoilage which involves rise in temperature and reactivation

of spoilage microbes that were inactivated due to the low pH. Activities of aerobic fungi and facultative anaerobic microbes like bacilli and enterobacteria increase and this results in complete spoilage of the biomass (Elferink et al., 2000). The degree of spoilage of an ensiled biomass will depend on quantity and activities of the spoilage microbes. However, if ensiling is done efficiently with the right chemical stabilizers, bacteria or acid treatments, samples can be preserved for a long period of time (Elferink et al., 2000, Albers et al., 2021)

Ensiling of macroalgae biomass is mainly achieved directly by adding organic acid or indirectly by adding a population of LAB. Formic acid is the most widely used acid in ensiling, but other organic acids may also be used (Albers et al., 2021). More specifically in S.L, research shows that formic acid or a mixture of formic and propionic acids most effectively preserves the macroalgae biomass (Albers et al., 2021).

There are some setbacks associated with ensiling caused by the chemical properties of the macroalgae such as the low DM content, high buffering ability from high sulfate and chloride levels (anion quantity) and different levels of fermentable sugars. Also, differences based on species, season of cultivation and time of harvest (Stévant, 2019). However, polyphenols present in seaweeds are reported to inhibit microbial activity and is a factor that influences the lactic acid fermentation in seaweeds species with high polyphenol amounts. S.L has been reported to exhibit positive fermentation potential during ensiling in comparison to other macroalgae types and can produce lactic acid from native populations of the bacteria (Stévant, 2019).

During ensiling, proteins and polysaccharides are highly sensitive to degradation (by proteases and glycosidases) and to oxidation and chemical hydrolysis. Moreover, for alginate, it has been reported that its subunits, Man A and Gulu A, can be broken down (depolymerisation) when the biomass is stored in water at RT, and this leads to loss of the components of the uronic acid. Thus, this affects the gel-forming ability of alginate as it depends on its molecular weight and content (Maneein et al., 2018).

1.7 Bioactivity of fucoidans in fish cells: potential for the aquaculture industry.

By definition, an immune modulator is a natural or synthetic compound or substance that regulates the immune system's resistance to diseases (Caipang et al., 2011). These immune modulators can be derived from many sources including brown macroalgae. They are often made up of polysaccharides and their mechanism of action involves recognising and activating the receptors of the immune system to generate an immune response (Caipang et al., 2011).

The immunomodulatory activities of fucoidans from brown macroalgae has been widely reported by numerous literature (Li et al., 2008, Caipang et al., 2011, Ale et al., 2011). Additionally, the effects of molecular weight of fucoidans on immune cells has been reported. In a murine model, Jang et al. (2014) treated spleen cells with fucoidans of lower molecular weight (LMW) and higher molecular weight (HMW). It was determined that HMW fucoidans improved spleen cells viability. Moreover, in functional analysis, it was determined that HMW fucoidans remarkably improved nitric oxide and interferon-γ production which activates macrophages, natural killer cells and neutrophils. In comparison, LMW fucoidans induced less immune activity and had a toxic effect in the spleen cells. Thus, they concluded that HMW fucoidans affects immune response better than LMW fucoidans, and that different molecular weight fucoidans exert different bioactive effects on immune cells.

Based on these results from mammalian models, it is of high interest to evaluate the effect of fucoidans in cells from fish of aquaculture interest. Currently, fish farmers use conventional disease prevention measures and treatments, including chemical disinfectants and pharmaceutical products such as antibiotics (used in high amounts), dangerously promoting the emergence of resistant pathogenic strains, as well as the water and environmental pollution associated with these antibiotic residues (Caipang et al., 2011, Flores-Kossack et al., 2020). A better alternative is the use of naturally derived immunomodulators such as fucoidans from macroalgae, which are environmentally friendly, and support the sustainability of the aquaculture industry. Studies investigating different concentrations of fucoidans (10 µg mL^{-1} and 100 µg mL^{-1}) at different time points (3 h and 24 h) on cell proliferation,

respiratory burst, and cellular myeloperoxidase of head kidney (H.K) leukocytes of Atlantic cod have been done (Caipang et al., 2011). They also tested the antibacterial functions of fucoidans by treating H.K leukocytes with a higher dose of the fucoidans. They concluded that, cell proliferation of H.K cells was much lower in the cells with the higher dose treatment for both time points, 3 h and 24 h. The cellular myeloperoxidase activity was improved in the cells treated with the higher dose of the fucoidans. Lastly bacterial growth was remarkably lower in both fucoidans treated cells than the controls. Considering this, it would be interesting to perform assays in Atlantic salmon, a fish species key for the Norwegian aquaculture industry and for the economy of the country.

Atlantic salmon constitutes the major segment of Norwegian seafood exports with approx. 534,000 tonnes exported in the first half of 2022, corresponding to about 48,4 billion Krones (Norwegian seafood council, 2022). However, diseases in Norway's Atlantic salmon farming have a detrimental effect on the fish welfare and can result in significant financial losses (Madhun et al., 2022). In 2019 and 2020, approx. 53.2 and 52.1 million, respectively, of Norwegian farmed salmon died between sea transfer and harvest, with at least 7 million due to algae bloom, bacterial and viral infections. This corresponds to over 860 million Krones in financial losses (Witzøe, 2019, Sommerset I, 2021). The most prevalent viral diseases in salmon farming are pancreas diseases, heart and skeletal muscle disease, cardiomyopathy syndrome caused by salmonid alphavirus, piscine orthoreovirus 1 and piscine myocarditis virus. Therefore, exploring the use of naturally-derived and environmentally friendly immunomodulators such as fucoidans from macroalgae, is an interesting platform to support the salmon immune system, and consequently, their robustness supporting the sustainability and growth of the aquaculture industry.

1.7.1 Basics of the Immune system of Atlantic salmon (*Salmo salar*)

The immune system of teleost fish like Atlantic salmon is fundamentally grouped into two branches – innate and adaptive immunity. Innate immunity is the first response against pathogenic intrusion, whereas the adaptive immunity is activated by a crosstalk with innate immunity, and involves antigen-

specific responses and a repertoire of receptors that discriminate between self- and nonself-antigens (Firdaus-Nawi et al., 2016, Uribe et al., 2011).

Innate immunity has humoral and cellular components. The humoral component includes interferons, transferrin, and lysozyme. The cellular part is composed of eosinophils, neutrophils, cytotoxic cells, and macrophages. Specifically, macrophages can detect, phagocytose, and kill pathogens and other harmful foreign materials. One of the cytokines mediating this response is the tumour necrosis factor alpha (TNFα). TNFα is a crucial multifunctional cytokine that drives inflammatory responses (Fitzgerald et al., 2001). Its expression and function are conserved in salmon, being one of the genes immediately up-regulated on the onset of infections. Considering the key role of TNFα in response to pathogenic attacks, the ability of fucoidans to stimulate higher TNFα gene and protein expression will be of great interest and a valuable product for the aquaculture and pharmaceutical industries.

Similarly, the adaptive immune system is also divided into 2 groups – humoral and cellular. The humoral is composed of immunoglobulins M, D and T, which fight against invasive extracellular pathogens, whereas the main components of the cellular immunity are T cells (lymphocytes) that focus on fighting specific foreign virus, bacteria or parasitic infected cells (Firdaus-Nawi et al., 2016). Together the innate and adaptive immune components function to protect the fish from pathogen attacks (Firdaus-Nawi et al., 2016).

1.7.2 Immune organs in Atlantic salmon

The major immunological organs in the teleost fish like salmon are kidney, thymus, spleen and mucosa-associated lymphoid tissues (MALT) (Press et al., 1999). The primary lymphoid organs in salmon are thymus and H.K while the spleen, H.K and MALT are secondary lymphoid organs (Soulliere et al., 2016). The salmon kidney is divided into 2 parts namely the anterior or H.K – a mammalian bone marrow equivalent, and the posterior or trunk kidney. The H.K is extremely important as it produces immune cells such as lymphocytes, blast cells, plasma cells, monocytes, and macrophages (Soulliere et

al., 2016). The posterior kidney is composed mainly of excretory tissues with few lymphoid tissues scattered within, and primarily serves as an excretory organ (Zwollo et al., 2005, Soulliere et al., 2016).

The salmon spleen is also a very important secondary lymphoid organ. The immune cells found in the spleen include monocytes, macrophages and lymphocytes (Uribe et al., 2011). Summarizing, both H.K and spleen are important immune organs whose leukocytes can be used in fucoidans bioactivity analysis due to their immune functions.

1.8 Aim of Thesis

The aim of this thesis is to evaluate the storage stability of brown macroalgae (S.L) biomass, preserved for a long period and to determine the effects of different pretreatment methods, on the general sensory properties and nutrient composition of the brown macroalgae. Another aim is to investigate the bioactivity of fucoidan-rich seaweeds extracts from differently treated samples. To achieve this, the S.L biomass was vacuum packed into bags with different treatments – ensiling additives, microbes, and chemical stabilizers, for a long period (approx. 2.5 months). After the bags were opened, observational analysis to check colour change, smell, growth of fungi and pH of the samples was conducted. Subsequently, chemical compositional analyses to determine the variation in monosaccharides released or carbohydrate composition between samples from the different treatments was conducted. Finally, the bioactivity of fucoidan-rich seaweeds extracts obtained from the different treatments was tested by incubating primary cells (leukocytes) from H.K of Atlantic salmon, RNA extraction from the treated cells and qPCR analysis.

2.0 MATERIAL AND METHODS

2.1 Sample collection

Brown macroalgae *Saccharina latissima* (S.L) cultivated outside Frøya was provided by Seaweed Solutions AS (Trondheim, Norway). The sugar kelp was harvested in June 2021, cut and frozen in bags before being transported to NMBU. Approximately 100 kg of the macroalgae was used for this work. A structural outline of the analyses conducted in this thesis is presented in **Figure 7.**

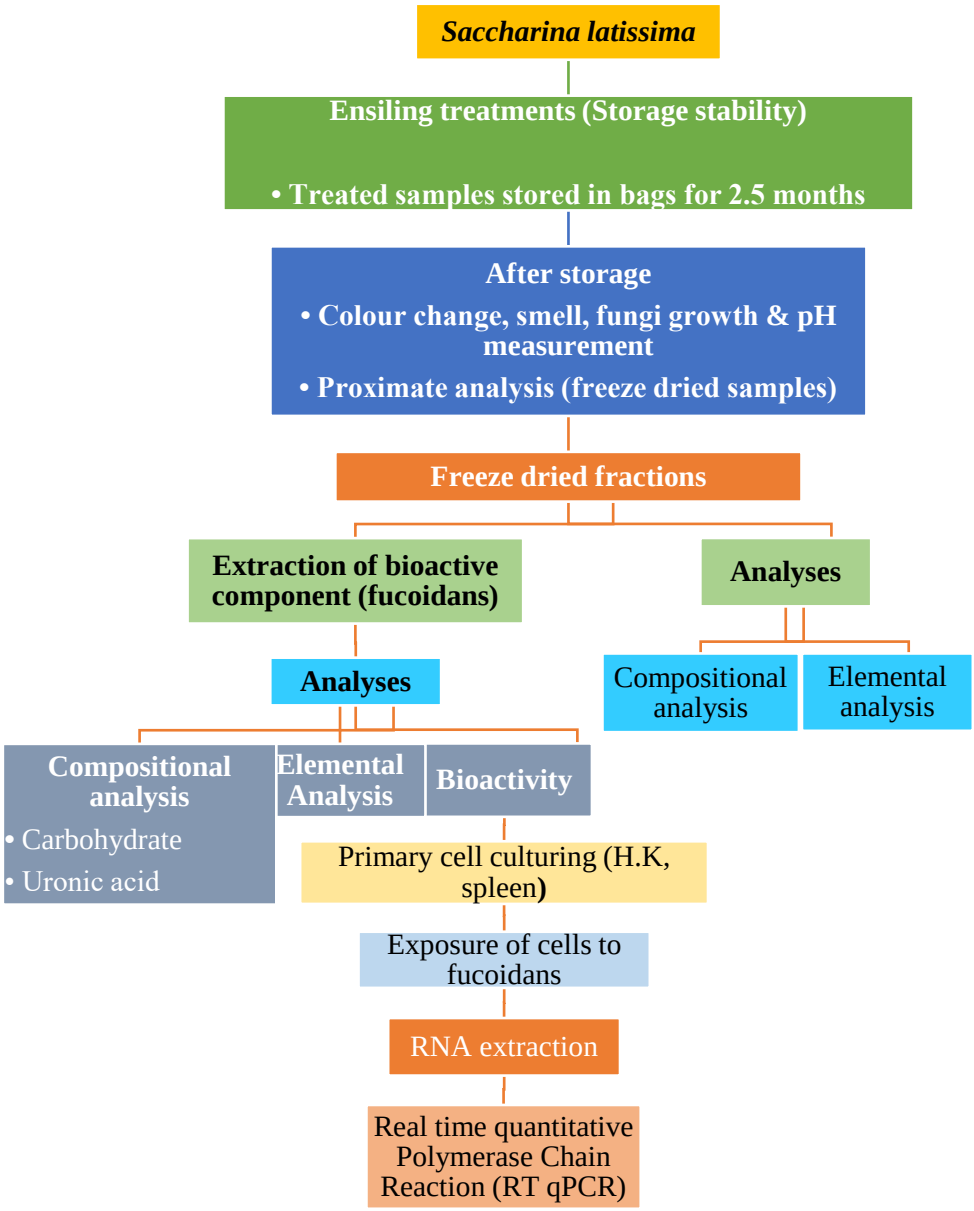


Figure 7: Structural outline of the experiments in this thesis.

2.2 Treatment and packing of macroalgae samples

The frozen S.L samples were grinded with a meat grinder and about 900 – 1000 g each was vacuum packed with the following ensiling treatments: Formic acid 85 % (VWR, USA) – 3 mL and 6 mL, GrasAAT® Lacto (ADDCON Nordic AS, Norway) – 3 mL and 6 mL, GrasAAT® Plus (ADDCON Nordic AS, Norway) – 3 mL and 6 mL, Kofasil® LP (ADDCON Nordic AS, Norway) – 3 mL and 6 mL, Sil-All® 4x4 (Lallemand, UK) – 2 mL and 4 mL, Magniva® Classic + (Lallemand, UK) – 2 mL and 4 mL, Magniva® Platinum 2 (Lallemand, UK) – 2 mL and 4 mL, *Lactobacillus sakei* R9207 (Lallemand culture collection, UK) – 2 mL and 4 mL, and *Pediococcus acidilactici* R9628 (Lallemand culture collection) – 2 mL and 4 mL. The last five ensiling products were in powder form and easily soluble in water. The powder was dissolved and diluted to give optimal amount of lactic acid bacteria (LAB; given as colony forming units (CFU) per gram of biomass) in 2 mL and 4 mL per kg of macroalgae biomass (as seen in **Table 3**)

Another treatment without any additive was made to allow for naturally occurring ensiling to occur due to the presence of the native LAB population in the brown macroalgae. Each treatment was quadruplicated and were stored at RT (as seen in **Table 4)**. Conversely, samples for the control treatment (containing no additive) were stored at -80 ℃. A vacuum packer was used to exclude air and seal the bags during packing. This was to enable anaerobic lactic acid fermentation to occur in the ensiled samples. **Table 4** gives an overview of how the packing of samples was performed.

Table 3: Overview of ensiling additives used in the experiment.

Ensiling additive	Ingredients	Dosage (per kg of biomass)
Formic acid 85 % [1]	Formic acid 85 %	3 mL and 6 mL
GrasAAT® Lacto [2]	Formic acid (E236): 62 % Sodium formate: 16 % Lactose: 1.5 % Caramel colour (E150)	3 mL and 6 mL
GrasAAT® Plus [2]	Formic acid (E236): 44 % Sodium formate: 20 % Propionic acid: 12 % Benzoic acid: 1.5 % Glycerol: 1 % Caramel colour (E150)	3 mL and 6 mL
Kofasil® LP [2]	Sodium nitrite (E250): 19% Hexamethylenetetramine: 14.5 % Sodium benzoate: 5 % Tartrazine (lemon yellow; E102)	3 mL and 6 mL
Sil-All® 4x4 [3]	Lactic acid bacteria (*Lactobacillus plantarum*, *Enterococcus faecium*, *Pediococcus acidilactici* and *Lactobacillus salivarius*) and enzymes (amylase, cellulase, hemicellulase, and pentosanase)	2 and 4 ml (LAB dosage: 1 000 000 and 2 000 000 CFU/g biomass)
Magniva® Classic + [3]	Lactic acid bacteria (*P. acidilactici*, *Pediococcus pentosaceus*, *L. plantarum*) and enzymes (β-glucanase and xylanase)	2 and 4 ml (LAB dosage: 1 000 000 and 2 000 000 CFU/g biomass)
Magniva® Platinum 2 [3]	Lactic acid bacteria (*Pediococcus pentosaceus*, *Lactobacillus hilgardii* and *Lactobacillus buchneri*)	2 and 4 ml (LAB dosage 500 000 and 1 000 000 CFU/g biomass)
L. sakei, strain R9207 [4]	Lactic acid bacteria (*Lactobacillus sakei*)	2 and 4 ml (LAB dosage 500 000 and 1 000 000 CFU/g biomass)
P. acidilactici, strain R9628 [4]	Lactic acid bacteria (*Pediococcus acidilactici*)	2 and 4 ml (LAB dosage 500 000 and 1 000 000 CFU/g biomass)

[1] VWR International, Norway

[2] ADDCON Nordic AS, Norway

[3] Kind gift from Lallemand Animal Nutrition, UK

[4] Kind gift from Lallemand culture collection, France

Table 4: Details of S.L samples used in this thesis. Different ensiling additives, volumes taken and the condition of storage (storage temperature) of the ensiled samples are shown. The samples were stored for 2 months and 14 days, from July to September.

Treatment	Additive	Vol. of additive per bag S.L (mL)	Storage (°C)	Bag Numbers
1	No additive	-	-80	1,2,3
2	No additive	-	RT	4,5,6,7,80,81
3	Formic acid 85%	3	RT	8,9,10,11
4	Formic acid 85%	6	RT	12,13,14,15
5	GrasAAT® Lacto	3	RT	16,17,18,19
6	GrasAAT® Lacto	6	RT	20,21,22,23
7	GrasAAT® Plus	3	RT	24,25,26,27
8	GrasAAT® Plus	6	RT	28,29,30,31
9	Kofasil® LP	3	RT	32,33,34,35
10	Kofasil® LP	6	RT	36,37,38,39
11	Sil-All® 4x4	2	RT	40,41,42,43
12	Sil-All® 4x4	4	RT	44,45,46,47
13	Magniva® Classic +	2	RT	48,49,50,51
14	Magniva® Classic +	4	RT	52,53,54,55
15	Magniva® Platinum 2	2	RT	56,57,58,59
16	Magniva® Platinum 2	4	RT	60,61,62,63
17	L. sakei R9207	2	RT	64,65,66,67
18	L. sakei R9207	4	RT	68,69,70,71
19	P. acidilactici R9628	2	RT	72,73,74,75
20	P. acidilactici R9628	4	RT	76,77,78,79

Where: RT – (Room temperature); Absence or lack of – (-).

2.3 Observational, proximate, and elemental analysis of ensiled macroalgae

After approx. 2.5 months, the packed bags of treated S.L samples were opened, and a physical check was conducted to determine any changes in colour, smell, or visible fungal growth. The pH was measured to evaluate the degree of acidity after fermentation due to ensiling. The treated samples were freeze-dried and finely grinded with a mixer mill MM301 (Retsch GmbH, Germany) and homogenised. The DM and moisture contents were determined by drying the samples in an oven (ThermoFischer Scientific, USA) at 103 °C overnight and the weights of the samples before and after drying were recorded. The ash content was determined by calcination of the dried samples in a muffle furnace set at

550 ℃ for 4 h. The amounts of C, N and S were determined using a Vario El Cube elemental analyser (Elementar Analysensysteme GmbH, Germany). The degree of sulfation was calculated using the formula proposed by Zayed et al. (2020). And finally, the percentage crude protein (CP) was determined using a nitrogen-to-protein conversion factor of 5 as proposed by Angell et al. (2016).

Figure 8: The categories of fungal growth observed after the packed bags were opened. (a) – no fungal growth, (b) – little fungal growth, (c) – medium fungal growth and (d) – much fungal growth.

2.4 Chemical compositional analysis of brown macroalgae

2.4.1 Acid hydrolysis

A modification of the NREL two-step sulfuric acid hydrolysis, as described by (Manns et al., 2014) was used to quantitatively determine the carbohydrate composition in ground freeze-dried S.L samples, freeze-dried fucoidan-rich seaweeds extracts samples and sugar recovery standards (fucose, mannitol,

galactose, xylose, mannose, glucose, mannuronic acid, guluronic acid, galacturonic acid and glucuronic acid). The first part of the two-step H_2SO_4 hydrolysis method involved measuring the samples into pressure tubes and adding 72 % (w/w) H_2SO_4 to the samples in the ratio 100 mg DM sample to 1 mL of H_2SO_4 at 30 °C for 1 h. This hydrolyzes the hemicelluloses and increases the monosaccharide yield (Quintero et al., 2011). For the second step, the acid was diluted using deionized water to a final concentration of 4 % (w/w) H_2SO_4 and put in an autoclave at 121 °C for 40 min. After the hydrolysis, the hydrolysates were cooled down at RT and filtered through a 0.22 µm steritop vacuum filter (Merck, Germany) before monosaccharides were quantified by HPAEC as described below.

2.4.2 Carbohydrate analysis

The HPLC system used for the analysis was the ICS-5000 HPAEC-PAD (Thermo Scientific, USA) equipped with the Dionex CarboPac PA1 column developed for use with pulsed amperometric detection. The mobile phase consisted of a multi-step gradient using three eluents: A: 0.1 M sodium hydroxide, B: 0.1 M sodium hydroxide and 1 M sodium acetate, C: deionized millipore water. Neutral sugars were eluted isocratic with 5 mM NaOH for 15 min followed by a gradient of 1 M NaOAc, 100 mM NaOH for 20 min for elution of uronic acids. Flowrate was 0.25 mL/min and 5 µL sample was injected for each run. Glucose, fucose, xylose, mannose, mannitol, galactose, and uronic acids at concentrations of 0.05, 0.025, 0.0125, 0.006, 0.003 and 0.001 g/L were used as standards for the calibration curve. The Dionex software Chromeleon 7.0 was used for identification and quantification of the monomeric sugars. Sugar recovery values were determined from parallel runs.

2.5 Fucoidans extraction

Samples from 6 of the treatments, formic acid 85 % - 6 mL, GrasAAT® Lacto – 6 mL, GrasAAT® Plus – 6 mL, Sil-All® 4x4 – 4 mL, no additive added (stored at RT and -80 ℃ respectively), were selected for fucoidans extraction and bioactivity tests. These treatments were selected based on their

ability to maintain the native physical (prevent spoilage) and chemical properties of the treated samples. Using a ratio of 1 kg to 20 L, 5 g of each sample was mixed with 100 mL (divided in 2 tubes) of 0.03 M HCl and incubated in a water bath (Techne SB16, UK) for 1 h at 70 °C. The test tubes containing the mixture were shaken every 15 min during incubation to ensure thorough mixing. After incubation, the solutions were centrifuged (Multifuge X1R, USA) at 3000 g for 20 min to remove the large residues. The supernatant obtained was mixed with 2 % w/v calcium chloride ($CaCl_2$) and stored at 4 °C overnight. This resulted in the precipitation of alginate. On the following day, the solutions were centrifuged (Multifuge X1R, USA) at 3000 g for 20 min and the precipitated alginate were collected. The supernatants were filtered with a 0.45 µm membrane filter (VWR, USA). The filtrates were pipetted into the concentrator body of a Vivaspin® 20 centrifugal concentrator (10 kDA cut-off membrane) and centrifuged for 20 min at 7000 g (Multifuge X1R, USA). The concentrates obtained were freeze dried.

2.6 Purification

The freeze-dried concentrates were dissolved in a sodium acetate buffer (pH of 6) in the ratio 100 mg to 10 mL. Cellic®CTec2 enzyme blend was added to each mixture in the ratio: 5 mg Cellic®CTec2 / g substrate and the solution was left shaking overnight in an incubator, to degrade the laminarin and cellulose. The next day, ethanol was poured into each solution to obtain a final concentration of 70 %, and the mixtures left overnight for precipitation of fucoidans. Finally, the solutions were centrifuged at 7000 g for 30 min and the pellets were collected as purified fucoidans. The pelleted fucoidans fractions were freeze-dried.

2.7 Leukocyte isolation from spleen and head kidney

Four Atlantic salmon were sacrificed using anesthesia tricaine methanesulfonate (MS222) and bled from the caudal vein. Fish were quickly transported on ice from the fish lab to the dissection lab. The spleen and H.K were extracted using scalpel and scissors and were put into glass tissue grinders (VWR,

USA) containing 5 mL of L-15 medium 10 % FBS 1 % penicillin-streptomycin (P/S) as shown in **Figure 9**. Tissue disaggregation was done on ice. The cell suspension was filtered through a 100 µm cell strainer (VWR, USA) adding 5 mL of media, to a final volume of 10 mL. The cell suspension was centrifuged at 250 g for 8 min and the supernatant discarded. An hypotonic lysis was done to eliminate erythrocytes (Hu et al., 2018) Briefly, the pellet was resuspended in 1 mL of media and 8 mL of ice-cold Milli Q water were added. The tube containing the cell suspension was gently inverted for 20 s and then 1 mL of ice-cold 10 x Phosphate Buffered Saline (PBS) was added to restore the isotonicity. The cell suspension was centrifuged at 250 g for 8 min, the supernatant was discarded, and the pellet was transferred to a biosafety cabinet to ensure sterile cell culture conditions. The pellets were resuspended in 5 mL of L-15 10 % FBS 3 x P/S and were incubated for 15 min. Afterwards, 15 mL of L-15 10 % FBS was added, and the cell suspension was centrifuged at 250 g for 8 min at 4 ºC. The pellets obtained were resuspended in 1 mL of L-15 10 % FBS 1 x P/S. An aliquot of 10 µL of the sample was taken and 10 µL of trypan blue added and thoroughly mixed for viability assessment. After mixing, 10 µL of the mixture was taken for cell counting in a Neubauer chamber, counting only clear and bright cells (as seen in Appendix C). Four quadrants were counted, and the mean value calculated. Based on the number of cells counted, the appropriate volume of media needed was determined and added to ensure equal distribution of the cells into 12-well plates.

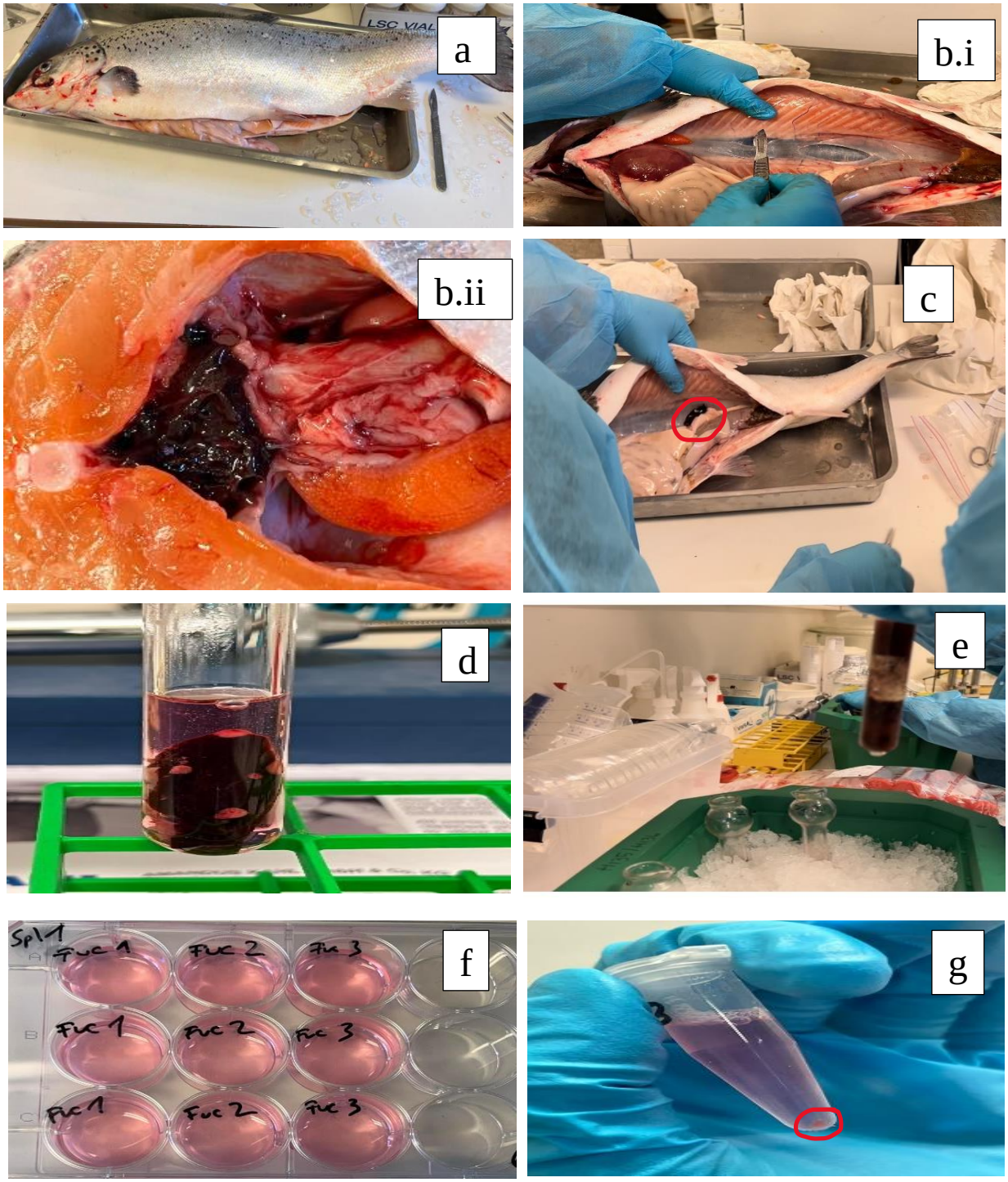


Figure 9: Flowchart of the activities involved in the cell culturing of leukocytes from H.K and spleen of Atlantic salmon. (a) dissected Atlantic salmon, (b.i) extension of the H.K to the backbone, (b.ii) H.K, (c) spleen, (d) extracted tissue (spleen) in medium before disaggregation, (e) disaggregation of tissue to release cells using grinder pestle, (f) fucoidans introduced to cells on plates, (g) recovered cells after treatment with fucoidans pelleted for RNA extraction.

2.8 Incubation of leukocytes with different fucoidans fractions

Seven hundred thousand cells per well were seeded in each well for this experiment. Three fucoidans fractions F1, F2 and F3 were used for the exposure experiment. Based on previous standardization protocols, 100 µg mL^{-1} of each fucoidans fraction was used for the exposure. Control cells were also included in the experimental design. The cells were incubated with fucoidans for 6 h and 24 h.

2.9 Leukocyte harvesting for RNA isolation

All surfaces of the fume hood and material to be used were disinfected with 70 % ethanol. After each respective time point, the wells were scraped with a cell scraper for 1 min. Visual inspection under microscope was done to ensure all cells detached. The cell suspension was gently resuspended and transferred to a 1.5 mL tube. The cells were centrifuged at 250 g for 8 min; the supernatant was discarded, and the pellet was resuspended in 500 µL of Qiazol. All samples were stored at -80 ℃ until RNA extraction.

2.10 RNA extraction

All surfaces of fume hood and material to be used were disinfected with 70 % ethanol. One hundred microliters of chloroform was added to each tube. The mixture was vortexed for 20 s and centrifuged at 13800 g for 15 min at 4 ℃ leading to the formation of 3 phases. The upper phase containing RNA was transferred into a new 1.5 mL tube and 250 µL of isopropanol was added and thoroughly mixed. The mixture was incubated for 10 min at RT and then centrifuged at 13800 g for 15 min at 4 ℃ which resulted in RNA precipitation as white pellet. The supernatant was removed after centrifugation and then spun a second time at 13800 g for 3 min at 4 ℃ which eliminated Qiazol or chloroform contamination. Excess liquid was removed without touching the pellet, and 200 µL 70 % ethanol in DEPC – treated H_2O/MQ H_2O was added. The mixture was centrifuged again at 13800 g for 10 min at

4 °C and the supernatant was removed without touching the pellet. The pelleted RNA was dried in a shorter time by centrifuging it at 13800 g for 3 min at 4 °C. Excess liquid was removed without touching the pellet and the tubes laid down on the side with open lid to air-dry the RNA pellet for 10 min at RT. The RNA was dissolved in 40 µL RNase-free water by pipetting up-and-down while kept on ice. The RNA was incubated for 10 min at 50 °C to help dissolve it further. The RNA obtained was kept on ice. The concentration of RNA was measured at 260 nm using the ratio 260nm/280nm and the ratio 260nm/230nm by NanoDrop. It was ensured that the 260/280 ratio was about 1.8 - 2.1, as lower values indicate protein contamination. The 260/230 ratio was between 1.8 - 2.4, as lower values indicate Qiazol contamination. The RNA was aliquoted and stored at -80 °C.

2.11 cDNA preparation

A 13 µL master mix containing 0.5 µL Oligo d(T)20 primer (ThermoFischer Scientific, USA), 0.5 µL dNTP mix (ThermoFischer Scientific, USA), template RNA and DEPC-treated H_2O was prepared in PCR tubes for each sample. The mixture in PCR tubes were heated at 65 °C for 5 min and then incubated on ice to allow for primer annealing to RNA template. A 7 µL reverse transcription reaction mix containing 5× SSIV Buffer (4 µL), 100 mM DTT (1 µL), 0.2 µL RNaseOUT™ Recombinant RNase Inhibitor, 0.2 µL SuperScript® IV Reverse Transcriptase (200 U/µL) and 1.6 µL DEPC-treated H_2O, all from ThermoFischer Scientific, USA, was prepared and added to the RNA mix. A CFX Opus 384 Real-Time PCR System thermal cycler (Bio-Rad, USA) was programmed according to the following temperature-time conditions and the samples were run: annealing at 55 °C for 10 min, reverse transcription at 80 °C for 10 min. The cDNA samples obtained were ready for qPCR.

2.12 Quantitative polymerase chain reaction (qPCR)

A 7.5 µL master mix containing 5 µL SybrGreen fluorescent dye (ThermoFischer Scientific, USA), 0.75 µL from each primer (for *efa1* and *tnfa* respectively) and 1.75 µL H_2O, was prepared for each

sample. The master mix was put into wells of a 384 well PCR plate and 2.5 µL of the samples added to each master mix. The samples were made by diluting 10 µL of cDNA (obtained from the RNA extraction) in 40 µL of H_2O to form a 1:5 dilution. The plates were vortexed for 50 s and centrifuged (Eppendorf, Germany) to remove air bubbles and the mixture was collected at the bottom of the wells. A CFX Opus 384 Real-Time PCR System thermal cycler (Bio-Rad, USA) was programmed according to the following temperature-time conditions and the samples were run: initial denaturation at 95 ℃ for 7 min for 1 cycle, denaturation at 95 ℃ for 10 s for 25 to 35 cycles, annealing at 72 ℃ for 20 s and extension at 72 ℃ for 20 s. Relative expression (fold change) was calculated using the $2^{-\Delta\Delta Ct}$ method described by Pfaffl (2001). The measured threshold cycle (Ct) values of the housekeeping gene *ef1*a was used to normalize the measured Ct values of the target gene *tnfa* (ΔCt). The *tnfa* gene expression from control cells was used to calculate the ΔΔCt. The statistical analysis used to evaluate significant differences was independent sample t-test using SPSS statistical software. Statistical differences between samples are defined as (*) $p< 0.05$ and (**) $p< 0.001$.

2.13 Statistical analysis

Independent sample t-test from IBM SPSS statistical software, USA, was used to evaluate if fucoidans extracted from samples from the various treatments affect the gene (*tnfa*) expression,

3.0. RESULTS

3.1 Observational and elemental analysis of treated S.L

An observational analysis was conducted on the ensiled S.L samples to determine colour change, smell, fungal growth, and pH after approx. 2.5 months of storage. The outcome is presented in **Table 5**.

S.L samples were subjected to 20 different treatments to determine the effective pretreatment methods for longer term storage. Treatment 1 (no additive added, storage at -80 ℃) was the control. Treatment 2 also had no additive added, but the samples were stored at RT. Treatments 3 to 20 contained commercial ensiling additives, microbes and chemical stabilizers as shown in **Table 5**.

In general, treatments 1 – 8, 11 and 12 produced positive results during physical observation, with no visible signs of fungal growth, colour change or development of pungent smell. The pH of the samples in these treatments was generally low (thus < 4.0), except for samples stored in the freezer at -80 ℃ (treatment 1). The remaining treatments produced less favourable effects after storage, with visible signs of spoilage – fungal growth, colour change and development of pungent smell, despite some of the treatments (13 – 20) having a pH below 4.

Table 5. Smell, colour change, fungal growth, and pH measurements of treated S.L samples after approx. 2.5 months of storage.

Treatment	Additive	Smell	Colour change	Fungal growth	pH	Bag Numbers
1	No additive	-	-	-	6.6	1,2,3
2	No additive	-	-	-	3.7	4,5,6,7,80,81
3	Formic acid 85%	+	-	-	3.4	8,9,10,11
4	Formic acid 85%	+	-	-	3.1	12,13,14,15
5	GrasAAT® Lacto	+	-	-	3.6	16,17,18,19
6	GrasAAT® Lacto	+	-	-	3.4	20,21,22,23
7	GrasAAT® Plus	+	-	-	3.7	24,25,26,27
8	GrasAAT® Plus	+	-	-	3.5	28,29,30,31
9	Kofasil® LP	++	++	+++	6.2	32,33,34,35
10	Kofasil® LP	++	++	+	6.2	36,37,38,39
11	Sil-All® 4x4	+	-	-	3.6	41,42,43
12	Sil-All® 4x4	+	-	-	3.6	44,45,46,47
13	Magniva® Classic +	++	+	++	3.6	48,49,50,51
14	Magniva® Classic +	++	+	++	3.6	52,53,54,55
15	Magniva® Platinum 2	++	+	++++	3.6	56,57,58,59
16	Magniva® Platinum 2	++	+	+++	3.7	60,61,62,63
17	L. sakei R9207	++	+	++	3.6	64,65,66,67
18	L. sakei R9207	++	+	+	3.7	68,69,70,71
19	P. acidilactici R9628	++	+	++	3.7	72,73,74,75
20	P. acidilactici R9628	++	+	+++	3.7	76,77,78,79

Smell: (-) fresh, (+) slightly irritating smell, (++) pungent smell. **Colour change:** (-) no change, (+) light brownish, (++) deep brownish. **Fungal growth:** (–) no growth, (+) little growth, (++) medium growth and (+++) much growth (as seen in **Figure 8**).

3.2 Compositional analysis of treated, freeze-dried S.L samples.

Results for the quantitative determination of neutral sugars and uronic acids content of the treated samples are presented in **Table 6**. The total carbohydrate composition (g/kg of DM) of the samples from the various treatments ranged between 38 % to 59 % with 49 % being recorded for treatment 1 (control – stored at - 80 ℃ without any additive). Carbohydrate composition (g/kg of DM) of 45 % - 47 % were recorded for treatment 3 and 4 (formic acid 85 %, 3 mL and 6 mL) and 41 % for treatment 2 (no additive, storage at RT). Other research have reported similar results as seen in this work for carbohydrate composition (g/kg DM) in S.L with glucose ranging from 100.43 - 167.75, galactose between 14.9 to 22.33, fucose between 16.7 to 35.15 and sugar alcohol mannitol between 5.14 to 119.3 (g/kg DM) (Sharma et al., 2018).

In **Table 7**, the moisture content recorded for the treated samples was around 90 % before freeze drying but 10 % afterwards. The ash content was 43.3 % and 48.9 % for treatment 1 and treatment 2 respectively and varied between 45.1 % and 53.2 % for the remaining treatments. Using a Nitrogen-to-protein factor of 5, the samples had a low crude protein content mostly between 7.7 % to 10.5 % of DM. The nitrogen content in the samples was low. From the elemental analysis of the samples, the degree of sulfation (0.1 for all the samples) was determined from the carbon and sulfur contents.

Table 6: A: The average composition of uronic acids (g/kg DM). B: Average neutral sugars composition in the samples (g/kg DM). C: The sum of uronic acids and neutral sugars composition (g/kg DM) of the treated and freeze-dried samples.

Treatment	Additive	A: Uronic acids composition of samples (g/kg DM)				B: Neutral sugars composition of samples (g/kg DM)						C: Total composition (g/kg DM)
		ManA	GluA	GuluA	Sum	Glucose	Xyl/ Man	Galactose	Fucose	Mannitol	Sum	Sum
1	No additive, -80	108	26.9	55.62	**190.5**	149.33	7.86	17.32	21.23	106.69	**302.4**	**492.9**
2	No additive, RT	101.75	28.18	78.81	**208.7**	146.13	9.13	14.9	20.17	12.49	**202.8**	**411.5**
3	Formic acid 85%	112.31	26.95	54.36	**193.6**	142.43	7.57	16.62	16.7	89.77	**273.1**	**466.7**
4	Formic acid 85%	123.59	29	53.68	**206.3**	132.37	7.7	19.21	21.52	60.11	**240.9**	**447.2**
5	GrasAAT® Lacto	113.25	25.88	52.18	**191.3**	100.43	6.69	17.44	17.8	46.8	**189.2**	**380.5**
6	GrasAAT® Lacto	129.46	27.78	59.57	**216.8**	117.31	7.15	19.59	22.18	57.72	**223.9**	**440.7**
7	GrasAAT® Plus	138.15	26.94	64.06	**229.2**	127	8.07	18.92	27.94	62.74	**244.7**	**473.9**
8	GrasAAT® Plus	149.08	27.12	67.16	**243.4**	161.41	9.22	22.33	31.64	85.93	**310.5**	**553.9**
9	Kofasil® LP	78.1	27.11	81.94	**187.1**	137.84	8.29	18.46	27.72	57.77	**250.1**	**437.2**
10	Kofasil® LP	100.13	29.25	105.19	**234.6**	167.75	10.69	20.69	35.15	119.3	**353.6**	**588.2**
11	Sil-All® 4x4	124.21	28.15	94.22	**246.6**	153.39	9.88	18.07	32.38	12.75	**226.5**	**473.1**
12	Sil-All® 4x4	135.04	30.23	103.29	**268.6**	163.77	10.27	19.03	30.36	11.56	**235**	**503.6**
13	Magniva® Classic +	134.03	28.48	92.71	**255.2**	146.88	9.18	17.16	27.98	42.76	**244**	**499.2**
14	Magniva® Classic +	128.26	26.45	87.63	**242.3**	143.97	9.49	17.67	32.33	44.4	**247.9**	**490.2**
15	Magniva® Platinum 2	125.32	30.23	95.48	**251**	143.95	11.5	18.12	33.9	16.97	**224.4**	**475.4**
16	Magniva® Platinum 2	105.93	30.61	77.83	**214.4**	130.48	13.54	15.59	22	14.63	**196.2**	**410.6**
17	L. sakei R9207	142.72	32.17	104.8	**279.7**	177.83	15.95	19.65	28.58	11.16	**253.2**	**532.9**
18	L. sakei R9207	108.17	28.43	86.43	**223**	131.99	13.39	15.79	23.78	23.51	**208.5**	**431.5**
19	P. acidilactici R9628	110.29	29.73	88.63	**228.7**	146.83	15.63	16.28	22.6	9.72	**211.1**	**439.8**
20	P. acidilactici R9628	116.38	28.31	83.05	**227.7**	143	14.54	16.7	28.74	5.14	**208.1**	**435.8**

Galacturonic acid (**Gal A**) was not detected in any of the samples. (**Man A** – Mannuronic acid; **Glu A** – Glucuronic acid; **Gulu A** – Guluronic acid)

Table 7: Ash %, nitrogen (N %), carbon (C %), sulfur (S %), crude protein (%) and degree of sulfation (DS) of treated and freeze-dried S.L samples.

Treatment	Ash %	N %	C %	S %	Crude Protein %	Additive
1 (1,2,3)	43.3	1.6	23.0	0.9	8.1	No additives, -80
2 (4,5,6,7, 80,81)	48.9	1.6	20.7	1.1	7.9	No additives, RT
3 (8,9,10,11)	45.1	1.5	21.7	1.0	7.5	Formic acid 85 %, 3 mL
4 (12,13,14,15)	49.7	1.8	19.9	0.9	8.8	Formic acid 85 %, 6 mL
5 (16,17,18,19)	53.2	1.7	18.4	1.0	8.6	GrasAAT® Lacto, 3 mL
6 (20,21,22,23)	50.0	1.7	19.8	0.9	8.5	GrasAAT® Lacto, 6 mL
7 (24,25,26,27)	49.0	1.7	20.4	0.9	8.7	GrasAAT® Plus, 3 mL
8 (28,29,30,31)	49.2	1.6	20.2	0.9	8.1	GrasAAT® Plus, 6 mL
9 (32,33,34,35)	51.3	2.0	19.6	0.9	10.0	Kofasil® LP, 3 mL
10 (36,37,38,39)	47.3	2.1	21.3	1.0	10.5	Kofasil® LP, 6 mL
11 (40,41,42,43)	49.1	1.6	20.7	1.1	8.0	Sil-All® 4x4, 2 mL
12 (44,45,46,47)	50.0	1.6	20.4	1.3	8.0	Sil-All® 4x4, 4 mL
13 (48,49,50,51)	46.5	1.5	21.6	1.2	7.3	Magniva® Classic +, 2 mL
14 (52,53,54,55)	46.6	1.5	21.6	1.0	7.7	Magniva® Classic +, 4 mL
15 (56,57,58,59)	47.7	1.6	21.5	1.1	8.2	Magniva® Platinum 2, 2 mL
16 (60,61,62,63)	50.0	1.6	20.3	1.1	7.8	Magniva® Platinum 2, 4 mL
17 (64,65,66,67)	48.6	1.6	20.9	1.0	8.0	*L. sakei* R9207, 2 mL
18 (68,69,70,71)	48.1	1.5	21.0	1.0	7.7	*L. sakei* R9207, 4 mL
19 (72,73,74,75)	48.9	1.6	20.7	1.0	8.0	*P. acidilactici* R9628, 2 mL
20 (76,77,78,79)	49.3	1.6	20.5	1.0	8.1	*P. acidilactici* R9628, 4 mL

Degree of sulfation of all the samples = 0.1. Moisture content ~ 90 % before, ~ 10 % after freeze-drying.

3.3 Chemical composition and elemental analysis of fucoidans from treated S.L samples.

Figure 10 shows the carbohydrate composition of fucoidans extracted from treated samples from some selected treatments. F1 represents fucoidans from treatment 1 (control – samples stored at -80 ℃ without any additive), F2 – fucoidans from treatment 2 (no additive, storage at RT), F3 – fucoidans from treatment 4 (formic acid 85 % (6 mL)), F4 – fucoidans from treatment 6 (GrasAAT® Lacto (6 ml)), F5 – fucoidans from treatment 8 (GrasAAT® Plus (6 ml)) and F6 – fucoidans from treatment 12 (Sil-All® 4x4 (4 mL)). The treatments were selected based on their positive outcome after storage – thus exhibiting no visible signs of spoilage or fungal growth, no colour change or development of pungent smell. **Figure 10** shows that F3 had the highest fucose (the major constituent of fucoidans) concentration, followed by F6, F2, F5, F4 and F1 of 523.6, 455.9, 385.7, 356.0, 333.1 and 271.0 g/kg DM respectively.

Table 8 shows nitrogen (N %), carbon (C %), sulfur (S %), crude protein (CP %) and DS of the extracted fucoidans. Elemental analysis of fucoidans is highly important for comparative analysis in determining the purity of different fucoidans fractions. N % is used to determine CP %, while C % and S % are used to determine DS. CP % and DS are essential factors in fucoidans purity assessments. According to Zayed et al. (2020), DS = 1/NSS, where "NSS is the number of sulfate esters per monosaccharides". NSS is calculated as follows: NSS = ((C % / 12)/ (S % / 32))/ 6; where "12 and 32 are the mass numbers of C and S and 6 is the number of C atoms per sugar monomer assuming all monomers in the polymer are hexoses." Thus, from **Table 8**, it can be deduced that F6 had the highest ratio of DS: CP %, followed by F3, F2, F4, F5 respectively with F1 having the least.

Based on these 2 factors – fucose concentration in the fucoidan and ratio of DS: CP %, F3 (highest), F2 (median) and F1 (lowest) were selected for fucoidans bioactivity analysis in H.K of salmon. This was done by exposing the H.K leukocytes with the F1, F2 and F3 and observing their effect on the expression of the *tnfa* gene.

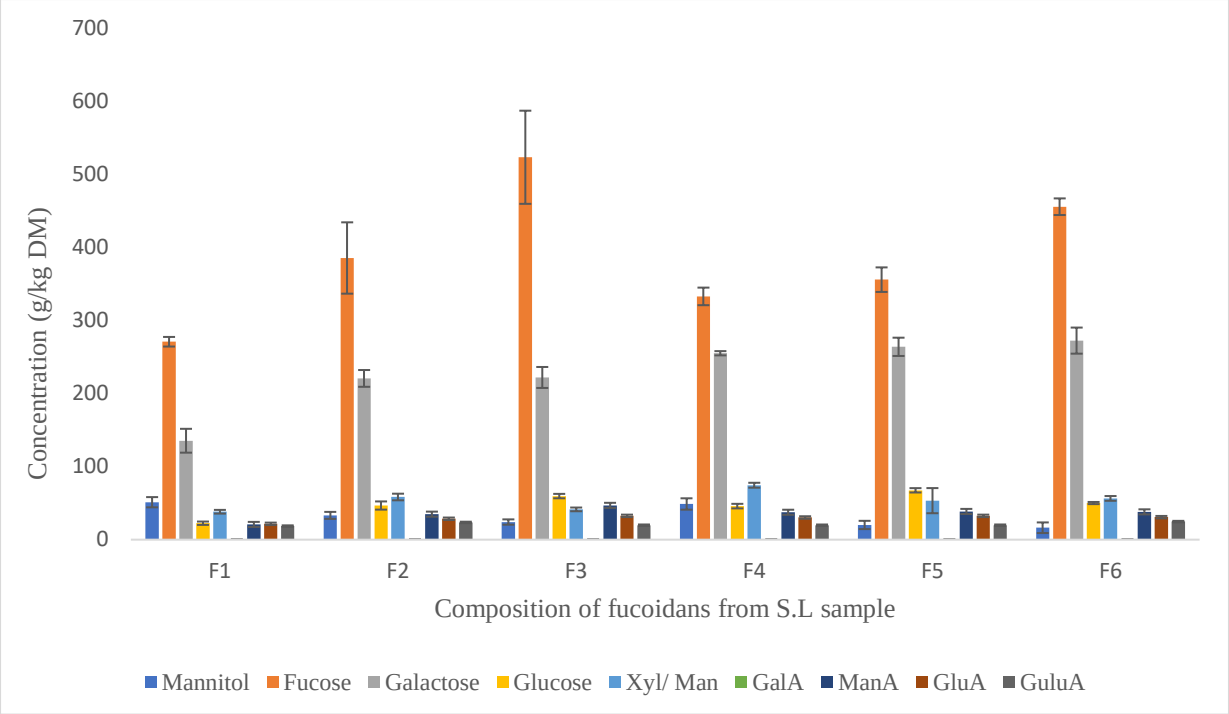


Figure 10: Bar graph showing the carbohydrate composition of fucoidans extract from treated and freeze-dried S.L samples. Where: **F1** – fucoidans from samples stored at -80 ℃ without any additive (control – treatment 1), **F2** – fucoidans from samples stored at RT, no additive added (treatment 2), **F3** – fucoidans from treatment 4 (formic acid 85 %; 6 mL), **F4** – fucoidans from treatment 6 (GrasAAT® Lacto; 6 ml), **F5** – fucoidans from treatment 8 (GrasAAT® Plus; 6 mL) and **F6** – fucoidans from treatment 12 (Sil-All® 4x4; 4 mL).

Table 8: The nitrogen (N %), carbon (C %), sulfur (S %), crude protein (%) and degree of sulfation (DS) of fucoidans from selected treated samples.

Fucoidans	Additive	N %	C %	S %	Degree of sulfation	Crude Protein (N % X 5)
F1	No additive, - 80 ℃	0.24	27.9	0.1	0.01	1.2
F2	No additive, RT	0.76	24.1	2	0.19	3.8
F3	Formic acid 85%, RT (6 mL)	0.9	24.4	3.2	0.30	4.5
F4	GrasAAT® Lacto, RT – (6 mL)	1.07	24.4	2.8	0.26	5.4
F5	GrasAAT® Plus, RT – (6 mL)	0.56	24.7	1.4	0.13	2.8
F6	Sil-All® 4x4, RT – (4 mL)	0.6	23.6	2.4	0.23	3.0

3.4 Bioactivity of fucoidans on H.K leukocytes

Fucoidans from F1 (treatment 1 – no additive, stored at -80 °C), F2 (treatment 2 – no additive, stored at RT) and F3 (treatment 4 – formic acid 85 % (6 mL), stored at RT), were used in bioactivity analysis in H.K leukocytes isolated from 4 salmon. Leukocytes were exposed to the fucoidans and incubated for 6 h and 24 h. Using the house-keeping gene *efa1*, the effects of fucoidans on the gene of interest *tnfa* is shown in **Figure 11**. It can be observed that there was an up-regulation of the *tnfa* gene for F1, F2 and F3 during both time points – 6 h and 24 h, but it was significantly higher ($p \leq 0.001$) for the 24 h time point.

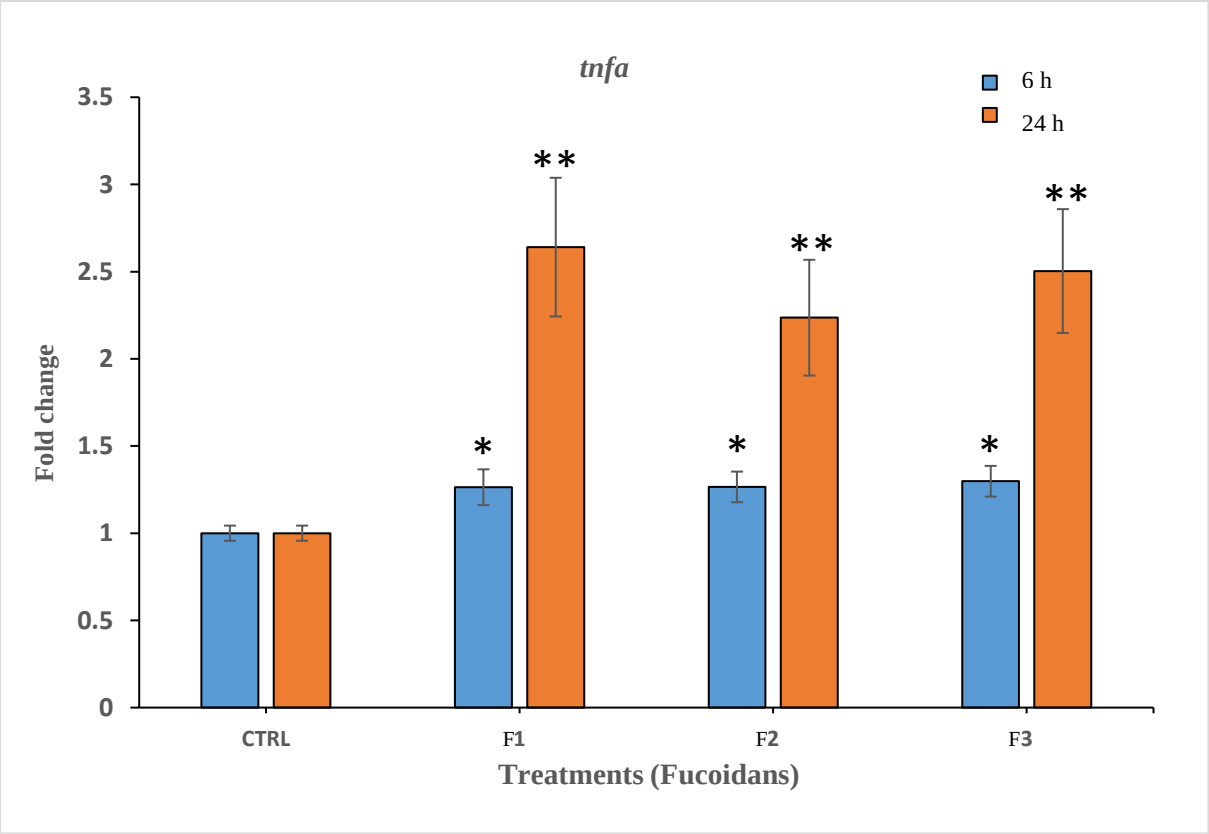


Figure 11: Bar graph showing effect of fucoidans, F1 – fucoidans from samples stored at -80 °C without any additive (control – treatment 1), F2 – fucoidans from samples stored at RT, no additive added (treatment 2), and F3 – fucoidans from treatment 4 (formic acid 85 %; 6 mL), on *tnfa* expression in H.K leukocytes isolated from salmon. It can be observed that there was up-regulation of the *tnfa* expression in H.K leukocytes treated with fucoidans for both 6 h and 24 h. The statistical analysis used is independent sample t-test, (*) $p < 0.05$. (**) $p \leq 0.001$ (as seen in Appendix A and B).

4.0 DISCUSSION

There is growing focus and interest in brown macroalgae, as a renewable source of biomolecules such as fucoidans, laminarin and alginate. Fucoidans are reported to exhibit many useful bioactive functions like inhibiting proliferation of cancer cells, anti-aging, Alzheimer's prevention, anti-viral, antioxidant, prebiotic, and antibacterial functions (Li et al., 2008, Holdt et al., 2011). Thus, fucoidans have enormous potential as a renewable ingredient in food and feed applications and as a substrate for conversion to chemicals and pharmaceutical products by enzymes and microbes (Allahgholi et al., 2020, Holdt et al., 2011). To ensure large scale or industrial utilization of brown macroalgae biomass, it is important that, an efficient, cost effective and low energy consuming pretreatment or preservation method is developed and established to ensure all year-round supply of the biomass, especially because harvesting of the macroalgae in Norway is currently done seasonally (usually between the months of April – June). Freezing, drying and heat treatments such as blanching and pasteurization – the commonly used methods – require large energy input and can be very costly (Løvdal et al., 2021, Øverland et al., 2019). Therefore ensiling, the biochemical process that is based on storage under anaerobic conditions and bacterial fermentation to inhibit further degradation, has been proposed by researchers as a suitable alternative for macroalgae preservation (Franco et al., 2016). Ensiling can occur when samples are stored vacuum packed and starter culture bacteria such as LAB are added to break down polysaccharides in macroalgae to produce organic acids. It can also occur by the direct addition of soluble substrates such as formic and acetic acids which rapidly lower the pH of the ensiled samples. The reduction in pH prevents growth of spoilage microbes and therefore preserves the samples, leaving valuable molecules like fucoidans. Ensiling has been proposed as a suitable alternative also due to its successful application in preserving grass silages (Uchida et al., 2013). The use of ensiling additives also ensures better control of the fermentation process and pH reduction, resulting in good product quality (Yitbarek et al., 2014)

The objective of this work was to investigate the effects of different pretreatments methods mainly containing LAB and/or variable combinations of acids and chemical stabilizers on the general sensory characteristics and chemical composition of brown macroalgae S.L as well as investigate fucoidans quality and bioactivity on H.K leukocytes of salmon.

4.1 Observational and elemental analysis of treated macroalgae

The treatments in this work that successfully preserved the macroalgae after vacuum packing and long-term storage were treatments 1 (control – no additive added, stored at -80 ℃), 2 (no additive added, stored at RT), 3 (formic acid 85 %, 3 mL added), 4 (formic acid 85 %, 6 mL added) , 5 (GrasAAT® Lacto, 3 mL added), 6 (GrasAAT® Lacto, 6 mL added), 7 (GrasAAT® Plus, 3 mL added), 8 (GrasAAT® Plus, 6 mL added), 11 (Sil- All® 4x4, 2 mL added) and 12 (Sil- All® 4x4, 4 mL added). In these, there were no observed fungal growth, colour change or development of pungent smell and the physical characteristics of the samples before ensiling were preserved. Furthermore, higher volumes of the additives in these treatments generally caused a greater decrease in the pH of the treated samples than lower volumes. For instance, in treatments 3 and 4, 5 and 6, and 7 and 8 respectively, higher volumes of the additives added resulted in greater reduction in the pH of the samples. Thus, in treatment 3 (formic acid 85 %, 3 mL added), the pH recorded was 3.4 compared to the pH of 3.1 recorded in treatment 4 (formic acid 85 %, 6mL added). Similar results were recorded for GrasAAT® Lacto (treatments 5 and 6) and GrasAAT® Plus (treatments 7 and 8) for higher volumes of additives versus lower volumes. The reason for this is that, the higher volume either directly decreased the pH as in the case of formic acid 85 % or indirectly functioned by increasing the LAB population which lead to a higher production of lactic and other organic acids (Leibeinsperger et al., 1988, Elferink et al., 2000).

In ensiling, the decrease in pH of biomass and exclusion of oxygen inhibits growth of microbes and thereby prevents spoilage. According to Albers et al. (2021), acid additives are found to be extremely effective, with formic acid or mixture of formic acid and propionic acid giving the best preservation results. Formic acid added during ensiling decreases the initial pH, altering the initial water activity and buffering the biomass during fermentation. The water activity is altered due to change in concentration of solute in the liquid fraction of the biomass upon addition of the acid (Leibeinsperger et al., 1988). This direct effect of formic acid is unlike what is observed when endogenous bacteria or enzymes are added, as the latter exert their effect over a period through a mechanism of actions that can take some time.

In general, organic acids are used extensively for storing forage and could also be used for macroalgae as they produce good results in aerobic stability and general product quality (Latre et al., 2009). Formic acid 85 % is composed of 84 - 85 % formic acid and can be highly corrosive to the skin but its application in ensiling is widely accepted and safe for consumption (Norgesfôr, 2022). As can be confirmed in this work, formic acid effectively preserves macroalgae biomass because of its ability to rapidly reduce the pH of samples upon addition. Albers et al. (2021) have reported similar results, showing that formic and propionic acids mixture decreased the pH of ensiled S.L samples from 6.15 to 4.42 – 4.50. An acidic pH below 4.48 to 4.10 is efficient in preventing growth of spoilage microbes in S.L (Maneein et al., 2018). However, in preservation for longer periods, a pH below 4 is suitable as no spoilage or sugar loss was detected in S.L stored for 6 months under anaerobic conditions with addition of sulfuric and formic acids (Albers et al., 2021). Furthermore, Albers et al. (2021) measured the weights of ensiled samples to determine the presence of spoilage microbes – which would decompose the macroalgae biomass into CO_2 – and observed slight decrease in weight with about 99.2 % to 99.4 % of weight retained. They also observed less reduction in weight in samples with lower pH value of 4.32 than in 4.57. Thus, they concluded that proper ensiling and biomass preservation is best achieved when pH of ensiled sample is lower than 4. This conclusion was also supported by the observation that, when the pH was above 4 there was higher loss of biomass into the effluent liquid in the form of water particles and dissolved compounds with biomass retention around 0.62 to 0.66. These values are below efficiency and occurs whenever there are microbial activity or spoilage, with the presence of butyric acid a prime indicator of bacterial growth (Albers et al., 2021).

Other effective preservation treatments in this work were GrasAAT® Lacto, GrasAAT® Plus and Sil-All® 4x4. GrasAAT® Lacto contains lactose, which is essential to LAB during fermentation, leading to the release of lactic acid which increases the acidity of the ensiled samples. This is referred to as the 'lactose effect' and it occurs in the initial stages of addition of this treatment before the release of sugars from the macroalgae biomass (Norgesfôr, 2022). The major components of GrasAAT® Lacto are 62 % formic acid, 16 % sodium formate and 1.5 % lactose (Norgesfôr, 2022). The presence of formic acid in GrasAAT® Lacto is also essential in reducing the pH. GrasAAT® Plus is made up of 44 % formic acid,

20 % sodium formate, 12 % propionic acid, 1.5 % benzoic acid and 1 % glycerol (Norgesfôr, 2022). The presence of the 'mold inhibiting' propionic and benzoic acids prevent yeast and mold growth and thus inhibits spoilage. Combining these two acids is more potent than using only propionic acid. This is referred to as the 'benzo effect' and requires dilution with water to ensure an even distribution of the benzoic acid as it can form crystals and reduce efficacy (Norgesfôr, 2022). Sil-All® 4x4 is also used mainly in ensiling to enhance the fermentation process which promotes aerobic stability, preservation, and nutrient retention. This additive is composed of 4 bacteria strains *L. plantarum, Enterococcus faecium, P. acidilactici* and *L salivarius*; and 4 enzymes (Latre et al., 2009). Sil-All® 4x4 is very effective in whole silage preservation because of its ability to reduce the pH to around 3.75 – which inhibits the activities of many spoilage microbes (Latre et al., 2009). Similarly in this work, Sil All® 4x4 effectively preserved the ensiled macroalgae samples with a recorded pH of 3.6.

The heterofermentative LAB present in Sil-All® 4x4 breakdowns glucose and other six carbon sugars into lactic acid, acetic acid, ethanol and CO_2 through the metabolic process of lactic acid fermentation (Boumba et al., 2008, Monteiro et al., 2021). Effective fermentation preserves the ensiled macroalgae biomass by producing lactate which rapidly decreases the pH. pH level less than 4.3 inactivates most spoilage microbes at refrigeration temperatures while more acidic pH less than 3.7 is ideal for storage at ambient temperatures (Løvdal et al., 2021).

Additionally, in this work, the pH of the samples without additives and stored at -80 ℃ (treatment 1) was 6.6, which agrees with literature. Albers et al. (2021) recorded that the pH of native S.L was between 6.5 – 7.0 with a pH of 6.9 recorded for -80 ℃ frozen samples. Despite no addition of additives and a near neutral pH, freezing effectively preserved the samples. This is because freezing immobilizes the water and nutrient components around the seaweed biomass while also inactivating spoilage microbes thus effectively protecting against microbial growth and spoilage (Løvdal et al., 2021). However, there are reports that freezing reduces the sensorial, appearance and nutritional properties of the macroalgae biomass in addition to its high cost, high energy consumption and environmental footprints (Løvdal et al., 2021). Thus, this method is not ideal especially in developing large-scale seaweeds biorefinery and processing for the future.

Conversely the following treatments were unsuccessful in preserving the macroalgae samples despite their successful application in preserving grass – Kofasil® LP, Magniva® Classic +, Magniva® Platinum 2, *L. sakei R9207* and *P. acidilactici R9628*. Kofasil® LP, is mainly used to ensile light or medium dried grass and is composed of salts such as sodium nitrite, hexamethylenetetramine and sodium benzoate (Norgesfôr, 2022). Magniva® classic + is composed of a mixture of bacteria – *L plantarum* and *P pentosaceus* – and enzymes – cellulase, hemicellulose, amylase and pentosanase (Hubbard, 2020). Magniva® plantinum 2 is mainly made up of *L hilgardii, L buchneri* and *P pentosaceus* and is most efficient for high moisture corn or grass silage samples which have a DM above 30 % and have high sugar contents (Pobednov et al., 2021, Lallemand, 2022). Two salt tolerant microbes *L. sakei R9207* and *P. acidilactici R9628* from Lallemand culture collection were included in this work because of the high salt content of brown macroalgae. *L. sakei* is known to be tolerant to sodium chloride, nitrite, acidic conditions and produces the bacteriocin sakacin K (Leroy et al., 1999). However, studies have also found that sodium chloride can inhibit the growth of *L. sakei* and cause an accompanying decrease in the release of the bacteriocin sakacin K. The production of sakacin K occurs through primary metabolic reactions and thus any reduction in growth of the bacteria cells results in reduction in sakacin K release. Thus, Leroy et al. (1999) recorded that *L. sakei* growth and tolerance to salt was reduced as the salt concentration were increased. *P. acidilactici R9628* is a very suitable silage inoculant for ensiling as it displays favourable properties such as rapid acid production through fast and efficient sugar to lactate conversion and the ability to resist wide range of pH and temperature (Fitzsimons et al., 1992). *P. acidilactici R9628* can also activate the naturally occurring community of *L. plantarum* present in silage samples which influence lactic acid production, pH reduction and prevention of spoilage microbes. According to Fitzsimons et al. (1992) inoculating *P. acidilactici R9628* in macroalgae samples with low level of WSC, produces no observable stimulation of native *L. plantarum* population and thus *P. acidilactici R9628* is efficient in samples with sufficiently high WSC composition.

In Kofasil® LP (pH 6.1 – 6.7) – 3 mL and 6 mL, the pH of the treated samples after storage was still very high and thus favourable for spoilage by yeast and molds (Albers et al., 2021, Løvdal et al., 2021).

However, for samples treated with Magniva® Classic + (pH 3.5 – 3.6) – 2 mL and 4 mL, Magniva® Platinum 2 (pH 3.6 – 3.69) – 2 mL and 4 mL, *L. sakei* (pH 3.4 – 3.89) – 2 mL and 4 mL, and *P. acidilactici R9628* (pH 3.3 to 4.2) – 2 mL and 4 mL, spoilage occurred despite the low pH observed. This may likely be because of the high salt content and low amount of WSC present in the brown macroalgae (Black, 1955, Leroy et al., 1999). Thus, *P. acidilactici* and *L. sakei* are known to be less efficient, particularly in high salt concentrations as found in brown macroalgae (Fitzsimons et al., 1992, Leroy et al., 1999)

4.2 Analysis of treated S.L samples

As seen in **Table 6**, the chemical compositions of the treated samples were within the range of reported values in literature. For instance, total uronic acids for all treatments (despite being preserved or unpreserved) was between 19.1 % to 28 % of DM with Man A having the highest proportion, then Gulu A, with minute amount of Glu A and no Gal A detected, as reported in literature (Manns et al., 2014, Rhein-Knudsen et al., 2017)**.** Furthermore, the neutral monomers composition in the samples in this work were between 18.9 % to 30.2 % of DM, which were also within the range of reported values in literature (Sharma et al., 2018)**.** In this work, the two-step sulfuric acid hydrolysis was used to hydrolyse polysaccharide components of S.L and the quantification of neutral monosaccharides and sugar alcohols was done by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC – PAD). Variable yields of different monosaccharides were detected in the treated samples. The neutral monosaccharides detected in this work were glucose, galactose, xylose, mannose, fucose and mannitol. The main polysaccharides present in brown macroalgae namely fucoidan, laminarin, cellulose and alginate are hydrolysed through the sulfuric acid hydrolysis to produce neutral monosaccharides and uronic acids (Nguyen et al., 2020, Manns et al., 2014). Furthermore, the total carbohydrate composition (g/kg DM) for all samples in the various treatments was approximately 50 %, which is also consistent with findings by Olsen et al. (2021).

The most abundant free sugar in brown macroalgae is mannitol unlike grass silages which have high amount of glucose (Enquist-Newman et al., 2014). As seen in **Table 6,** both treatment 1 and 2 had similar carbohydrate compositions except for the markedly lesser mannitol amount in treatment 2 compared to treatment 1. Freezing at -80 ℃ (treatment 1) prevents both chemical reactions and growth of microbes due to the low temperature and conversion of water to ice (Albers et al., 2021). Conversely, in treatment 2, despite no additives added, the presence of native LAB population and WSC (although in small amounts in brown macroalgae) likely caused fermentation to occur (Herrmann et al., 2015, Black, 1955). This is evident by the lower pH (a change from native pH around 6.5 – 7.0 to 3.7) recorded and no visible signs of spoilage. Thus the low level of mannitol seen in treatment 2 is due to fermentation which utilized the free mannitol present to produce lactate and other organic acids (Maneein et al., 2018, Monteiro et al., 2021)

4.3 Analysis of fucoidans from treated S.L samples.

The fucoidans with the highest fucose concentration was extracted from the sample treated with formic acid 85 % (F3), followed by F6 (Sil-All 4x4), then F2 (samples with no additives added – stored at RT), F5 (GrasAAT® Plus), F4 (GrasAAT® Lacto) and F1 (freezing at -80 ℃) had the least fucose concentration. Furthermore, elemental analysis of extracted fucoidans is imperative in determining the purity of different fucoidans fractions from different species of macroalgae or different treatments (Zayed et al., 2020). To assess fucoidans purity or quality using elemental analysis, a reduction in N % amount compared to an increase in S % is indicative of the removal or lack of proteins and a corresponding increase in the sugar monomer-sulfate ratio. Thus, a lower % CP (% N * 5) relative to a higher DS in the fucoidans is indicative of a higher fucoidans purity (Zayed et al., 2020). Therefore, from this work, F6 had the highest ratio of DS: CP % (and in effect highest purity of extracted fucoidans), followed by F3, F2, F5, F4 and F1 having the least.

4.4 Bioactivity of fucoidans on H.K leukocytes

TNFα is an important multifunctional cytokine that regulates paracrine and endocrine immune responses and inflammation. Thus, it plays a crucial role in sepsis and in inflammatory responses (Fitzgerald et al., 2001). TNFα is mainly secreted by activated macrophages and monocytes, but is also produced in limited quantities by B cells, natural killer, and T cells. In fish species, the *tnfa* gene was first identified and sequenced in Japanese flounder fish and has since then been identified in other teleost fish like salmon. Other members of the necrosis family include the *TNF-ß* and lymphotoxin ß (LT-ß) (Morrison et al., 2007).

Being a pro-inflammatory cytokine, *tnfa* is one of the genes expressed immediately on the onset of infections in salmon, being a key gene and cytokine secreted in response to pathogenic attacks and inflammation (Zou et al., 2016, Morrison et al., 2007).

Zou et al. (2016) reported that, *in vitro* exposure of trout H.K leukocytes to TNFα resulted in secretion, stimulation, and upregulation of *tnfa* and other genes associated with inflammation and antimicrobial immune functions such as interleukin 1B (IL-1B) and cyclooxypenase-2. Furthermore, Amoebic gill disease (A.G.D) – the fish disease that is caused by the ectoparasite *Neoparamoeba perurans* resulted in inflammatory mucoid lesions causing up-regulation of the expression of *il-1b* and inducible nitric oxide synthase (*iNOS*) genes (Morrison et al., 2007, Kube et al., 2012). Up-regulation of *iNOS* is initiated by TNFα and interferon gamma (IFN-γ). The result of this is stimulation of nitric oxide dependant cytotoxicity that fights against the anaerobic parasitic amoebozoan causing the disease. Morrison et al. (2007) also reported identification and up-regulation of two *tnfa* transcripts – 1 and 2 – in the leukocytes of salmon upon stimulation. Regardless of the transcript up-regulated, it did not affect A.G.D pathogenesis, perhaps due to ineffective signalling or not enough cells at the infected areas to produce *tnfa*. Nonetheless, there is a correlation between TNFα production and inflammation onset in salmon (Morrison et al., 2007, Zou et al., 2016)

In this study, results obtained from the qPCR analysis showed that, there was an up-regulation of the *tnfa* gene expression in H.K leukocytes treated with fucoidan-rich seaweeds extracts for 6 h and 24 h,

with a substantial up-regulation at 24 h. The time-dependent response of the *tnfa* gene expression to fucoidans need to be further elucidated, expanding the incubation time points used. Moreover, although the fucoidans were extracted from different sources (i.e., samples from different pretreatments), with varying purity levels, they all induced an up-regulation of the *tnfa* gene as seen in **Figure 11**. However, a more definitive conclusion of the immune stimulatory functions of fucoidans cannot be drawn based on just one gene *(i.e., tnfa)* analyzed in this work. Therefore, more genes will be analysed at a later stage. Considering the biological functions of *tnfa*, stimulation of salmon cells with fucoidans could prime the immune responses of salmon against pathogens (Apostolova et al., 2020, Jayasinghe et al., 2022), leading to a better and faster immune response against pathogens. Together with the results establishing vacuum packing and formic acid-based additives as the best pretreatment methods for long term storage of macroalgae biomass, the immune modulatory effects of fucoidans constitute a desirable and marketable added-value to fucoidans (Morrison et al., 2007).

In conclusion, this thesis provides valuable information for the long-term storage of macroalgae biomass as novel pretreatments method were investigated. The results obtained contribute to ensuring an all-year-round supply of chemically intact macroalgae biomass, as this will be useful for larger-scale and industrial utilization of the biomass. Furthermore, this work also highlights that fucoidans isolated from long-term stored S.L biomass retains their bioactivity.

5.0 CONCLUSION

From this work, it can be concluded that treatments 1, 2 (only vacuum-packing), 3, 4, 5, 6, 7, 8 (formic acid-based additives), 11 and 12 (Sil All® 4x4, which contains 2 LABs and 4 enzymes) were effective in preserving the S.L samples over a long period of time (approx. 2.5 months). Also, different volumes of the additives had effect on pH reduction and preservation. In general, higher volume of the additives (6 mL or 4 mL) caused a lower pH than lower volumes (3 mL or 2 mL). Furthermore, while freezing is an effective method for brown macroalgae preservation, the high cost and energy requirement makes it unsustainable. Hence ensiling (with chemical additives, microbes, and stabilizing agents) is a less costly and sustainable means of preservation. Furthermore, higher volume of formic acid 85 % was the most effective method based on the fucose content and purity of fucoidans extracted, followed by Sil All® 4x4 and storage at RT (without any additive but vacuum packed). From fucoidans bioactivity analysis, it can be concluded that fucoidans extracted from long-term stored S.L are indeed bioactive as observed with the up-regulation of *tnfa*. Finally, the effect of fucoidans on the gene expression of *tnfa* in salmon immune cells (H.K leukocytes) is time-dependent, as cells exposed for 24 h had greater up-regulation of the gene expression than at 6 h.

6.0 FURTHER WORK AND PERSPECTIVES

Regarding storage techniques, a longer duration (i.e., 6 months or a year) for the 3 most effective techniques used in this work, namely storage with formic acid 85 %, Sil-All® 4x4 and no additive (storage at RT), should be investigated. The outcomes of those studies will positively impact the macroalgae biomass harvesting and storing system in Norway, avoiding losses due to deterioration of the biomass (caused by environmental factors), and contributing to the profitability of the macroalgae industry.

Further work should also be done analyzing variations in the chemical composition of brown macroalgae caused due to different species, time of cultivation and harvesting, and environmental factors, to determine the most optimal conditions for growing and harvesting, as well as standardizing the procedures for extraction of compounds from macroalgae.

Lastly, further studies using salmon should be done to investigate the effects of fucoidans on other genes of immunological relevance, as the bioactivity has already been probed in this fish. In addition, thorough work on the mechanism of action of fucoidans (receptors involved in recognition, cell signalling, effector molecules) is necessary, to fully understand its biological modulating effects, providing crucial insights for potential additional applications.

REFERENCES

AGULHON, P., ROBITZER, M., DAVID, L. & QUIGNARD, F. O. 2012. Structural regime identification in ionotropic alginate gels: influence of the cation nature and alginate structure. *Biomacromolecules,* 13**,** 215-220.

AHMAD, F., SULAIMAN, M. R., SAIMON, W., YEE, C. F. & MATANJUN, P. 2016. Proximate compositions and total phenolic contents of selected edible seaweed from Semporna, Sabah, Malaysia. *Borneo science*.

AHMAD, T. B. S. 2015. *Methods for quantification and extraction of fucoidan, and quantification of the release of total carbohydrate and fucoidan from the brown algae Laminaria hyperborea.* NTNU.

ALBERS, E., MALMHÄLL-BAH, E., OLSSON, J., STERNER, M., MAYERS, J. J., NYLUND, G. M., RUPAR-GADD, K., ABDOLLAHI, M., CVIJETINOVIC, S. & WELANDER, U. 2021. Influence of preservation methods on biochemical composition and downstream processing of cultivated Saccharina latissima biomass. *Algal Research,* 55**,** 102261.

ALBOOFETILEH, M., REZAEI, M. & TABARSA, M. 2019. Enzyme-assisted extraction of Nizamuddinia zanardinii for the recovery of sulfated polysaccharides with anticancer and immune-enhancing activities. *Journal of Applied Phycology,* 31**,** 1391-1402.

ALE, M. T., MARUYAMA, H., TAMAUCHI, H., MIKKELSEN, J. D. & MEYER, A. S. 2011. Fucose-containing sulfated polysaccharides from brown seaweeds inhibit proliferation of melanoma cells and induce apoptosis by activation of caspase-3 in vitro. *Marine drugs,* 9**,** 2605-2621.

ALE, M. T. & MEYER, A. S. 2013. Fucoidans from brown seaweeds: An update on structures, extraction techniques and use of enzymes as tools for structural elucidation. *Rsc Advances,* 3**,** 8131-8141.

ALLAHGHOLI, L., SARDARI, R. R., HAKVÅG, S., ARA, K. Z., KRISTJANSDOTTIR, T., AASEN, I. M., FRIDJONSSON, O. H., BRAUTASET, T., HREGGVIDSSON, G. O. & KARLSSON, E. N. 2020. Composition analysis and minimal treatments to solubilize polysaccharides from the brown seaweed Laminaria digitata for microbial growth of thermophiles. *Journal of Applied Phycology,* 32**,** 1933-1947.

ANGELL, A. R., MATA, L., DE NYS, R. & PAUL, N. A. 2016. The protein content of seaweeds: a universal nitrogen-to-protein conversion factor of five. *Journal of Applied Phycology,* 28**,** 511-524.

APOSTOLOVA, E., LUKOVA, P., BALDZHIEVA, A., KATSAROV, P., NIKOLOVA, M., ILIEV, I., PEYCHEV, L., TRICA, B., OANCEA, F. & DELATTRE, C. 2020. Immunomodulatory and anti-inflammatory effects of fucoidan: A review. *Polymers,* 12**,** 2338.

BLACK, W. 1955. The preservation of seaweed by ensiling and bactericides. *Journal of the Science of Food and Agriculture,* 6**,** 14-23.

BOUMBA, V. A., ZIAVROU, K. S. & VOUGIOUKLAKIS, T. 2008. Biochemical pathways generating post-mortem volatile compounds co-detected during forensic ethanol analyses. *Forensic science international,* 174**,** 133-151.

CAI, J., LOVATELLI, A., AGUILAR-MANJARREZ, J., CORNISH, L., DABBADIE, L., DESROCHERS, A., DIFFEY, S., GARRIDO GAMARRO, E., GEEHAN, J. & HURTADO, A. 2021. Seaweeds and microalgae: an overview for unlocking their potential in global aquaculture development. *FAO Fisheries and Aquaculture Circular*.

CAIPANG, C. M. A., LAZADO, C. C., BERG, I., BRINCHMANN, M. F. & KIRON, V. 2011. Influence of alginic acid and fucoidan on the immune responses of head kidney leukocytes in cod. *Fish Physiology and Biochemistry,* 37**,** 603-612.

COUNCIL, N. S. 2022. *Norwegian seafood exports exceed NOK 70 billion in the first half of the year.* [Online]. Available: https://en.seafood.no/news-and-media/news-archive/norwegian-seafood-exports-exceed-nok-70-billion-in-the-first-half-of-the-year/ [Accessed].

DE CASTRO, G. M. C., BENEVIDES, N. M. B., CABRAL, M. C., DE SOUZA MIRANDA, R., GOMES FILHO, E., ROCHA, M. V. P. & ARAÚJO, M. L. H. 2017. Optimized acid hydrolysis of the polysaccharides from the seaweed Solieria filiformis (Kützing) PW Gabrielson for bioethanol production. *Acta Scientiarum. Biological Sciences,* 39**,** 423-430.

DENIAUD-BOUËT, E., HARDOUIN, K., POTIN, P., KLOAREG, B. & HERVÉ, C. 2017. A review about brown algal cell walls and fucose-containing sulfated polysaccharides: Cell wall context, biomedical properties and key research challenges. *Carbohydrate polymers,* 175**,** 395-408.

DENIAUD-BOUËT, E., KERVAREC, N., MICHEL, G., TONON, T., KLOAREG, B. & HERVÉ, C. 2014. Chemical and enzymatic fractionation of cell walls from Fucales: insights into the structure of the extracellular matrix of brown algae. *Annals of Botany,* 114**,** 1203-1216.

ELFERINK, S., DRIEHUIS, F., GOTTSCHAL, J. C. & SPOELSTRA, S. F. 2000. Silage fermentation processes and their manipulation. *FAO Plant Production and Protection Papers***,** 17-30.

ENQUIST-NEWMAN, M., FAUST, A. M. E., BRAVO, D. D., SANTOS, C. N. S., RAISNER, R. M., HANEL, A., SARVABHOWMAN, P., LE, C., REGITSKY, D. D. & COOPER, S. R. 2014. Efficient ethanol production from brown macroalgae sugars by a synthetic yeast platform. *Nature,* 505**,** 239-243.

FIRDAUS-NAWI, M. & ZAMRI-SAAD, M. 2016. Major Components of Fish Immunity: A Review. *Pertanika Journal of Tropical Agricultural Science,* 39.

FITZGERALD, K. A., O'NEILL, L. A., GEARING, A. J. & CALLARD, R. E. 2001. *The cytokine factsbook and webfacts*, Elsevier.

FITZSIMONS, A., DUFFNER, F., CURTIN, D., BROPHY, G., O'KIELY, P. & O'CONNELL, M. 1992. Assessment of Pediococcus acidilactici as a potential silage inoculant. *Applied and Environmental Microbiology,* 58**,** 3047-3052.

FLORES-KOSSACK, C., MONTERO, R., KÖLLNER, B. & MAISEY, K. 2020. Chilean aquaculture and the new challenges: Pathogens, immune response, vaccination and fish diversification. *Fish & shellfish immunology,* 98**,** 52-67.

FLÓREZ-FERNÁNDEZ, N., LÓPEZ-GARCÍA, M., GONZÁLEZ-MUÑOZ, M. J., VILARIÑO, J. M. L. & DOMÍNGUEZ, H. 2017. Ultrasound-assisted extraction of fucoidan from Sargassum muticum. *Journal of Applied Phycology,* 29**,** 1553-1561.

FORBORD, S., MATSSON, S., BRODAHL, G. E., BLUHM, B. A., BROCH, O. J., HANDÅ, A., METAXAS, A., SKJERMO, J., STEINHOVDEN, K. B. & OLSEN, Y. 2020. Latitudinal, seasonal and depth-dependent variation in growth, chemical composition and biofouling of cultivated Saccharina latissima (Phaeophyceae) along the Norwegian coast. *Journal of Applied Phycology,* 32**,** 2215-2232.

FRANCO, R. T., BUFFIÈRE, P. & BAYARD, R. 2016. Ensiling for biogas production: Critical parameters. A review. *Biomass and Bioenergy,* 94**,** 94-104.

HAKIM, M. M. & PATEL, I. C. 2020. A review on phytoconstituents of marine brown algae. *Future Journal of Pharmaceutical Sciences,* 6**,** 1-11.

HERRMANN, C., FITZGERALD, J., O'SHEA, R., XIA, A., O'KIELY, P. & MURPHY, J. D. 2015. Ensiling of seaweed for a seaweed biofuel industry. *Bioresource technology,* 196**,** 301-313.

HOLDT, S. L. & KRAAN, S. 2011. Bioactive compounds in seaweed: functional food applications and legislation. *Journal of applied phycology,* 23**,** 543-597.

HU, Y., MAISEY, K., SUBRAMANI, P. A., LIU, F., FLORES-KOSSACK, C., IMARAI, M., SECOMBES, C. J. & WANG, T. 2018. Characterisation of rainbow trout peripheral blood leucocytes prepared by hypotonic lysis of erythrocytes, and analysis of their phagocytic activity, proliferation and response to PAMPs and proinflammatory cytokines. *Developmental & Comparative Immunology,* 88**,** 104-113.

HUBBARD. 2020. Available: https://www.hubbardfeeds.com/species/beef/forage/magniva-classic# [Accessed].

JAMES, P. & WANG, X. 2020. *Kelp farming: a great opportunity for northern Norway and the world* [Online]. Available: https://thefishsite.com/articles/kelp-farming-a-great-opportunity-for-northern-norway-and-the-world#:~:text=Recently%2C%20seaweed%20farming%20has%20expanded,and%20commonly%20cultivated%20commercial%20species. [Accessed].

JANG, J.-Y., MOON, S.-Y. & JOO, H.-G. 2014. Differential effects of fucoidans with low and high molecular weight on the viability and function of spleen cells. *Food and chemical toxicology,* 68**,** 234-238.

JAYASINGHE, A. M. K., KIRINDAGE, K. G. I. S., FERNANDO, I. P. S., HAN, E. J., OH, G.-W., JUNG, W.-K. & AHN, G. 2022. Fucoidan Isolated from Sargassum confusum Suppresses

Inflammatory Responses and Oxidative Stress in TNF-α/IFN-γ-Stimulated HaCaT Keratinocytes by Activating Nrf2/HO-1 Signaling Pathway. *Marine Drugs,* 20**,** 117.
KADAM, S. U., TIWARI, B. K. & O'DONNELL, C. P. 2015. Extraction, structure and biofunctional activities of laminarin from brown algae. *International Journal of Food Science & Technology,* 50**,** 24-31.
KIM, J. K., YARISH, C., HWANG, E. K., PARK, M. & KIM, Y. 2017. Seaweed aquaculture: cultivation technologies, challenges and its ecosystem services. *Algae,* 32**,** 1-13.
KUBE, P. D., TAYLOR, R. S. & ELLIOTT, N. G. 2012. Genetic variation in parasite resistance of Atlantic salmon to amoebic gill disease over multiple infections. *Aquaculture,* 364**,** 165-172.
LALLEMAND. 2022. Available: https://lallemandanimalnutrition.com/en/europe/our-products/product-details/magniva-platinum-2/ [Accessed].
LATRE, J., WAMBACQ, E., NOLLET, J. & HAESAERT, G. Effects of silage additives on aerobic stability of maize silage. Proceedings XVth International Silage Conference, July, 2009. 27-29.
LEIBEINSPERGER, R. & PITT, R. 1988. Modeling the effects of formic acid and molasses on ensilage. *Journal of Dairy Science,* 71**,** 1220-1231.
LEROY, F. & DE VUYST, L. 1999. The presence of salt and a curing agent reduces bacteriocin production by Lactobacillus sakei CTC 494, a potential starter culture for sausage fermentation. *Applied and Environmental Microbiology,* 65**,** 5350-5356.
LI, B., LU, F., WEI, X. & ZHAO, R. 2008. Fucoidan: structure and bioactivity. *Molecules,* 13**,** 1671-1695.
LI, Y., ZHENG, Y., ZHANG, Y., YANG, Y., WANG, P., IMRE, B., WONG, A. C., HSIEH, Y. S. & WANG, D. 2021. Brown Algae Carbohydrates: Structures, Pharmaceutical Properties, and Research Challenges. *Marine Drugs,* 19**,** 620.
LØVDAL, T., LUNESTAD, B. T., MYRMEL, M., ROSNES, J. T. & SKIPNES, D. 2021. Microbiological food safety of seaweeds. *Foods,* 10**,** 2719.
MADHUN, A. S., KARLSEN, Ø., NILSEN, R. & KVAMME, B. O. 2022. Annual report on health monitoring of wild anadromous salmonids in Norway 2021-Screening of Atlantic salmon (Salmo salar) postsmolts for viral infections. *Rapport fra havforskningen*.
MANEEIN, S., MILLEDGE, J. J., NIELSEN, B. V. & HARVEY, P. J. 2018. A review of seaweed pre-treatment methods for enhanced biofuel production by anaerobic digestion or fermentation. *Fermentation,* 4**,** 100.
MANNS, D., DEUTSCHLE, A. L., SAAKE, B. & MEYER, A. S. 2014. Methodology for quantitative determination of the carbohydrate composition of brown seaweeds (Laminariaceae). *Rsc Advances,* 4**,** 25736-25746.
MIYASHITA, K., MIKAMI, N. & HOSOKAWA, M. 2013. Chemical and nutritional characteristics of brown seaweed lipids: A review. *Journal of Functional Foods,* 5**,** 1507-1517.
MONTEIRO, P., LOMARTIRE, S., COTAS, J., PACHECO, D., MARQUES, J. C., PEREIRA, L. & GONÇALVES, A. M. 2021. Seaweeds as a Fermentation Substrate: A Challenge for the Food Processing Industry. *Processes,* 9**,** 1953.
MORONEY, N. C., O'GRADY, M. N., LORDAN, S., STANTON, C. & KERRY, J. P. 2015. Seaweed polysaccharides (laminarin and fucoidan) as functional ingredients in pork meat: An evaluation of anti-oxidative potential, thermal stability and bioaccessibility. *Marine drugs,* 13**,** 2447-2464.
MORRISON, R., ZOU, J., SECOMBES, C., SCAPIGLIATI, G., ADAMS, M. & NOWAK, B. 2007. Molecular cloning and expression analysis of tumour necrosis factor-α in amoebic gill disease (AGD)-affected Atlantic salmon (Salmo salar L.). *Fish & Shellfish Immunology,* 23**,** 1015-1031.
MORRISSEY, J., KRAAN, S. & GUIRY, M. D. 2001. *A guide to commercially important seaweeds on the Irish coast*, Irish Bord Iascaigh Mhara/Irish Sea Fisheries Board.
NGUYEN, T. T., MIKKELSEN, M. D., TRAN, V. H. N., TRANG, V. T. D., RHEIN-KNUDSEN, N., HOLCK, J., RASIN, A. B., CAO, H. T. T., VAN, T. T. T. & MEYER, A. S. 2020. Enzyme-assisted fucoidan extraction from brown macroalgae Fucus distichus subsp. evanescens and Saccharina latissima. *Marine Drugs,* 18**,** 296.
NORGESFÔR. 2022. Available: https://www.norgesfor.no/produkter/butikk-driftsmidler/driftsmidler/ensileringsmidler/ [Accessed].

OLSEN, Y., SURILAYANI, D. & HANDÅ, A. Carbohydrate of the Brown Seaweed, Saccharina latissima: A Review. 2nd and 3rd International Conference on Food Security Innovation (ICFSI 2018-2019), 2021. Atlantis Press, 180-182.
ØVERLAND, M., MYDLAND, L. T. & SKREDE, A. 2019. Marine macroalgae as sources of protein and bioactive compounds in feed for monogastric animals. *Journal of the Science of Food and Agriculture,* 99**,** 13-24.
PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic acids research,* 29**,** e45-e45.
POBEDNOV, Y. A., MAMAEV, A., OSIPYAN, B., KUCHIN, I. & YURTAEVA, K. 2021. Study of factors determining aerobic stability of silage from corn harvested in the phase of waxy ripeness of the grain. *Problems of Productive Animal Biology*.
PRESS, C. M. & EVENSEN, Ø. 1999. The morphology of the immune system in teleost fishes. *Fish & shellfish immunology,* 9**,** 309-318.
QUINTERO, J. A., RINCÓN, L. E. & CARDONA, C. A. 2011. Production of bioethanol from agroindustrial residues as feedstocks. *Biofuels.* Elsevier.
RHEIN-KNUDSEN, N., ALE, M. T., AJALLOUEIAN, F. & MEYER, A. S. 2017. Characterization of alginates from Ghanaian brown seaweeds: Sargassum spp. and Padina spp. *Food Hydrocolloids,* 71**,** 236-244.
RHEIN-KNUDSEN, N., ALE, M. T. & MEYER, A. S. 2015. Seaweed hydrocolloid production: an update on enzyme assisted extraction and modification technologies. *Marine drugs,* 13**,** 3340-3359.
RIOUX, L.-E. & TURGEON, S. L. 2015. Seaweed carbohydrates. *Seaweed sustainability.* Elsevier.
SHAO, Z. & DUAN, D. 2022. The Cell Wall Polysaccharides Biosynthesis in Seaweeds: A Molecular Perspective. *Frontiers in Plant Science,* 13.
SHARMA, S., NEVES, L., FUNDERUD, J., MYDLAND, L. T., ØVERLAND, M. & HORN, S. J. 2018. Seasonal and depth variations in the chemical composition of cultivated Saccharina latissima. *Algal research,* 32**,** 107-112.
SOMMERSET I, B. J. B., BORNØ B, HAUKAAS A AND BRUN E (EDS.). 2021. The Health Situation in Norwegian Aquaculture 2020. . *Norwegian Veterinary Institute*
SOULLIERE, C. & DIXON, B. 2016. Immune system organs of bony fishes. *Reference Module in Life Sciences.* Elsevier.
STÉVANT, P. 2019. Seaweeds in food applications: effects of processing on product quality.
TANAKA, Y., ASHAARI, A., MOHAMAD, F. S. & LAMIT, N. 2020. Bioremediation potential of tropical seaweeds in aquaculture: Low-salinity tolerance, phosphorus content, and production of UV-absorbing compounds. *Aquaculture,* 518**,** 734853.
THORSNÆS, G. 2022. *Norway's geography in Store norske lexikon at snl.no* [Online]. Available: https://snl.no/Norges_geografi [Accessed 17 September 2022].
UCHIDA, M. & MIYOSHI, T. 2013. Algal fermentation—The seed for a new fermentation industry of foods and related products. *Japan Agricultural Research Quarterly: JARQ,* 47**,** 53-63.
URIBE, C., FOLCH, H., ENRÍQUEZ, R. & MORAN, G. 2011. Innate and adaptive immunity in teleost fish: a review. *Veterinarni medicina,* 56**,** 486.
WHITE, N. & MARSHALL, C. 2007. Saccharina latissima. Sugar kelp.
WISE, T. A. 2013. Can we feed the world in 2050. *A scoping paper to assess the evidence. Global Development and Environment Institute Working Paper*.
WITZØE, A. 2019. *The algae bloom has cost Norwegian salmon farmers at least € 84 million* [Online]. Available: https://salmonbusiness.com/the-algae-bloom-has-cost-norwegian-salmon-farmers-at-least-e-84-million/ [Accessed].
YITBAREK, M. B. & TAMIR, B. 2014. Silage additives. *Open Journal of Applied Sciences,* 2014.
YUAN, Y. & MACQUARRIE, D. J. 2015. Microwave assisted step-by-step process for the production of fucoidan, alginate sodium, sugars and biochar from Ascophyllum nodosum through a biorefinery concept. *Bioresource technology,* 198**,** 819-827.
ZAYED, A., EL-AASR, M., IBRAHIM, A.-R. S. & ULBER, R. 2020. Fucoidan characterization: Determination of purity and physicochemical and chemical properties. *Marine Drugs,* 18**,** 571.
ZOU, J. & SECOMBES, C. J. 2016. The function of fish cytokines. *Biology,* 5**,** 23.

ZWOLLO, P., COLE, S., BROMAGE, E. & KAATTARI, S. 2005. B cell heterogeneity in the teleost kidney: evidence for a maturation gradient from anterior to posterior kidney. *The Journal of Immunology,* 174**,** 6608-6616.

APPENDIX

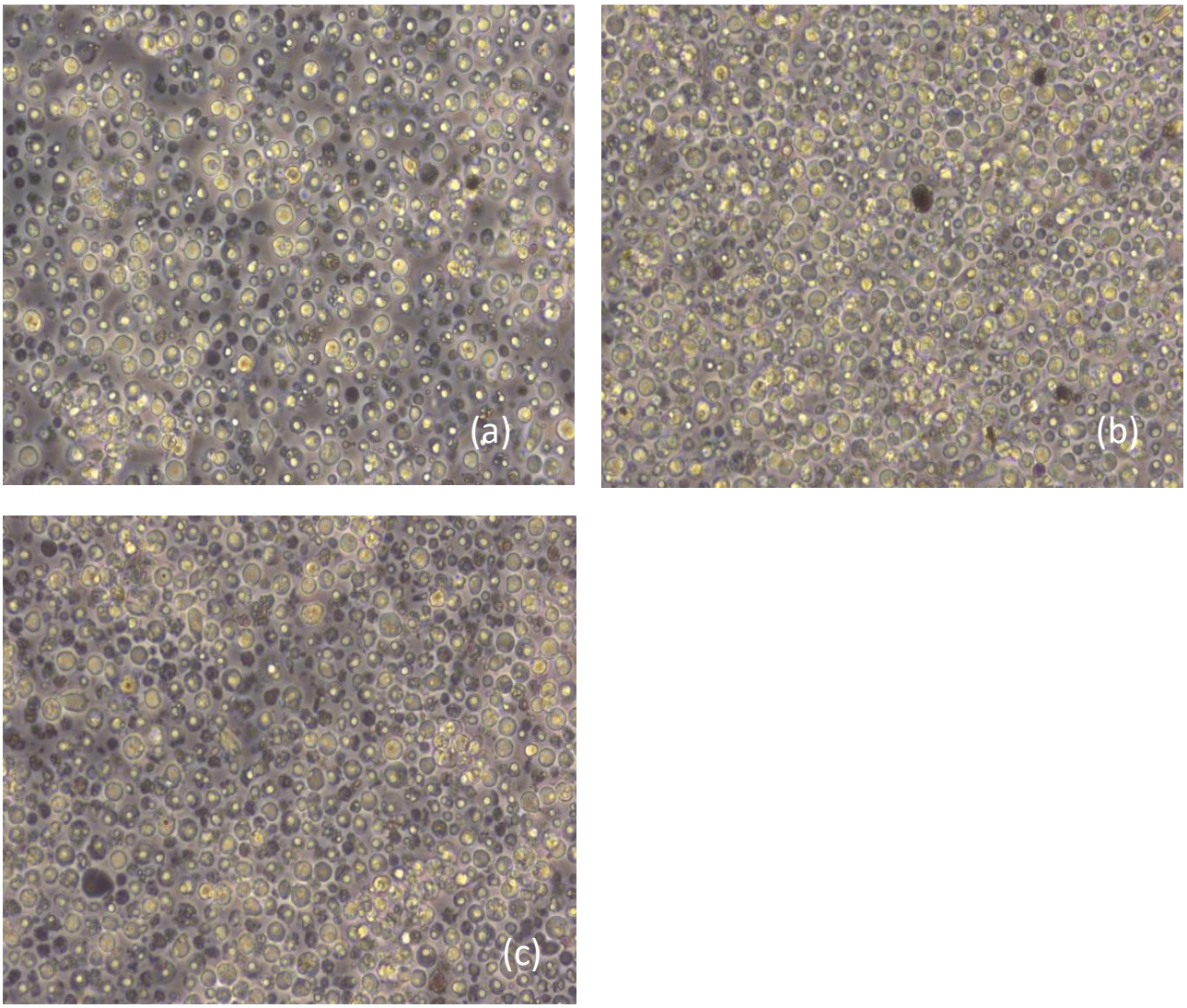
Appendix A: Average fold change, standard deviation (SD) and standard error (SE) for F1, F2 and F3.

	Time interval	Average fold change	SD	SE
F1	6	1.26	0.39	0.10
F1	24	2.64	1.53	0.39
F2	6	1.26	0.34	0.08
F2	24	2.23	1.28	0.33
F3	6	1.29	0.34	0.08
F3	24	2.50	1.37	0.35
Control		1	0.08	0.04

Appendix B: Independent sample t-test for F1, F2 and F3 (fucoidans effect on *tnfa* gene for 6 hrs and 24 hrs)

	t-test for equality of means (p-value <0.05)	
	Time intervals	
	6 hrs	24 hrs
F1	0.015	<0.001
F2	0.007	0.001
F3	0.004	<0.001

Appendix C: H.K leukocytes from salmon used in the analysis, observed under a microscope.



Appendix C: H.K leukocytes from salmon observed under a microscope (10 x) after trypan blue exclusion. (a) – H.K leukocytes from fish 2 treated with F1 for 24 h. (b) – H.K leukocytes from fish 2 treated with F2 for 24 h. (c) – H.K leukocytes from fish 2 treated with F3 for 24 h. Note the bright cells in all cases (live cells).

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

Postboks 5003
NO-1432 Ås
Norway