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# **The effect of *Laminaria hyperborea* and its bioactive components on the intestinal health of Atlantic salmon**

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## List of abbreviations

|                                |  |
|--------------------------------|--|
| <b>ANF</b>                     | Anti-nutritional factor                |
| <b>BW</b>                      | Body weight                            |
| <b>CP</b>                      | Crude protein                          |
| <b>DC</b>                      | Dendritic cells                        |
| <b>DI</b>                      | Distal intestine                       |
| <b>DM</b>                      | Dry matter                             |
| <b>FCR</b>                     | Feed conversion ratio                  |
| <b>FM</b>                      | Fish meal                              |
| <b>FUC</b>                     | Fucoidan                               |
| <b>HPLC</b>                    | High performance liquid chromatography |
| <b>IFN-<math>\gamma</math></b> | Interferon gamma                       |
| <b>IL</b>                      | Interleukin                            |
| <b>iNOS</b>                    | Inducible nitric oxide synthase        |
| <b>LAM</b>                     | Laminarin                              |
| <b>LF</b>                      | Laminarin and Fucoidan                 |
| <b>LH</b>                      | <i>Laminaria hyperborea</i>            |
| <b>LPS</b>                     | Lipopolysaccharide                     |
| <b>MW</b>                      | Molecular weight                       |
| <b>MWCO</b>                    | Molecular weight cut off               |
| <b>NK</b>                      | Natural killer                         |
| <b>NO</b>                      | Nitric oxide                           |
| <b>SBM</b>                     | Soy bean meal                          |
| <b>SBMIE</b>                   | Soy bean meal-induced enteropathy      |
| <b>SGR</b>                     | Specific growth rate                   |
| <b>TLR</b>                     | Toll-like receptors                    |
| <b>TNF-<math>\alpha</math></b> | Tumor necrosis factor alpha            |





## Abstract

The rapid expansion of the aquaculture industry is imposing an increasing demand on the available feed resources for aquafeeds, particularly fishmeal. Plant-derived ingredients have been widely investigated in recent years as alternative protein and energy sources. Nevertheless, anti-nutritional factors present in plant ingredients can lead to reduced growth performance and affect gut health and function. In recent years, research has shown that certain feed ingredients and additives prevent or ameliorate the negative effects of some plant-derived anti-nutrients on gut health. Marine algae contain a variety of novel polysaccharides, which are highly bioactive and can be used as a new line of functional dietary supplements. In this study, the salmonid soy bean meal induced enteritis model has been used to investigate the effect of dietary inclusion of brown seaweed *Laminaria hyperborea* (LH) and two extracted bioactive compounds, fucoidan and laminarin, on the intestinal health of Atlantic salmon. The LH extract was obtained by incubation of ground LH in 0.03 M HCl for an hour at 70°C. CaCl<sub>2</sub> was used to precipitate alginate from the extract. Crude fucoidan and laminarin were obtained by step-wise filtration of the extract through spiral membranes with molecular weight cut offs at 100, 50, 25, 10 and 2 kDa. Six diets were formulated and extruded for this experiment. The dietary treatments were a fish meal (FM)-based diet as a negative control, a soybean meal (SBM)-based diet as a positive control, a test diet containing 5% LH, as well as three test diets containing either fucoidan or laminarin or both in concentration of 0.075%. All diets except for the fish meal based diet were formulated to contain 20% SBM. There were triplicate tanks per dietary treatment and 18 tanks in total with 12 fish per tank. At the end of the experimental period, the fish were sacrificed and the distal intestine (DI), liver, mid kidney, spleen, skin, feces and blood were collected from 4 fish per tank. There was no significant difference in the growth performance of fish fed the experimental diets. However, a large intra-treatment

variation in the performance of fish was observed. Fish fed the FM-based diet showed significantly lower signs of inflammation compared to the fish fed other dietary treatments, while fish fed the SBM-based diet and the test diets with LH and the bioactive components showed clear signs of enteritis. To conclude, the dietary inclusion of LH and the bioactive compounds had no significant amelioration effect on inflammation.

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Rouzbeh Keihani



## 1. Introduction

Rapid expansion of the aquaculture industry is imposing an increasing demand on available feed resources for aquafeeds, particularly fishmeal. To keep pace with this demand, there is an urge to develop new ingredients that can be both economically and nutritionally viable and sustainable for inclusion in aquafeeds. Plant-derived ingredients have been widely investigated during recent years as alternative protein and energy sources, due to their availability and competitive prices. Nevertheless, the inclusion level of these ingredients in aquafeeds, and particularly in the diets of carnivores such as salmonids, are limited by the presence of anti-nutritional factors (ANFs), unbalance amino acid profiles and/or high cost of processing for protein concentration and elimination of anti-nutrients (Francis et al., 2001; Gatlin et al., 2007; Hardy, 2010). Many of the ANFs in plant ingredients can affect fish gut health and function in a dose-dependent manner. They may lead to reduced growth performance, alteration of gut histology and microbiota as well as weakening of the immune defense mechanisms (Krogdahl et al., 2000; Francis et al., 2001; Oliva, 2012).

In recent years, it has been shown that certain feed ingredients and additives prevent or ameliorate the negative effects of some plant ingredients on gut health. Romarheim et al. (2011), for example, showed that inclusion of 300 g/kg of a bacterial meal in the diets of Atlantic salmon prevents soybean meal induced enteritis. Likewise, Grammes et al. (2013) reported that inclusion of the yeast *Candida utilis* and the microalgae *Chlorella vulgaris* counteract the negative effect of SBM on the intestinal homeostasis. In another study by Vasanth et al. (2015), the dietary inclusion of microbial feed additive (Bactocell®) alleviated oxazolone induced inflammation in Atlantic salmon. Furthermore, different studies have shown that inclusion of functional dietary supplements (probiotics and prebiotics) in aquafeeds can be an effective approach in improving fish performance,

immunity, overall health and resistance to disease (Nayak, 2010; Reverter et al., 2014; Song et al., 2014; Carbone & Faggio, 2016).

Marine macroalgae or seaweeds contain a variety of novel polysaccharides, which are highly bioactive and can be used as a new line of functional dietary supplements. Brown seaweeds, for example, are valuable sources of functional compounds such as alginates, polyphenols, laminarin and fucoidan (Freile et al., 2008; Plaza et al., 2008; Manilal et al., 2009; Holdt & Kraan, 2011; Yu & Gu, 2015). *Laminaria hyperborea* (LH) is one of the main seaweed species that is harvested commercially for the production of alginate in the Europe. It is a large brown seaweed growing mostly in shallow coast waters of Norway, Scotland and Ireland, and its stipe can reach up to 3m in length (Andersen et al., 1996; Werner & Kraan, 2004). It is generally localized all along the coast of Norway with the highest concentration on the west coast. The production and harvesting of this seaweed are closely monitored and regulated to ensure a sustainable harvesting practice (Werner & Kraan, 2004; Schiener et al., 2015). The utilization of LH in Norway has a long tradition. It dates back prior to the establishment and development of alginate industry when it was mostly used as animal feed additive and fertilizer. Nowadays, the harvesting is mostly mechanized and the industry is capable of providing more raw materials for different application (Vea & Ask, 2011). In Norway, more than 160,000 tons of LH are harvested annually (Werner & Kraan, 2004).

This section briefly reviews two important bioactive compounds in LH that are pertinent to this study.

### ***1.1 Fucoidans***

Fucoidans are high molecular weight sulfated fucose polysaccharides mostly found in the fronds and to a lesser extent in the stipes of most brown seaweeds. Depending on the source, they may occur in various structural forms and chemical composition. The term fucose-containing sulfated

polysaccharides may be alternatively used to refer to these compounds in general (Ale & Meyer, 2013; Ehrig & Alban, 2014). The principal structure of fucoidan is consisted of a backbone of (1→3)  $\alpha$ -L-fucopyranose. The backbone may be ramified by alternating (1→4) or/and (1→2)-linked  $\alpha$ -L-fucopyranose residues. Sulfate ( $\text{SO}_3^-$ ) and sometimes acetate ( $\text{CH}_3\text{COO}^-$ ) may substitute the L-fucopyranose residues, mostly on the C-2 or C-4 and occasionally C-3 (Figure 1). Structure may also contain minor amount of monosaccharides such as mannose, xylose, glucose and/or glucuronic acid (Bilan et al., 2006; Li et al., 2008; Ale et al., 2011).

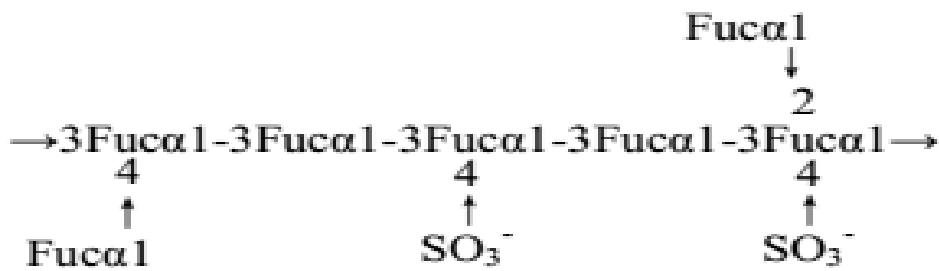


Figure 1. The average structure of fucoidan as reported by Patankar et al. (1993)

Along with the complexity of the structure, which results in occurrence of fucoidans with different molecular weights, the nature and strength of biological responses of these compounds differ notably (Cumashi et al., 2007; Zhang et al., 2015). Therefore, generalization of bioactivities is impractical. A wide variety of biological activities, including immunomodulatory anti-inflammatory, antitumor, antiviral, antithrombotic, anticoagulant, and antioxidant activities have been reported regarding fucoidans (Witvrouw & De Clercq, 1997; Pereira et al., 1999; Omar et al., 2013; Kwak, 2014). It has been suggested that the degree of sulfonation, position of the sulfate groups and molecular size are influential in at least some bioactive properties (Pereira et al., 1999;

Luyt et al., 2003; Zhang et al., 2015). Studies indicate that fucoidans are capable of modulating the function of different immune cells and cytokine expression. However, the mechanism and direction of modulation can differ according to the present immune balance and the type of challenge. The antitumor activity of fucoidans is assigned to its ability to increase macrophage phagocytosis activity and the production of macrophage-related proinflammatory molecules such as interleukin (IL)-2, IL-12 and interferon gamma (IFN- $\gamma$ ), which in turn lead to rapid amplification of natural killer (NK) and T cell population (Ale et al., 2011; Zorofchian et al., 2014). It has been reported that fucoidan stimulates maturation of human antigen presenting dendritic cells (DCs). Fucoidan induces DCs to turn into a potent producer of tumor necrosis factor alpha (TNF- $\alpha$ ) and IL-2, a combination that eventually drives immune system toward the production of T helper type 1 (Yang et al., 2008). Halling et al. (2015) reported enhanced phagocytic activity and significant increase in IL-2 expression in the spleen cells of mouse fed 1.7 and 5 mg /kg body weight (BW) fucoidan from LH. Studies also indicate that fucoidan can be both stimulator and inhibitor of nitric oxide (NO) production. In a study by Kar et al. (2011) the oral administration of fucoidan (25 mg/kg/day, 3 times weekly) increased the production of nitric oxide (NO) by macrophage in the parasite infected rats and led to elimination of parasite. On the contrary, fucoidan suppressed inducible nitric oxide synthase (iNOS) expression and NO production in rat glioma cells by inhibiting the production of proinflammatory TNF- $\alpha$ - and IFN- $\gamma$  (Do et al., 2010). It also prevented aspirin induced gastric ulceration in a similar manner when orally administrated to the rat at 0.02 g/kg BW daily (Choi et al., 2010). The bifunctional effect of fucoidan on NO production has been also reported by Yang et al. (2006). In their study, exposing quiescent macrophages to a low concentration of fucoidan caused a slight rise in the basal expression level of iNOS. Contrarily, fucoidan inhibited the release of NO from mouse



macrophage-like cell line (RAW264.7 cells) after lipopolysaccharide (LPS) stimulation. It has been also reported that fucoidan inhibits the activity of the adhesion molecules selectins. So, it attenuates inflammation by preventing the recruitment of inflammatory cells into affected sites (Cumashi et al., 2007; Fitton et al., 2015). This anti-inflammatory property also tested by Kyung et al. (2012) after creation of an inflammatory condition by injection of carrageenan into the air pouch of rats. They reported that infiltration of inflammatory cells into air pouch was potently blocked by fucoidan intake of 18 mg/kg BW. Additionally, oral intake of fucoidan could ameliorate inflammatory bowel diseases by down regulating of IL-6 as well as the protection of epithelial barrier function by inducing the expression of some tight junction proteins (Iraha et al., 2013). Isnansetyo et al. (2016) tested the effect of fucoidan on non-specific immune response of tilapia. The result showed that intraperitoneal injection of fucoidan at doses of 0.4–0.6 mg/kg fish lead to significant increases in phagocytic activity and leukocyte count in the blood samples of tilapia.

## ***1.2 Laminarin***

Laminarin is the storage polysaccharide of brown seaweed. It is a low molecular weight  $\beta$ -glucan consisted of (1,3)-  $\beta$ -D-glucopyranose residues with some branching. Two type of chains namely M and G have been observed in laminarin structure. M chains contain mannitol while G chains have a glucose unit at reducing end (Figure 2). Laminarin is usually composed of 20-25 glucose units and the average molecular weight is about 5 kDa (range from 2–7 kDa) (Graiff et al., 2015; Kadam et al., 2015b). The composition of laminarin varies depending on species, age of seaweed and the environmental factors such as temperature, salinity and sea current. The variation is mostly observed in M:G ratio, degree of polymerization and branching. The structural variation affects both chemical and biological properties of laminarin. Degree of branching, for instance, affects

solubility of laminarin in water. Laminarins with high degree of branching are soluble in cold water whereas those with low levels of branching are only dissolved in warm water (Rioux et al., 2007).

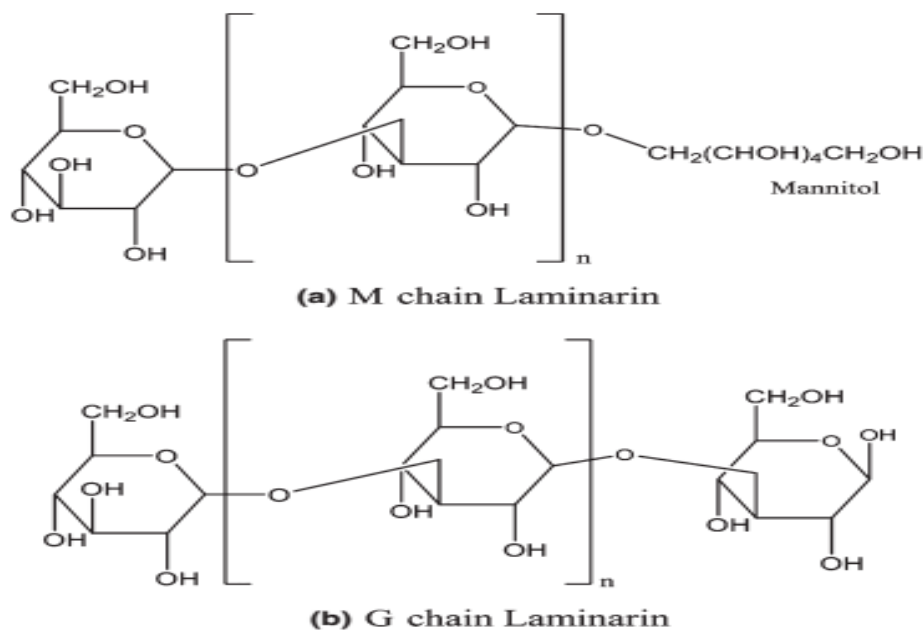


Figure 2. Chemical structure of M and G chains in laminarin (Kadam et al., 2015b)

A diverse range of biological activities have been reported for laminarin. The proliferation of RAW 264.7 cells in the presence of laminarin led to elevated inflammatory mediators such as  $H_2O_2$ , NO, transcription factors, immune response gene and different cytokines in the mouse macrophages. It was concluded that laminarin reinforces the immune reaction via transcription factor pathways (Lee et al., 2012). Neyrinck et al. (2007) studied the effect of dietary laminarin on the LPS induced systematic inflammation in the rats. They reported that the laminarin fed group had significantly lower concentrations of serum TNF- $\alpha$ , monocytes and nitrite ( $NO_2$ ) comparing with the control group. They also observed reduced monocyte/neutrophil concentration in the liver of the laminarin fed group. It was concluded that the observed results could be either due to the direct effect of  $\beta$ -glucans on the immune cells or an indirect effect originating from fermentation in the gut. Maternal

supplementation of 1 g laminarin per day (Heim et al., 2015) and 300 ppm per day to weaned piglets (Walsh et al., 2013; Heim et al., 2014) resulted in improved intestinal health, including longer villi, lower population of *Enterobacteriaceae* and down regulation of colonic IL-6 and iliac IL-8. The laminarin fed piglets also had superior growth performance during growing finisher period comparing to the control group. The authors suggested that the anti-inflammatory effect might be due to change in the intestinal microbiota. Smith et al. (2011) also reported that dietary inclusion of 300 ppm laminarin led to the reduction in the *Enterobacteriaceae* populations in the intestines of pigs with a mean BW of 17.9 kg. Furthermore, laminarin exerted a proinflammatory effect by enhancing IL-6 and IL-8 cytokine expression of colonic tissue challenged by LPS in vitro. The fish grouper (*Epinephelus coioides*) fed 0.5% and 1% laminarin in the diet showed improved growth rate and feed conversion ratio (Yin et al., 2014). It has been also reported that feeding laminarin to the rats could modulate intestinal metabolism and mucus composition. Moreover, it caused a significant increase in the levels of short chain fatty acids, especially butyrate (Devillé et al., 2007).

### **1.3 Extraction methods**

Various methods have been employed for extraction of both fucoidan and laminarin. Even so, no standard method for extraction has been devised. The extraction process is most often consisted of grinding, solid liquid extraction with solvents such as hot water, acid, base, CaCl<sub>2</sub> or /and ethanol at temperatures ranging from ambient to 100° C (Black et al., 1951; Black et al., 1952; Yvin et al., 1999; Devillé et al., 2004; Bilan et al., 2006; Hahn et al., 2012; Ale & Meyer, 2013; Kadam et al., 2015b). Pretreatment with a mixture of CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O or acetone may also be used to remove lipids, proteins and phenol compounds (Chizhov et al., 1999; Rocha et al., 2005; Cumashi et al., 2007). So far the treatment with diluted acid at ambient or slightly elevated temperature seems to

be the preferred first step in extraction procedure. After initial treatment of seaweed, several steps may be taken for separation and purification of the target compounds. The molecular weight (MW) of laminarin is lower than other major polysaccharides present in the seaweeds. Therefore, simple dialysis with an appropriate molecular weight cut-off (MWCO) is most often sufficient to isolate laminarin. The quantity of laminarin is usually determined by total acid hydrolysis of a sample into glucose. The quantity of glucose is then assumed to be the equivalent of laminarin content (Kadam et al., 2015b), although a small portion of glucose may be originated from cellulose. Alginate and fucoidan are, in contrary, high MW polysaccharides.  $\text{CaCl}_2$  is most often used for removal of alginate from extracted solution through precipitation. Nevertheless, the quantity of alginate can be very high in some species (e.g. up to 40% of the dry matter in LH) (Horn et al., 1999) and the efficiency of  $\text{Ca}^{2+}$  for precipitation of alginate is limited by the structural characteristic of alginate, particularly the amount of guluronic acid. Therefore, further purification of fucoidan may be achieved by ethanol precipitation of negatively charged fucoidan in presence of cation ions such as sodium (Shaklee et al., 2012). The charged sulfur ester group of fucoidan also gives the possibility to separate and fractionize it by cetyl-trimethyl-ammonium-bromide (CATB) (Halling et al., 2015) as well as anion exchange chromatography method (Béress et al., 1993; Pereira et al., 1999; Anastyuk et al., 2012; Manns et al., 2014).

As the bioactivities of fucoidan and laminarin are dependent on the chemical structure, efforts have been made to optimize the extraction process to achieve the highest possible pure yield while preserving the structural integrity during extraction. It was shown that the type and concentration of solvent, temperature and duration of treatment play roles in conservation of structural integrity and the magnitude of yield. Increasing HCl concentration, especially in combination with high temperature and long extraction time, may negatively affect yield and lead to release of sulfate.

Long extraction time and elevated temperatures alone are most often favorable for increasing the total yield of polysaccharides; nevertheless, such conditions are accompanied by reduced concentration of sulfated fucose (Ponce et al., 2003; Ale et al., 2012; Ale & Meyer, 2013; Guo et al., 2013). Additionally, new methods such as microwave assisted extraction (Rodriguez-Jasso et al., 2011), enzyme assisted extraction (Athukorala et al., 2006) and extraction from live harvested seaweed (Hjelland et al., 2012) have been practiced for the same purpose. The yield is also affected by the original quantities of polysaccharides in the seaweeds, which is species, seasonal and geographical dependent (Figure 3) (Ehrig & Alban, 2014; Schiener et al., 2015). The amount of laminarin can be very low or absent during growing season and reach up to 30-35% of dry weight in LH during autumn (Kadam et al., 2015a). The highest alginate content in LH has been observed during summer (Schiener et al., 2015). In *Laminariaceae*, the fucose content follows a similar pattern as laminarin. It is minimum around March and April and increases towards the end of growing season (Black, 1954; Ehrig & Alban, 2014).

#### ***1.4 SBM-induced enteropathy: a model for studying the effect of functional additives***

Animal models are important tools for studying intestinal inflammatory diseases (Jiminez et al., 2015), and many such models are being used to evaluate health effects of e.g., dietary additives. Soybean meal-induced enteritis or SBM-induced enteropathy (SBMIE) is a well-described and reproducible condition in the distal intestine (DI) of salmonids, and have been shown to be a very useful tool to study intestinal inflammation (Romarheim et al., 2011; Grammes et al., 2013; Kortner et al., 2016). SBMIE is a subacute enteritis which is identified by infiltration of various inflammatory cells into lamina propria, atrophy of the mucosal folds and decreased numbers of vacuoles in the enterocytes (Baeverfjord & Krogdahl, 1996; Urán et al., 2009; Krogdahl et al., 2010). In Atlantic salmon, the condition occurs at SBM inclusion levels as low as 10% of the diet.

The pathohistological symptoms intensify and appear faster as the level of SBM in the diet increases (Krogdahl et al., 2003). SBMIE model has been successfully used previously to demonstrate the effect of functional feed additives (Grammes et al., 2013; Romarheim et al., 2013b).

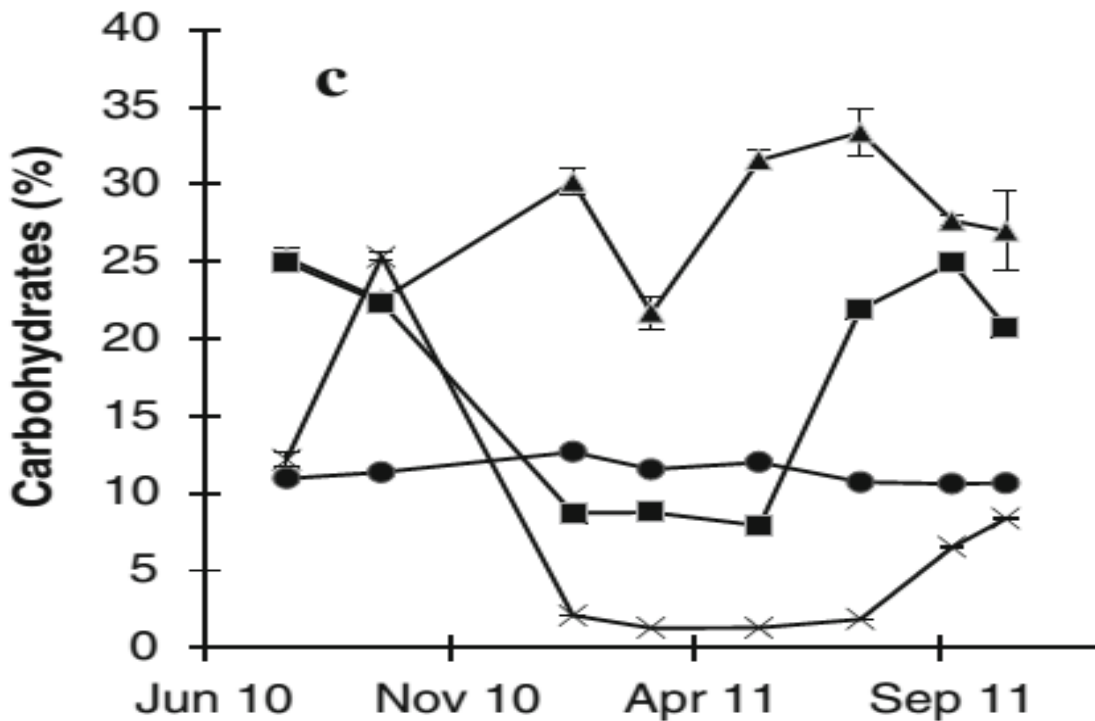


Figure 3. Seasonal variation of polysaccharide contents of *L. hyperborea* as demonstrated by Schiener et al. (2015). Alginate (▲), mannitol (■), cellulose (●), laminarin (x). Carbohydrate yields are expressed as a percentage of the dry weight.

The aim of this study was to develop a method for extraction of fucoidan and laminarin from LH, and to investigate the effect of dietary inclusion of dried LH and the extracted fucoidan and laminarin on the intestinal health of Atlantic salmon using the SBMIE model.

## **2. Materials and Methods**

### ***2.1 Fucoidan and laminarin extraction***

Dried winter harvested *L. hyperborea* (LH) was provided by Seaweed Energy Solutions. Three kg of dried LH were ground to pass through a 1mm sieve (Fritsch universal cutting mill pulverisette 19 (2800 - 3400 rpm) fitted with a disk milling cutter rotor). The milled LH was mixed with 0.03M HCl (Ale et al., 2012) in the ratio of 1 kg to 20L in several plastic containers and incubated for 1h at 70°C in an oven. The containers were shaken by hand every 15min during incubation. After incubation, large particles were removed by passing the extract through a nylon filter and the filtrate was collected. Thereafter, the filtrate was mixed with 1M CaCl<sub>2</sub> at a ratio of 1:1 and the solution was stored at 4°C overnight, resulting in precipitation of alginate. The next day, solution was filtered again and the precipitated alginates were removed. The obtained liquid was then fractionalized by a filtration unit (GEA, Membrane Filtration Pilot Plant L) equipped with spiral membranes (Dairy Hygienic Spiral Membrane Elements, Alfa Laval UF-pHt Series). The filtrate was passed through spiral membrane with molecular weight cut offs (MWCO) of 100, 50, 25, 10 and 2 kDa sequentially. The filtrate of each membrane was collected and filtered with the smaller MWCO membrane while the retentate of each membrane was collected as a fraction and freeze dried for further analysis.

### ***2.2 Purification***

As the reported laminarin MW is below 10 kDa, the 2 kDa fraction was assumed to be the crude laminarin extract. Based on chemical analyses (see below), the 100 kDa fraction was verified to contain the highest amount of fucoidan. The fucoidan in the 100 kDa fraction was further purified following a procedure similar to Shaklee et al. (2012). Freeze dried powder from the 100 kDa was

dissolved in MQ water at a ratio of 1:100 on a magnetic stirrer for 2h at 40°C. Thereafter, NaCl was added to the solution in a concentration of 20 g/l. The solution was stirred for 10 min and finally the pH of solution was adjusted to 5.6-6.00 by the addition of sodium hydroxide. Then, ethanol (94%) was poured into the solution at a ratio of 1:1, and the mixture was left overnight for precipitation of fucoidan. The next day, the solution was centrifuged for 20 min at 3000 RPM and the pellet was collected as purified fucoidan and freeze dried.

### ***2.3 Chemical composition of seaweed and fractions***

A two-step sulphuric acid hydrolysis based on National Renewable Energy Laboratory (NREL) method (Sluiter et al., 2005) was used for quantitative determination of the carbohydrate composition. This method has been shown to be an appropriate method for quantitative determination of the carbohydrate composition of brown seaweeds (Manns et al., 2014; Sharma & Horn, 2016). Dry ground LH samples, freeze dried fractions, reference samples of fucoidan (F8190 Fucoidan from *Fucus vesiculosus*; purity $\geq$ 95%; Sigma-Aldrich) and laminarin (L9634 laminarin from *Laminaria digitata*; Sigma-Aldrich) and sugar recovery standards (glucose, fucose, xylose and galacturonic acid) were first exposed to 72% (w/w) H<sub>2</sub>SO<sub>4</sub> at 30°C for exactly 1 h. In the next step, samples were hydrolyzed by 4% (w/w) H<sub>2</sub>SO<sub>4</sub> at 121°C in an autoclave for 40 min. After 40 min, the hydrolysates were left to cool down, filtered through Duran sintered filter (4 $\mu$ m Sigma Aldrich), and finally diluted with deionized water. Before injection into the high performance liquid chromatography (HPLC) system, the samples were filtered once more through a 0.22 ml micro-filter. For the purpose of this analysis, the HPLC system was equipped with a 300 $\times$ 7.8 mm Rezex ROA-Organic Acid H<sup>+</sup> analytical column fitted with cation-H cartridge guard column. The column temperature was set to 65°C and 5 mM H<sub>2</sub>SO<sub>4</sub> was used as the mobile phase at a flow rate of 0.6 ml per min. Glucose, fucose, xylose and mannitol at concentrations of 0.10, 0.25, 0.50, 1.00,



and 2.50 g/l were made as calibration standards. The Dionex software Chromeleon 7.2 was used for identification and quantification of monomeric sugars.

Uronic acids were quantified using a modified version of a method used by Manns et al. (2014). Uronic acids were separated by a Dionex ICS-3000 HPAEC-PAD on a Dionex CarboPac PA1 with multi-step gradient using the three eluents: A: 0.1M sodium hydroxide, B: 0.1M sodium hydroxide and 1M sodium acetate, C: deionized Millipore water. All eluents were filtered and ultrasonicated to avoid formation of CO<sub>2</sub>. The chromatographic elution and detection was carried out at a flow rate of 0.25 ml per min and pulsed amperometric detection (PAD) respectively. Thermo Scientific® Dionex Chromeleon Console 7 was used for identification and quantification of the total uronic acids.

Carbon, hydrogen, nitrogen and sulfur contents in LH and fractions were determined by CHNS elemental analyzer (Vario El Cube, Elementar, Germany).

#### ***2.4 Diets preparation and chemical analyses***

Six diets were formulated (Table 1) and extruded in form of 3 mm pellets at the Center for Feed technology at Norwegian University of Life Science, Aas, Norway. The diets included a fish meal (FM)-based diet as a negative control, a soybean meal (SBM)-based diets as a positive control, a test diet containing 5% LH, as well as three test diets containing 0.075% of either fucoidan (FUC) or laminarin (LAM) or both (LF). All diets except for the FM-based diet were formulated to contain 20% SBM, a level which is known to cause SBM induced enteritis in a short time. Fucoidan and laminarin were added to the diets by dissolving them in 400ml of water and vacuum coating into SBM-based extruded pellets. Afterward, pellets were dried by a force air setup and vacuum coated again with fish oil.

Table 1. Formulation and chemical composition of the experimental diets.

| Diets                         | FM  | SBM  | LH   | FUC  | LAM  | LF        | L.H <sup>1</sup> |
|-------------------------------|-----|------|------|------|------|-----------|------------------|
| Ingredient (g/kg diet)        |     |      |      |      |      |           |                  |
| Soybean meal <sup>2</sup>     |     | 200  | 200  | 200  | 200  | 200       |                  |
| Corn gluten meal              |     | 60   | 35   | 60   | 60   | 60        |                  |
| Wheat flower                  | 169 | 181  | 132  | 181  | 181  | 181       |                  |
| Wheat gluten                  | 151 | 150  | 170  | 150  | 150  | 150       |                  |
| Fish meal <sup>3</sup>        | 425 | 193  | 193  | 193  | 193  | 193       |                  |
| Fish oil <sup>4</sup>         | 240 | 186  | 191  | 186  | 186  | 186       |                  |
| Fuoidan (crude extract)       | -   | -    | -    | 0.75 | -    | -         |                  |
| Laminarin (crude extract)     | -   | -    | -    | -    | 0.75 | -         |                  |
| Laminarin & fuoidan           | -   | -    | -    | -    | -    | 0.75&0.75 |                  |
| <i>Laminaria hyperborea</i>   | -   | -    | 50   | -    | -    | -         |                  |
| L-Threonine <sup>5</sup>      | 1   | 2    | 3    | 2    | 2    | 2         |                  |
| L-Lysine <sup>6</sup>         | 0.5 | 7    | 2    | 7    | 7    | 7         |                  |
| Methionine <sup>7</sup>       |     | 2    | 7    | 2    | 2    | 2         |                  |
| Monocalcium phosphate         | 6   | 11   | 11   | 11   | 11   | 11        |                  |
| Choline chloride <sup>8</sup> | 2   | 2    | 2    | 2    | 2    | 2         |                  |
| Premix <sup>9</sup>           | 6   | 6    | 6    | 6    | 6    | 6         |                  |
| Chemical composition (g/kg)   |     |      |      |      |      |           |                  |
| Dry matter                    | 963 | 941  | 940  | 932  | 936  | 940       | 842              |
| Crude protein                 | 445 | 409  | 414  | 407  | 397  | 414       | 106              |
| Gross energy MJ/kg            | 24  | 22.4 | 22.7 | 22   | 23   | 22.4      | 11.3             |
| Crude lipid                   | 253 | 182  | 182  | 181  | 182  | 167       | 6.6              |
| Starch                        | 113 | 144  | 115  | 162  | 155  | 164       |                  |
| Total ash                     | 90  | 63   | 70   | 64   | 62   | 63        | 278              |

<sup>1</sup>composition of *Laminaria hyperborea*. <sup>2</sup>Soy bean meal, Non-GMO, Denofa AS. <sup>3</sup>LT fishmeal, Norsildmel, Egersund, Norway. <sup>4</sup>NorSalmOil, Norsildmel, Egersund, Norway. <sup>8</sup>L-Threonine, CJ Biotech CO., Shenyang, China. <sup>6</sup>L-Lysine CJ Biotech CO., Shenyang, China. <sup>7</sup>Rhodimet NP99, Adisseo ASA, Antony, France. <sup>8</sup>Choline chloride, 70 % Vegetable, Indukern s.a., Spain. <sup>9</sup>Premiks fisk, Mineralnæring AS, Hønefoss, Norway. Per kg feed; Retinol 3150.0 IU, Cholecalciferol 1890.0 IU,  $\alpha$ -tocopherol SD 250 mg, Menadione 12.6 mg, Thiamin 18.9 mg, Riboflavin 31.5 mg, d-Ca-Pantothenate 37.8 mg, Niacin 94.5 mg, Biotin 0.315 mg, Cyanocobalamin 0.025 mg, Folic acid 6.3 mg, Pyridoxine 37.8 mg, Ascorbate monophosphate 157.5 g, Cu: CuSulfate 5H<sub>2</sub>O 6.3 mg, Zn: ZnSulfate 151.2 mg, Mn: Mn(II)Sulfate 18.9 mg, I: K-Iodide 3.78 mg, Ca 1.4 g.

The chemical analyses of diets were performed in the laboratory of Department of Animal and Aquacultural Sciences (IHA) at Norwegian University of Life Science, Aas, Norway. Dry matter was obtained by drying samples at 103°C ±2 until constant weight and ash content by incineration at 550°C. Protein content was calculated based on nitrogen content (N×6.25) obtained by the Kjeldahl method. Crude fat was analyzed using the Accelerated Solvent Extractor (ASE200) from Dionex, and gross energy by bomb calorimetry (Parr 1271 Bomb calorimeter, Parr, Moline, IL, USA). Starch was analyzed as glucose after removal of fat by acetone and hydrolysis with amylase and amyloglucosidase (Total starch assay kit [AA/AMG], Megazyme International Ireland).

### ***2.5 Experimental setup and fish rearing condition***

The experiment was conducted at the Norwegian Institute for Water Research (NIVA) located in Solbergstrand, Drøbak, Norway. Unvaccinated Atlantic salmon with an average initial weight of 250 g were randomly allocated into tanks (12 fish/tank; equivalent of 3 kg biomass/tank). Overall, eighteen 300L fiberglass circular tanks were used in this experiment. Seawater (35‰) with temperature ranges of 8-9°C was pumped from sea to a header tank and distributed to the experimental tanks. Fish were fed in triplicate (each diet to three tanks) with automatic belt feeders approximately 20% above the daily consumption (twice per day; 1h each) for a duration of 28 days. The approximate daily feed intake was monitored by collecting the uneaten feed after each feeding session (twice/day) and weighing it immediately. The oxygen level was monitored regularly, and it remained above 85% saturation the entire experiment. A constant 24h light regime was used during the experiment.

### ***2.6 Sampling***

On day 28, four fish per tank were randomly selected for sampling. Fish were anaesthetized with

tricaine methanesulfonate (MS-222; 50 mg/l water) and killed by a blow to the head. Weights and lengths of the selected fish were measured individually. The rest of fish in each tank were killed and the bulk weights were recorded to have the final weight of each tank. The distal intestine (DI), liver, mid kidney, spleen, skin, faeces and blood were collected from the selected fish. For histology, the tissues were removed and fixed in 10% neutral buffered formalin (NBF) for two days before further processing and embedding in paraffin according to standardized routines. For RNA extraction, the tissues were fixed in 1.5 ml of RNAlater<sup>®</sup> (Sigma Aldrich) for 24h at room temperature and then stored in -80°C. The other samples were promptly snap frozen in liquid nitrogen after removal from fish. The blood samples were immediately centrifuged at 1800g for 10 min and aliquots of plasma pipetted out into the PCR strips. All samples were kept in isopor boxes containing dry ice during sampling and finally stored at -80°C. In this thesis, only the histology results of DIs will be presented.

### ***2.7 Histology of the distal intestine***

The paraffin embedded DIs were cut and the sections stained by hematoxylin and eosin. A scoring scale of 0-2 (Romarheim et al., 2013a) was used and sections were scored for the following parameters:

“(1) Lamina propria – accumulation of leucocytes in the lamina propria.

(2) Changes in the epithelium – reduced supranuclear vacuolization, reduced cellular height and increased cytoplasmic basophiles.

(3) Atrophy – reduced height of the intestinal folds”

For each parameter, score 0 was given to normal section, 0.5 to slight change, 1 to moderate, 1.5 to distinct change and 2 to the greatest relative change in the DIs sections. The average of each parameter in a dietary treatment generated a total histological score for that treatment.

## ***2.8 Calculations and statistics***

All uneaten feed was collected during experiment and oven dried at 103°C for 24h to obtain the dry matter (DM) intake. The feed conversion ratio (FCR) was calculated as  $FCR = \text{dry matter intake} / \text{weight gain}$ . Specific growth rate (SGR) was calculated as  $SGR\% = 100 \times [(\ln \text{ final weight}) - (\ln \text{ initial weight})] / \text{number of days}$ . The data were analyzed by SAS software. GLM was used as statistical model for growth performance parameters and the least square means were reported. Additionally, Tukey's studentized range test was used for comparison of means. For histology, Kruskal–Wallis ANOVA by ranks was used. The level of significance was set at  $P < 0.05$ .

## **3. Results**

### ***3.1 Chemical analysis of seaweed and fractions***

The results of HPLC and element analysis (CHNS analysis) of the seaweed fractions are presented in Table 2 and 3, respectively. The result of proximate analysis of LH is presented in Table 1. The used LH in this experiment contained approximately 16.4% laminarin (equivalent of glucose content), 4.3% fucose, 9.5% of alginate (as uronic acids), 6% protein (based on amino acid content), 1.7% nitrogen (10.6% CP), 0.66% crude lipid and 28% ash. The monomeric sugars of polysaccharides were observed in all fractions. The amount of fucose-containing compounds reduced remarkably in retentates of membranes under 100 kDa. There were small amounts of freeze dried materials (ca. 2 g) from both retentates of 50 kDa and 25 kDa. Therefore, the majority of fucose-containing compounds were recovered from retentate of 100 kDa. Comparing with fucoidan from Sigma, our purified fucoidan had lower content of fucose (370 vs.499 g/kg). On the other hand, very small amount of glucose and less amount of alginate were detected in our purified fucoidan. Interestingly, the composition of 100 kDa fraction and fucoidan from Sigma were very

close, and the main notable difference was in fucose contents of two samples. Considering the amount of fucose in our fraction, it can be inferred that we included about 277 mg of fucose per kg of feed. The highest amount of glucose was observed in 10 kDa fraction (834 g/kg). The 2 kDa fraction as the target laminarin fraction had a glucose content of 579 g/kg. This amount was higher than laminarin from Sigma (367 g/kg). Additionally, there was a lower amount of alginate in our laminarin fraction (64 g/kg) compared with laminarin from Sigma (93 g/kg). The amount of included laminarin in our diet can be estimated as 434 mg/kg of diet. The CHNS analysis showed that the purified fucoidan fraction contained 90 g/kg of sulfur while the sulfur content in Sigma fucoidan was about 64 mg/kg. There was lesser amount of sulfur in our laminarin fraction than in Sigma laminarin (7 vs.34 g/kg). There were detectable amounts of xylose and nitrogen in all of extracted fractions as well. However, the N-content in the fractions was much lower than in the parent meal of LH.

Table 2. Laminarin, fucose, uronic acid xylose and mannitol contents of *L. hyperborea* (LH) and fractions

| Monosaccharide contents<br>(g/kg sample) | Glucose <sup>1</sup> | Fucose <sup>1</sup> | Xylose <sup>1</sup> | Mannitol <sup>1</sup> | Uronic acid <sup>2</sup> |
|--|----------------------|---------------------|---------------------|-----------------------|--------------------------|
| Samples/Fractions                        |                      |                     |                     |                       |                          |
| LH                                       | 164                  | 43                  | 30                  | 44                    | 95                       |
| 100 kDa                                  | 49                   | 346                 | 90                  | ND <sup>4</sup>       | 93                       |
| 50 kDa                                   | 4                    | 237                 | 52                  | ND                    | 132                      |
| 25 kDa                                   | 1                    | 76                  | 33                  | ND                    | 109                      |
| 10 kDa                                   | 834                  | 17                  | 21                  | 17                    | 92                       |
| 2 kDa                                    | 579                  | 7                   | 15                  | 24                    | 64                       |
| Purified fucoidan                        | 3                    | 370                 | 94                  | ND                    | 44                       |
| Laminarin Sigma <sup>3</sup>             | 367                  | ND                  | 8                   | 5                     | 93                       |
| Fucoidan Sigma <sup>3</sup>              | 51                   | 499                 | 87                  | ND                    | 95                       |

<sup>1</sup>Determined by HPLC. <sup>2</sup>Determined by HPAEC. <sup>3</sup>F8190 fucoidan from *Fucus vesiculosus*; purity ≥ 95% and L9634 laminarin from *Laminaria digitata* both purchased from Sigma-Aldrich. <sup>4</sup>ND: Not detected.

Table 3. Carbon (C), hydrogen (H) nitrogen (N), sulfur (S) contents, C to N (C:N) and C to S (C:S) ratios of *L. hyperborea* (LH) and fractions.

| Elements (g/kg)              | C   | H  | N   | S  | C:N   | C:S  |
|------------------------------|-----|----|-----|----|-------|------|
| Samples/Fractions            |     |    |     |    |       |      |
| LH                           | 303 | 50 | 17  | 16 | 17.8  | 18.7 |
| 100 kDa                      | 237 | 56 | 4   | 88 | 59.1  | 2.7  |
| 50 kDa                       | 178 | 48 | 3   | 78 | 60.1  | 2.3  |
| 25 kDa                       | 109 | 30 | 5   | 71 | 22.6  | 1.5  |
| 10 kDa                       | 368 | 65 | 1   | 8  | 441.8 | 47.7 |
| 2 kDa                        | 373 | 65 | 1   | 7  | 360.7 | 52.3 |
| Purified Fucoidan            | 224 | 50 | 5   | 90 | 48.1  | 2.5  |
| Fucoidan Sigma <sup>1</sup>  | 252 | 46 | 0   | 67 | -     | 3.8  |
| Laminarin Sigma <sup>1</sup> | 220 | 41 | 1.5 | 34 | 150   | 6.5  |

<sup>1</sup>F8190 fucoidan from *Fucus vesiculosus*; purity $\geq$ 95% and L9634 laminarin from *Laminaria digitata* both purchased from Sigma-Aldrich.

### 3.2 Fish growth

Fish performance for each treatment including initial and final weight, feed intake, FCR and SGR is presented in Table 4. There were nine mortalities during the experiment due to disease prevalence (all dead fish had lesions on skins and fin rots). The mortalities were mostly at the beginning of experiment and occurred in the FM, LH, FUC and LAM treatments. The weights of these fish were removed from initial weights. A tank from the FUC treatment had high mortality rate (3 fish) and there was a problem with feed collection from a tank with the LF treatment. In both cases, problems reflected in unusually high FCR values (FUC<sup>3</sup> FCR=2.6 LF<sup>4</sup> FCR=2.24; see Table 6 in the appendix for more information). Therefore, growth performances of the LF and FUC groups are uncertain. Overall, there was considerable variation in fish performances of different tanks belonging to the same dietary treatments. The disease incidence during experiment and a short experimental period are the major likely reasons behind these variations. However, there were no statistically significant differences between performance parameters of fish from

different dietary treatments. Performing the statistical test after removal of data of problematic tanks did not result in any significant differences as well (see Table 5 in the appendix). Therefore, the decision was made to keep all tanks in calculation to keep the balance design.

Table 4. Initial and final weights, feed intake, FCR and SGR of fish fed experimental diets<sup>1</sup>.

| Diets                                 | FM   | SBM  | LH   | FUC  | LAM  | LF   | SEM <sup>2</sup> | P value |
|---------------------------------------|------|------|------|------|------|------|------------------|---------|
| <b>Performances</b>                   |      |      |      |      |      |      |                  |         |
| Initial weight (g/fish)               | 261  | 257  | 260  | 266  | 265  | 253  | 3.29             | 0.11    |
| Final weight (g/fish)                 | 333  | 315  | 307  | 316  | 319  | 292  | 7.90             | 0.06    |
| Feed intake (g DM/ kg initial weight) | 245  | 250  | 269  | 294  | 278  | 274  | 16.35            | 0.35    |
| FCR                                   | 0.94 | 1.12 | 1.41 | 1.65 | 1.29 | 1.69 | 0.24             | 0.26    |
| SGR %                                 | 0.98 | 0.81 | 0.66 | 0.68 | 0.74 | 0.58 | 0.11             | 0.25    |

<sup>1</sup>Values are least square means <sup>2</sup>pooled standard error of mean

### ***3.3 Histology of the distal intestine***

The histological scores are presented in Figure 4. The images of DIs from fish fed with the different dietary treatments are presented in Figures 4 and 5. Variations in fish performances were also reflected in histological parameters (Figure 9 in the appendix). Fish from the FM treatment had the lowest signs of enteritis. The differences between the SBM, LH, LAM and LF groups were non-significant for all evaluated inflammation parameters. Regarding change in the epithelium and atrophy, fish fed the FM diet showed significantly lower signs of enteropathies compared to fish fed the other diets. The degrees of leucocytes infiltrations were not significantly different among fish fed the FM, SBM and LAM diets. On the contrary, fish fed LH (P= 0.002), FUC (P= 0.007) and LF (P= 0.037) showed significantly higher sign of leucocytes infiltration compared to the FM fed control group. There were signs of inflammation in some fish in FM treatment (mainly, but not only, in degree of leucocytes infiltration), which is an indication that the disease most likely affected the histological parameters of the intestine in this group.



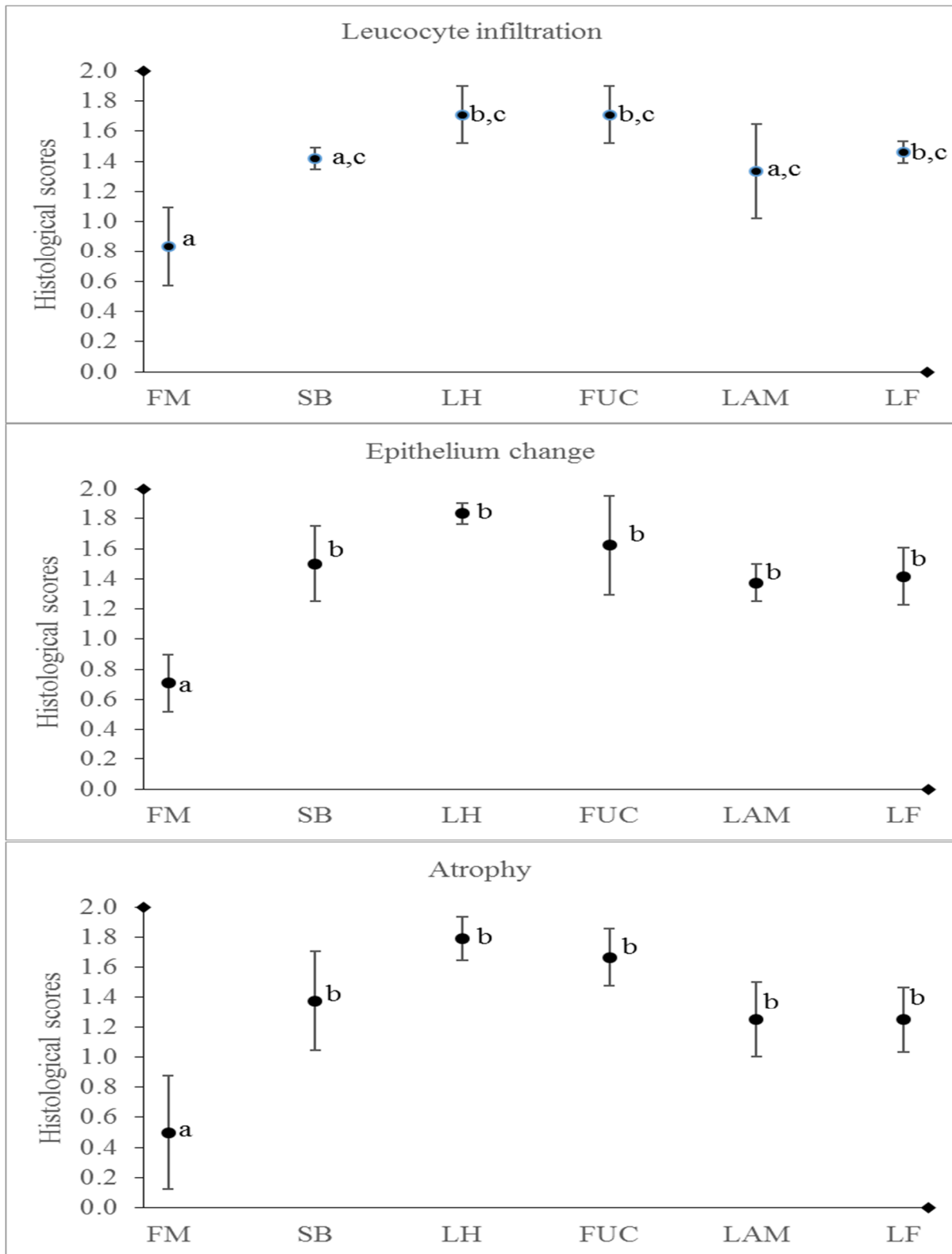


Figure 4. Histological parameters including leucocyte infiltration epithelium changes and atrophy of folds in the fish fed different exponential diets. Values are means (n=12), and the standard deviations of means are represented by vertical bars. Means with different letters are significantly different (P<0.05)

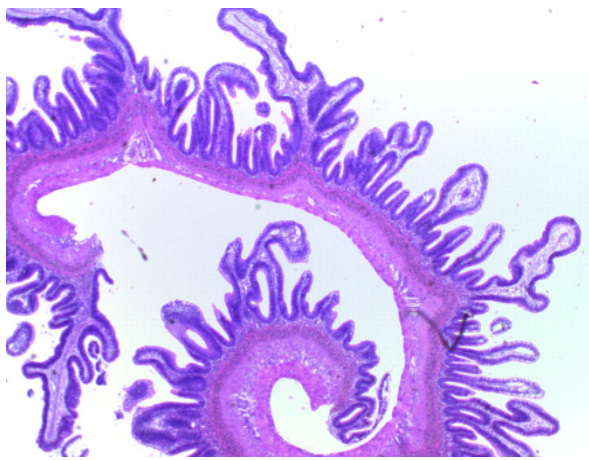
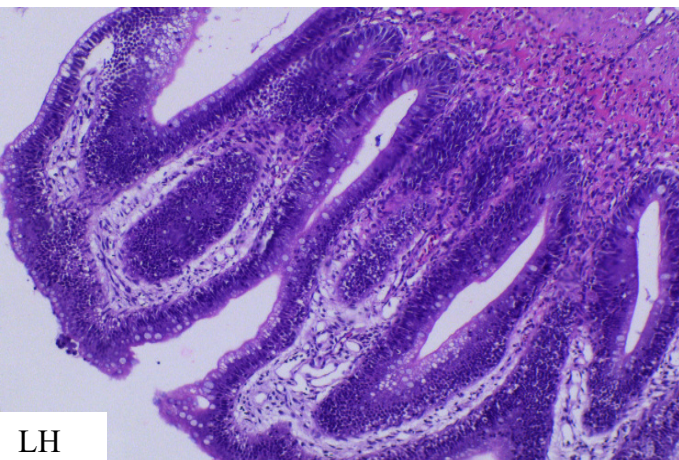
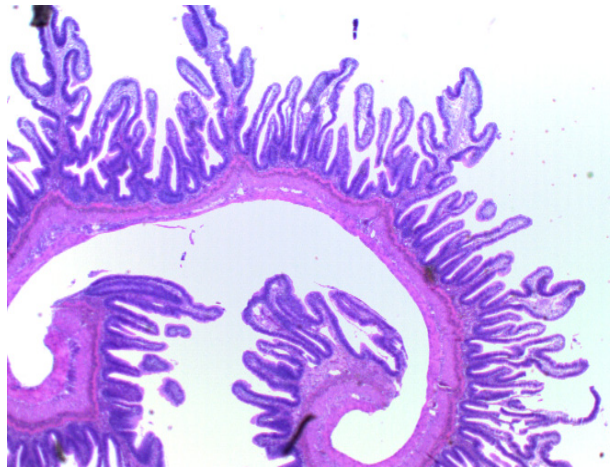
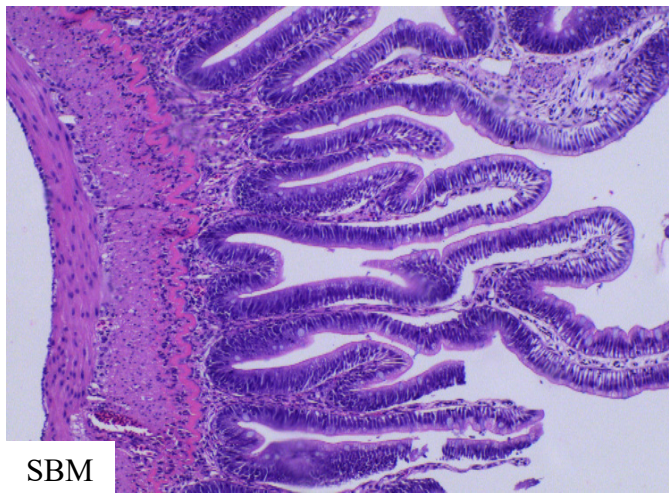
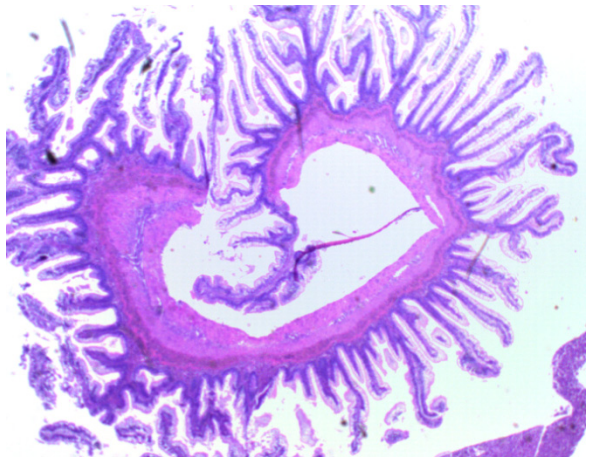
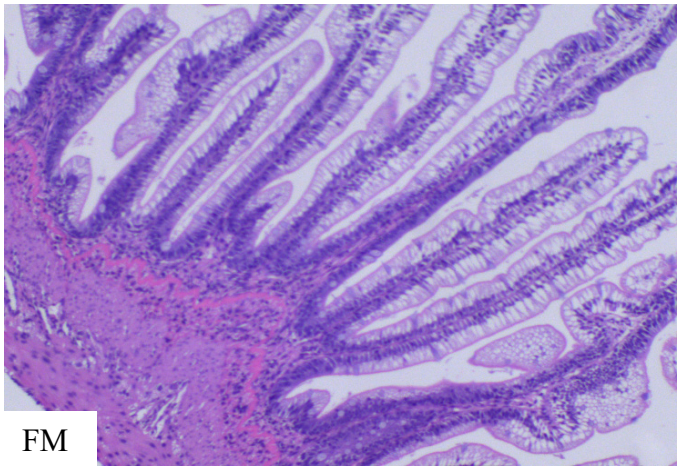


Figure 5. Distal intestinal (DI) morphology in Atlantic salmon fed the FM, SBM and LH diets. DIs of fish fed the SBM containing diets present the typical features of enteritis, including atrophy, shabby structure of folds, lack of vacuolization and infiltration of leucocytes ( $\times 2.5$  and  $\times 10$ )

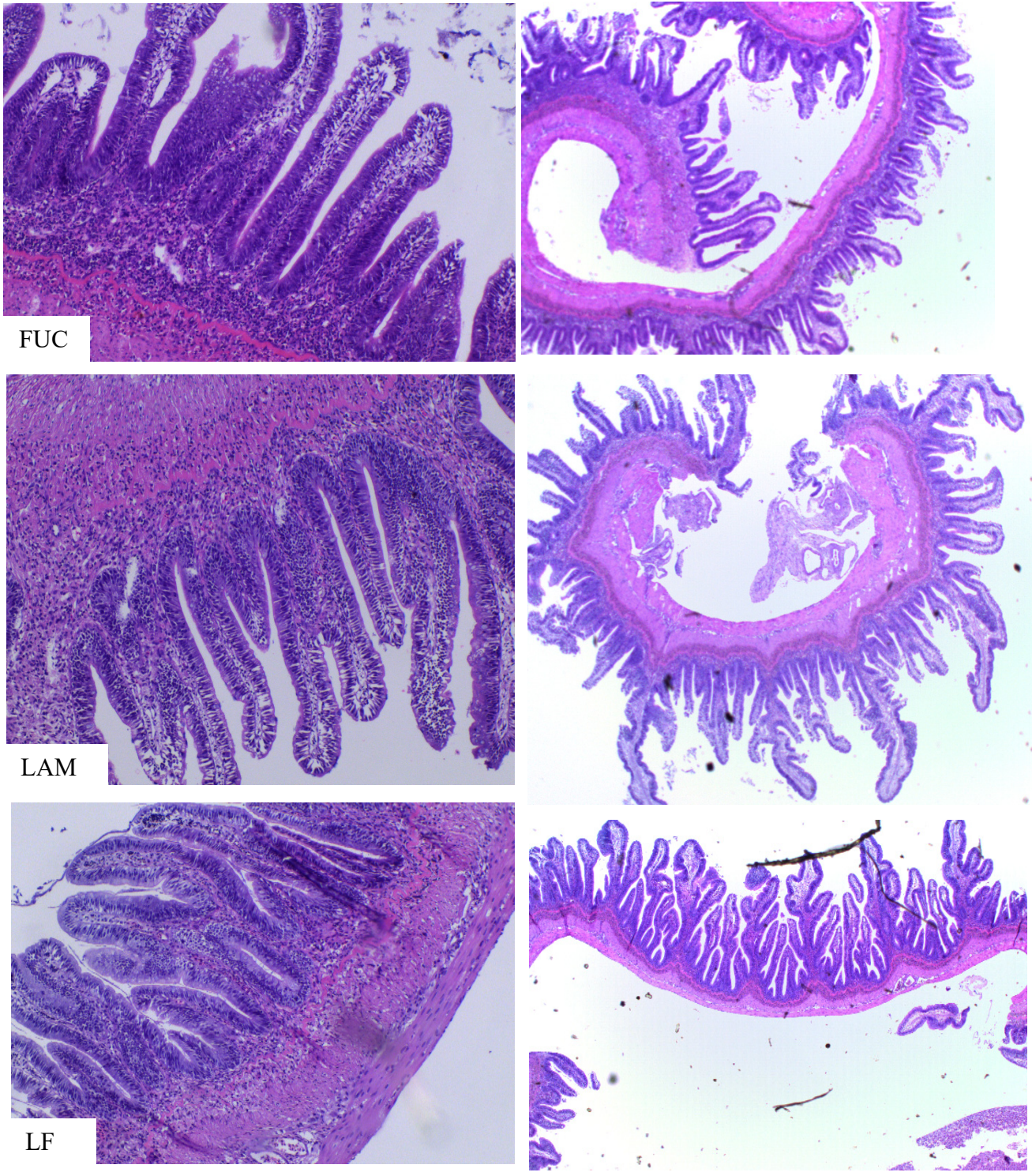


Figure 6. Distal intestinal (DI) morphology in Atlantic salmon fed the FUC, LAM and LF diets. DIs of fish fed the SBM containing diets present the typical features of enteritis, including atrophy, shabby structure of folds, lack of vacuolization and infiltration of leucocytes (×2.5 and ×10)

## 4. Discussion

The main objective for the present study was to assess the effect of the macroalgae LH and its extracted bioactive components on gastro-intestinal health of Atlantic salmon in a short-term experiment of 28 days. Additionally, we developed a method and successfully extracted fucoidan and laminarin from brown seaweed at a medium-scale to ensure enough materials for use in the fish diets.

Concerns over impurity of fucoidan is mainly based on contamination with other major polysaccharides in the brown seaweeds, mainly alginate and laminarin. Analyses showed that our fucoidan extract was mostly devoid of other major polysaccharides found in the brown seaweeds, and comparable to the counterpart from Sigma. It has been reported that Sigma-Aldrich uses the triple-step HCl extraction method devised by Black et al. (1952). The fucoidan obtained by this method has been reported to contain 44% fucose; 26% total sulfate, and 31% ash (Ale & Meyer, 2013). We used a mild extraction process aiming at preserving the structure integrity of compounds over total yield. In our study, fucose was found in all fractions, but in lower levels than in the high-MW fraction. The existence of fucoidan with different MWs in a specific seaweed has been reported previously (Shaklee et al., 2012) and efforts have been made to fractionize these compounds based on MW and test their bioactivity separately (Crocì et al., 2011). However, it must be noted that not all of the fucose containing polysaccharides are fucoidans. Most of the fucoidans were recovered from 100 kDa during our extraction. It indicates that most of the isolated fucoidans had a high MW. This MW is in agreement with the result of a study by Halling et al. (2015). They reported that fucoidan extracted from leachwater of live LH can have a MW of up to 1200 kDa. Additionally, their fucoidan fraction contained fucose (36%), galactose (3-4%), mannose (<0.5%) and 38-40% ester sulfate (calculated as  $\text{SO}_4^{2-}$ ). The amount of fucose in our

purified fucoidan fraction was about 37%. This amount is in close agreement with the reported amount (36%) by Halling et al. (2015). CHNS analysis quantified 90 g/kg sulfur in the purified fucoidan fraction. Converting this amount to sulfate equivalent, 27% of our fraction is ester sulfate ( $\text{SO}_4$  molecular weight is about 96 g/mole; there are  $90/32 \approx 2.81$  units of sulfur;  $2.81 \times 96 = 270 \text{ SO}_4^{2-}$  g/kg sample). The somewhat lower sulfate content in our fraction compared to the fraction obtained by Halling et al. (2015) is probably due to use of acid treatment during our extraction.

It has been reported that the fucoidan fraction of some seaweeds may be contaminated by 2-10% protein (Ponce et al., 2003; Li et al., 2008; Mak et al., 2014). This report was confirmed in our study as well. There were small amounts of nitrogen in our fucoidan fraction ( $0.5 \times 6.25 \approx 3\%$  CP) as well as in the other fractions. It is noteworthy that some fucose containing polysaccharides can also have protein and amino-sugar in their structure (Nishino et al., 1994).

Sigma does not provide any information about purity and extraction method of laminarin. According to analysis, the laminarin extracted in our study appeared to have higher purity than Sigma laminarin. HPLC analysis showed a high level of glucose in 10 kDa fraction. It has been reported that MW of laminarin in LH range from 3 to 5 kDa (Graiff et al., 2015). Therefore, the source of glucose in 10 kDa fraction is potentially ambiguous. Although the origin of glucose in 10 kDa remains unclear and demands further investigation, it is worth mentioning that the only other major source of glucose in a brown seaweed is cellulose. The analysis showed that there are notable amounts of xylose in our fractions. As Halling et al. (2015) reported that there was no detectable level of xylose in their extracted fucoidan, it is likely that the xylose in our fractions originated from cellulose.

The result of element analysis in our study was in close agreement with the reported data by Graiff et al. (2015). They reported carbon and nitrogen contents of 35.5 and 1.45 g/kg, respectively for

LH.

All experimental diets contained 20% soybean meal in order to induce enteritis in the DIs of the fish. A condition that occurs within one week of feeding high levels of SBM. Weight gain and feed intake were registered mainly to monitor the health status of the fish and to confirm that all tanks had a similar feed intake.

The non-significant differences in growth performances signify that fish fed different diets performed similar. Additionally, it was evident from the numerical values that at least one tank from each of the dietary treatment showed similar or close performance to the SBM control group (see Table 6 in appendix). This trend gives the impression that a more uniform growth performance could have been achieved if fish had not been affected by the external factors. As most of the increased FCRs values were observed in the tanks with mortalities, it can be stated that either removal of mortalities from initial weight led to increased FCR or the ongoing disease caused retardation of growth. On the other hand, it has been previously shown that fish fed SBM perform poorer than those receiving FM diets (Olli et al., 1994; Refstie et al., 1998; Refstie et al., 2000; Romarheim et al., 2011). Nevertheless, there was a non-significant difference between performances of the SBM and FM fed groups in this study. This non-significant difference is probably originating from the performances of the fish in two of the FM tanks. One of the tanks had mortality (Table 6 in the appendix: FM<sup>1</sup>). There was no mortality in another tank (FM<sup>2</sup>); even so, the ratio of feed intake to gain weight was high (FCR=1.1), indicating the existence of a problem in the tank. Some histological abnormalities were also observed in the intestines of fish from these tanks (Figure 8 in appendix). It is noteworthy that the length of our study was short for a conclusive growth trial. Additionally, there were variations in protein content and gross energy of the diets that could affect fish growth performance.

In a normal condition a FM fed group should not show any sign of inflammation in the intestine. Nevertheless, the FM fed group in this study received high scores in the degree of leucocyte infiltration. This incidence caused a non-significant difference between the FM and SBM or LAM fed groups. Neither LH nor its bioactive compounds had notable amelioration effect on SBMIE development. However, some matters were observed in this study that are worth being further discussed. SBMIE in fish fed LH appeared high in all the evaluated parameters. Besides, the effect of SBM, there might be another likely reason for severity of inflammation in the LH fed group. The grinder that we used was incapable of grinding seaweed to fine particles, probably due to the high ash content. Coarse particles of LH had sharp and rough edges that could further damage the intestine. The particles of LH were visible in the faeces during faecal collection (Figure 9 in the appendix). Fish belonging to the fucoidan group also showed high levels of leucocyte infiltrations. Increase in infiltration of leucocytes after dietary intake of fucoidan is in contrast with earlier reports showing that fucoidan can decrease the infiltration of inflammatory cells (Cumashi et al., 2007; Fitton et al., 2015). However, the proinflammatory effect of fucoidan has been also reported. It has been shown that fucoidan administration can lead to rapid amplification of natural killer (NK) cells, T-cell population and overall increase in leucocyte number (Yang et al., 2008; Kar et al., 2011; Isnansetyo et al., 2016). Additionally, it has been reported that fucoidan has the ability to bind to Toll-like receptors (TLR)-2 and TLR-4. Therefore, it can lead to activation of transcription nuclear factor NF- $\kappa$ B pathway and stimulation of the immune defence system (Makarenkova et al., 2012). It was previously shown by Sahlmann et al. (2013) that responses to SBM per se can upregulate the p100 subunit of NF- $\kappa$ B. Consequently, a synergic effect of fucoidan and SBMIE causative agents on NF- $\kappa$ B is likely. On the other hand, the degrees of leucocyte infiltrations in DIs of fish fed the FUC containing diets (LH, FUC and LF) were

significantly higher than those fish fed the FM diet, but no such significant differences was observed between fish fed FM and LAM or SBM diets. This pattern might be an indication that the ongoing disease was capable of increasing the levels of leucocytes, and fucoidan intensified the recruitment of leucocytes in a such situation.

We chose to have the LF diet because it was previously reported that laminarin and fucoidan can neutralize each other effects (Walsh et al., 2013; O'Shea et al., 2014). This study cannot confirm or reject this property.

Finally, it must be mentioned that the effect of immunomodulatory compounds such as  $\beta$ -glucan and fucoidan are most often dose dependent (Novak & Vetvicka, 2008; Traifalgar et al., 2012; Song et al., 2014). Therefore, there is a probability that the dosage in our experiment was unsuitable to cause any remarkable effect.

## **5. Conclusion**

Although extraction of the bioactive components from seaweeds is practical, accurate identification and quantifications of the extracted polysaccharides are still a challenge. The assessment of bioactivities of fucoidan and laminarin is complicated due to their compositional variations. The incidence of disease during this study made the evaluation of effects of these compounds even more complicated. Based on the result of the current study, LH and the bioactive compounds, fucoidan and laminarin, from this seaweed did not ameliorate the SBMIE condition in Atlantic salmon. Further research and preferably a dose-response experiment is needed to define the actual effects of these compounds on the intestinal health of fish. Additionally, the SBMIE model may not be an appropriate model for demonstrating the beneficial effects of these bioactive compounds.



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## Appendix

Table 5. Initial weight, final weight, feed intake, FCR and SGR of fish fed the experimental<sup>1</sup> diets<sup>1</sup>

| Diets                                 | FM   | SBM  | LH   | FUC <sup>2</sup> | LAM  | LF <sup>2</sup> | SEM <sup>3</sup> | P value           |
|---------------------------------------|------|------|------|------------------|------|-----------------|------------------|-------------------|
| Performances                          |      |      |      |                  |      |                 |                  |                   |
| Initial weight (g/fish)               | 261  | 257  | 261  | 261              | 265  | 251             | 2.65             | 0.09              |
| Final weight (g/fish)                 | 333  | 315  | 308  | 324              | 319  | 298             | 7.47             | 0.12              |
| Feed intake (g DM/ kg initial weight) | 245  | 250  | 269  | 298              | 278  | 286             | 17.03            | 0.37              |
| FCR <sup>4</sup>                      | 0.94 | 1.12 | 1.41 | 1.18             | 1.29 | 1.41            | 0.09             | 0.04 <sup>4</sup> |
| SGR %                                 | 0.98 | 0.81 | 0.66 | 0.86             | 0.74 | 0.68            | 0.08             | 0.16              |

<sup>1</sup>Values are least square mean

<sup>2</sup>N=2; One tank has excluded

<sup>3</sup>Pooled standard error of mean

<sup>4</sup>Only the difference between FM and LH is significant



Figure 7. Photo of faeces from fish fed the LH diet. The black dots are large undigested particles of seaweed in the faeces.

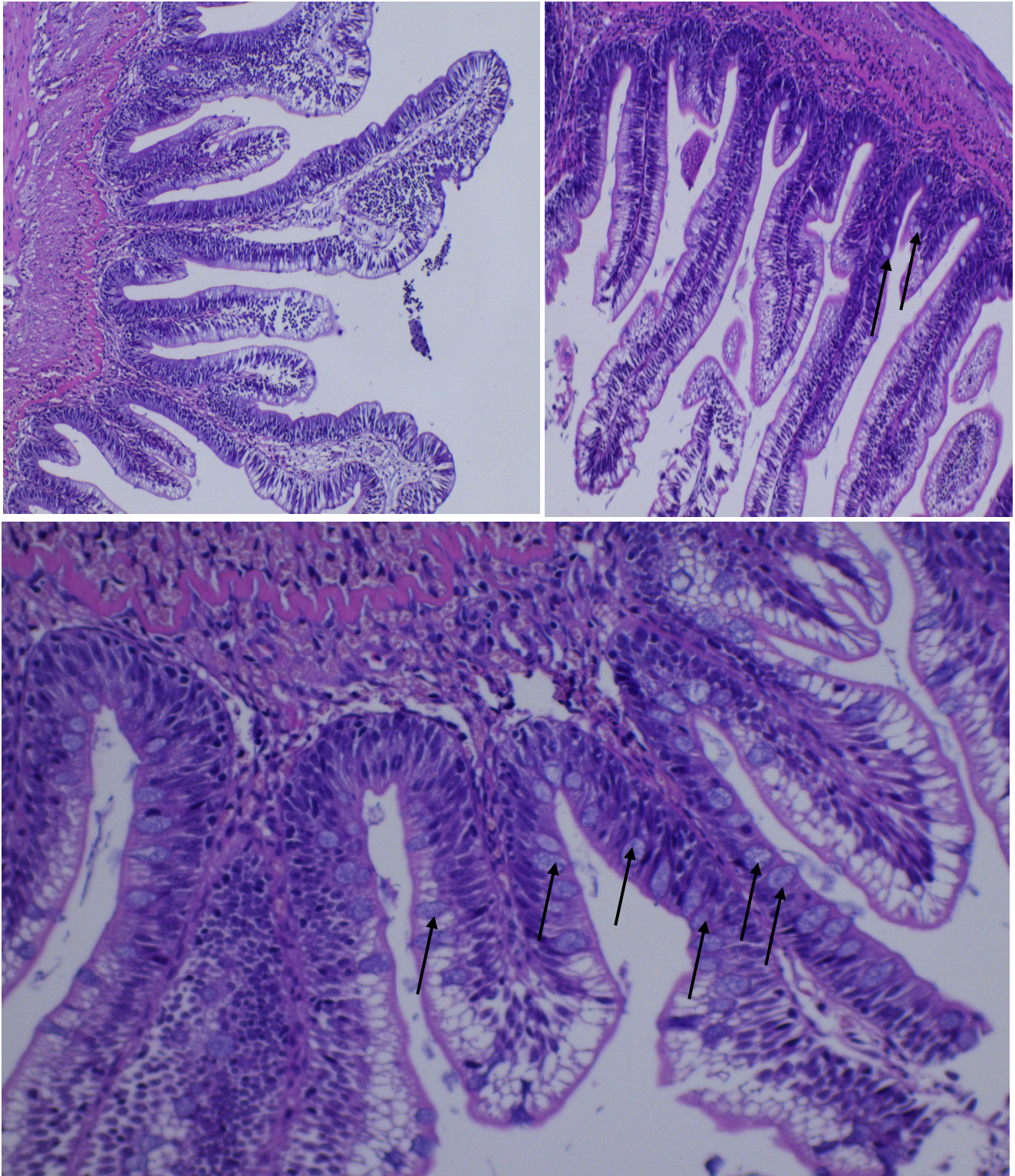


Figure 8. DIs sections from three different fish from FM<sup>1</sup> and FM<sup>2</sup> tanks showing signs of abnormality, including infiltration of immune cells, shabby structure of folds as well as increased number of goblet cells (Arrows) ( $\times 10$ )

Table 6. Initial weights (IW), final weights (FW), DM intake (DMI), FCR and SGR of each tank fed the experimental diets

| Diets         | FM   | FM <sup>1</sup> | FM <sup>2</sup> | SB   | SB   | SB   | SB   | LH <sup>1</sup> | LH   | LH <sup>1</sup> | FUC <sup>1</sup> | FUC <sup>3</sup> | FUC  | LAM  | LAM <sup>1</sup> | LAM <sup>1</sup> | LF   | LF   | LF <sup>4</sup> |
|---------------|------|-----------------|-----------------|------|------|------|------|-----------------|------|-----------------|------------------|------------------|------|------|------------------|------------------|------|------|-----------------|
| IW (g/fish)   | 262  | 266             | 255             | 258  | 253  | 260  | 263  | 258             | 262  | 268             | 277              | 254              | 265  | 266  | 266              | 266              | 251  | 252  | 257             |
| FW (g/fish)   | 356  | 335             | 309             | 310  | 308  | 325  | 307  | 311             | 304  | 328             | 300              | 321              | 334  | 312  | 312              | 312              | 302  | 294  | 282             |
| DMI (g/kg IW) | 287  | 236             | 240             | 241  | 254  | 259  | 238  | 249             | 260  | 289             | 215              | 278              | 286  | 248  | 237              | 264              | 256  | 256  | 220             |
| FCR           | 0.79 | 0.92            | 1.11            | 1.19 | 1.15 | 1.03 | 1.4  | 1.2             | 1.62 | 1.3             | 2.6              | 1.06             | 1.1  | 1.41 | 1.37             | 1.3              | 1.52 | 2.24 |                 |
| SGR %         | 1.24 | 0.92            | 0.78            | 0.74 | 0.80 | 0.89 | 0.63 | 0.75            | 0.60 | 0.80            | 0.32             | 0.93             | 0.93 | 0.65 | 0.64             | 0.74             | 0.62 | 0.37 |                 |

<sup>1</sup>One mortality in each tank

<sup>2</sup>Large FCR value

<sup>3</sup>Three mortalities in the tank

<sup>4</sup>Problem with feed collection due to floating pellet. The problem could affect other tanks in the same treatment as well.

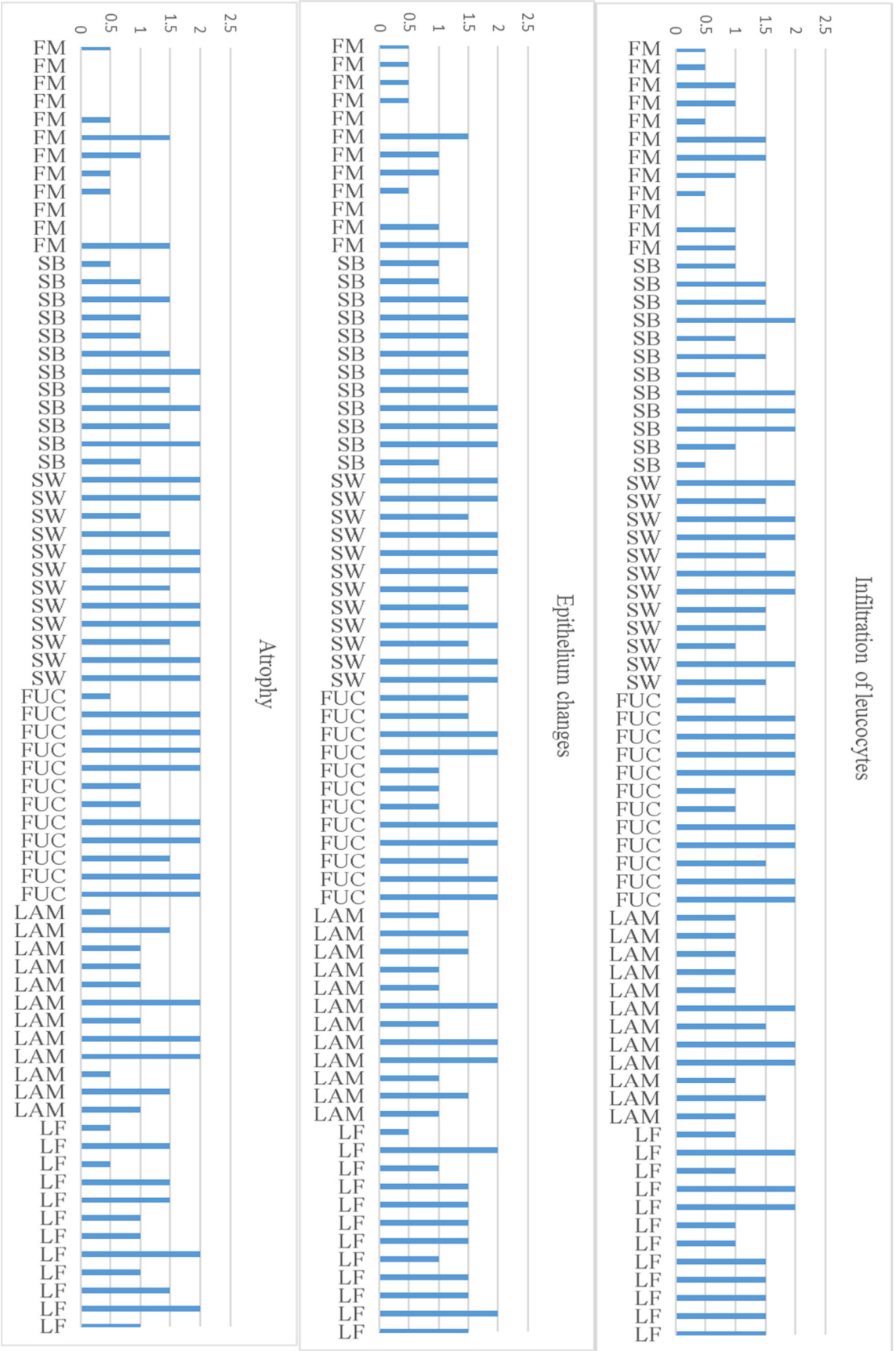


Figure 9. Histological scores for individual fish fed different experimental diets





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