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Novel functional feeds containing *Debaryomyces hansenii* or fucoidan improved growth performance and modulated hepatic osmoregulatory, immune and energy responses of Atlantic salmon during seawater transfer

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Master of Science in Aquaculture

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Abstracts

Atlantic salmon (*Salmo salar*) is the most important farmed specie in Norwegian aquaculture industry that undergoes profound physiological and immunological alterations before and after seawater transfer (SWT). At this stage, fish are vulnerable to stress, physical injury, and infectious diseases. Poor smoltification and inadequate timing of transfer to seawater (SW) is associated with high mortality, which represents significant economic losses for the industry. Nutritional interventions and modulation of immune system using functional feeds could be part of the solution to these challenges. The objective of this thesis was to characterize the effect of the functional feeds, containing *Debaryomyces hansenii* yeast and fucoidan extract from the brown algae *Saccharina latissima*, on growth performance and transcriptomic modulation in liver related to osmoregulatory, immune and energy responses of Atlantic salmon during SWT. The liver was chosen as an organ of interest due to its central role in metabolic processes and due to the limited knowledge existing of the liver's role regarding immunity during SWT. Atlantic salmon were fed either a control diet (C), a diet containing 0.2% of *D. hansenii* (D2) or a diet containing 0.2% fucoidan (D3) for 4 weeks in fresh water (FW) followed by 4 weeks in SW. The results showed that the growth performance was significantly enhanced for D2 and D3, especially in the FW phase. In addition, results from qPCR in liver showed up-regulation of energy related gene *COX1* after SWT, suggesting that fish in D2 and D3 groups were better prepared to regain homeostasis in SW phase. Fish fed D3 showed up-regulation of genes related to osmoregulatory functions 4 weeks post SWT, indicating that they were better equipped of handling the acclimation to SW. Moreover, the immunity related genes *hepcidin* and *Anxa1* were up-regulated in the D2 group in FW period, which might have been of advantage before SWT. Immunity related genes such as *C3* and *IL-10* were up-regulated for D3 group in SW phase, which might have been response to high pathogenic load. Both D2 and D3 enhanced immune modulating response with an increased gene expression of *C3* and *hepcidin*, as well as of anti-inflammatory genes, which suggest immune homeostasis. To conclude, use of functional feeds containing *D. hansenii* or fucoidan enhanced growth performance and modulated hepatic gene expressions. Future work should be carried out to study how the functional feeds affect other immune active organs by use of transcriptomics and other multi-omics approaches and to illuminate the underlying mechanism of the bioactive components of *D. hansenii* and fucoidan.

Abbreviations

C	Control commercial like diet
D2	Control diet + 0,2% *Debaryomyces hansenii*
D3	Control diet + 0,2% fucoidan extract from *Saccharina latissima*
DM	Dry matter
FW	Fresh water
IMM	Immunostimulant
NMBU	Norwegian University of Life Science
SEM	Standard error mean
SD	Standard deviation
SW	Seawater
SWT	Seawater transfer

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1. Introduction

1.1 Current situation in aquaculture industry

Aquaculture is the world's fastest growing food production sector and accounts for more than half of the fish supply today. With a growing population and limited food and feed resources, aquaculture is an essential contribution to global food security and nutrition (FAO, 2022). The total fisheries and aquaculture production continues to grow (Figure 1), although the capture fishers have stagnated due to the limited fishery stocks that mostly have been fished to their limit or beyond (FAO, 2022).

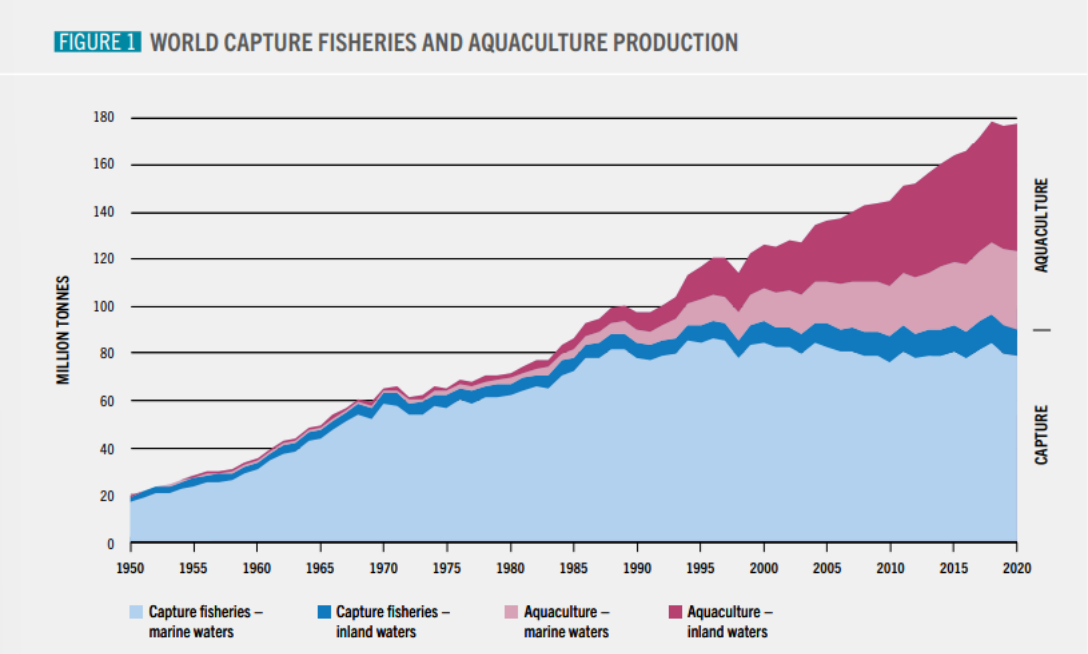

Figure 1. The worlds capture fisheries and aquaculture production from 1950 until 2020. The data is expressed in live weight equivalent (FAO, 2022).

Salmon aquaculture industry is one of the most lucrative industries worldwide. Norway is the world's largest producer of Atlantic salmon (*Salmo salar*) with a production of approximately 1.37 million tons in 2020 (Kontali, 2021) as compared with only 170,000 tons in 1990 (NSC, 2020; Ytrestøyl et al., 2015). Salmon is one of the most energy efficient farmed animals with a small carbon footprint and usage of fresh water. It is an excellent food product, rich in omega-

3 fatty acids and has a great economical impact which contribute to many direct and indirect jobs. The intensification of salmon farming is a great contributor to the success today (ISFA, 2018). However, this intensification together with other factors such as climate change and infectious diseases can contribute to multi-stressor condition and subsequent adverse effects on fish health, leading to reduced growth and poor welfare (Dawood & Koshio, 2016).

Atlantic salmon farming takes place in both fresh water (FW) and seawater (SW) phases. Atlantic salmon is an anadromous fish that need to undergo a parr-smolt transformation called smoltification (Stefansson et al., 2008). Smoltification is driven by hormones (Bjornsson et al., 2011) and triggered by changes in water temperature and photoperiod (Prunet et al., 1989), once the fish reaches a threshold size (Kendall et al., 2015). A fish in the smolt phase will for example have increased salinity tolerance, increased metabolism, decreased condition factor, schooling behavior, olfactory imprinting, and silvering (Bjornsson et al., 2011). The period of smoltification is stressful for the fish, making them more sensitive to handle stress (Barton & Iwama, 1991) and reducing their immune capacity (Maule et al., 1989).

A critical period in the salmon production cycle is seawater transfer (SWT), where a major part of the losses takes place during the first months in the sea (Aunsmo et al., 2008). Approximately 30 million fish died in the FW phase, while almost 54 million fish died in the SW phase in Norwegian farms in 2021 (Sommerset et al., 2022). An increased number of infectious diseases can often be seen after SWT (Johansson et al., 2016) caused by poor smoltification which means increased stress for fish and thereby increases the risk for health problem and death during the first part of release into SW. It is important to have good smolt quality to be able to improve fish growth performance, reduce mortality and improve fish welfare (Sommerset et al., 2022).

To prevent health problems, fish are vaccinated, either by injection, with dip, bath, or given orally in the feed. The vaccination usually give protection against infectious pancreas necrosis, furunculosis, cold-water vibriosis, and *Moritella viscosa* (Sommerset et al., 2022). Studies have however shown indications that vaccines can have limited efficiency (Figueroa et al., 2022; Rosaeg et al., 2021; Thorarinsson et al., 2021; Vargas et al., 2021). Antibiotics can also be used in aquaculture to prevent or control infectious diseases (Hernàndez-Serrano, 2005). Globally has an effort been made to minimize and eventually eliminate the use of antibiotics, since the use can spread drug resistant bacteria and thereby contribute to the antibiotic's resistance challenge (Dawood et al., 2015; Landers et al., 2012). In Norway, the use of antibiotic is almost non-existing, with only 0,8% of the Atlantic salmon being subjected to antibacterial treatment in 2020 (NORM-VET, 2021).

Nutritional intervention by use of functional feeds with its specific bioactive components could be a cost-efficient way to improve immune responses, prevent diseases and overall health of the fish. This could also be a way to reduce the use of antibiotics and compensate or improve for the limited efficiency of vaccines.

1.2 Novel functional feeds

Functional feeds are defined as feeds that has added benefits beyond the basic nutritional requirement. These feeds can promote fish performance and health (Holdt & Kraan, 2011). The inclusion of novel functional components in fish diets can thereby limit the use of chemotherapeutic and antibiotic treatment (Tacchi et al., 2011). Functional feeds can be divided into a few categories: immunostimulants (IMM), prebiotics, probiotics, vitamins, minerals, nucleotides, and plant or algal extracts (Tacchi et al., 2011).

The single largest expense for the Atlantic salmon farmers is the fish feed (Tacchi et al., 2011), often representing around 40% of the total cost for a Norwegian farmer (Fiskeridirektoratet, 2021). The salmon feed has changed from being mainly based on fishmeal and fish oil to being based on plant ingredients such as soy protein concentrate and rapeseed oil (Tacon & Metian, 2008; Ytrestøyl et al., 2015). This rapid change in feed formulation has resulted in numerous health issues for the fish (Kiron, 2012; Vandeningh et al., 1991). The fish's response to new feeds is not fully understood, however, it is known that many genes and proteins are modulated in different tissues of the fish (Froystad et al., 2008; Leaver et al., 2008; Martin et al., 2003).

Nutrition has major health implications for fish (Kiron, 2012; Trichet, 2010) and the interplay between nutrition and immune system is well recognized (Martin & Krol, 2017). Nutrition, should, therefore, be integrated into the health management strategies to improve fish health, reduce disease outbreaks and/or improve post-infection recovery (Martin & Krol, 2017; Tacchi et al., 2011). Nutrition can play an important role in the function of the immune response, and it can be enhanced by specific novel ingredients (Kiron, 2012; Tacchi et al., 2011).

IMM can be classified by their origin, mode of action and the way they are administrated (Dawood et al., 2018). IMM act directly on the immune system of the fish, increasing the disease resistance and the immunocompetence of the fish (Sakai, 1999). IMM can be provided to the fish during a time that the host has the best opportunity to resist the disease or during particularly stressful situations such as during the SWT (Tacchi et al., 2011). IMM can be injected or given orally in the feed (Dawood et al., 2018; Tacchi et al., 2011), either together with a vaccine antigen or without (Anderson, 1992; Dawood et al., 2018). IMM does not only effect the immune system, but they also enhance the growth performance of fish (Dawood et al., 2018; Lin et al., 2011). Bioactive components such as β-glucans, mannans, chitin and fucoidan can stimulate the immune system and act as IMM (Dawood et al., 2018).

1.2.1 Yeast

Classified in the fungi kingdom, yeast is a single-cell eukaryotic microorganism that is found almost everywhere in the environment. Yeast relies on living and dead organic material as sources of nutrients, being classified as heterotrophs. These microorganisms have cell walls and nuclear membranes but unlike plants no chloroplasts (Bennett, 1998). The use of yeast as a feed ingredient in animal feed has increased dramatically in recent years (Shurson, 2017). Yeast is a sustainable ingredient, due to its ability to convert low-value non-food biomass from the

agriculture industry and forestry into high-value nutrients. Thereby having less dependence on water, arable land, and changing climate conditions (Anwar et al., 2014; Couture et al., 2019; Lapena et al., 2020a; Lapena et al., 2020b).

Yeast production has recently become more efficient and less costly due to improvements in fermentation technology, making yeast more feasible ingredient for use in aquafeeds (Kim et al., 1998; Omar et al., 2012). Yeast has the potential to be both a nutritional and functional feed resource, proven to have a positive impact on fish growth performance and fish health (Agboola et al., 2021; Meena et al., 2013; Reyes-Becerril et al., 2008a; Robertsen et al., 1990; Sahlmann et al., 2019; Sarlin & Philip, 2011; Torrecillas et al., 2014). Furthermore, yeast can improve the palatability of fish feed as it contains stimulants with feed enhancing properties (Kasumyan & Doving, 2003).

The nutritional composition of yeast varies depending on the used strain, growth media, and condition for growing (Øverland et al., 2013). The immune effect varies depending on processing technology (Hansen et al., 2021), yeast strain, and inclusion level (Øverland & Skrede, 2017). Yeast crude protein content varies between 40-55% and the lipid content is usually low, around 2-5% with mainly unsaturated fatty acids (Halasz & Laztity, 1991). Overall yeast ash content is high and the carbohydrate content is moderate (Øverland et al., 2013) containing mainly polysaccharides (Halasz & Laztity, 1991). The cell wall of yeast represents 26-32% of the total dry weight, which contains the components mannan-oligosaccharides (MOS), β-glucan, and chitin (Figure 2) (Klis et al., 2002; Schiavone et al., 2014). Which are microbe-associated molecular patterns (MAMPs) (Navarrete & Tovar-Ramírez, 2013). MAMPs can act on host pattern recognition receptors (PRRs), activating cells of the immune system (Whyte, 2007).

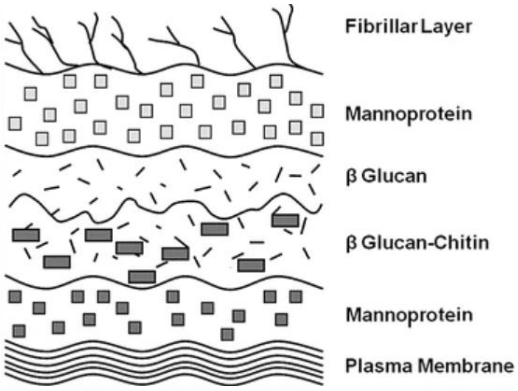

Figure 2. Yeast cell wall architecture schematically. Layers arrangement shows zones of enrichment, in reality can components such as mannoproteins be found distributed throughout the whole wall (Stewart, 2017).

MOS has been reported to have a positive effect on the health and growth performance of Atlantic salmon (Refstie et al., 2010). Digestion and gut health can be improved with MOS by the binding to and blocking of glycoprotein receptors on pathogens (Fernandez et al., 2002). MOS can for example bind to enteropathogenic bacteria and thereby prevent host colonization (Torrecillas et al., 2014). It can also enhance the growth of beneficial bacteria in the gut, and by functioning as a prebiotic it can improve the feed efficiency in Atlantic salmon (Grisdale-

Helland et al., 2008) and immune status and growth in Rainbow trout (*Oncorhynchus mykiss*) (Staykov et al., 2007; Yilmaz et al., 2007). Another yeast cell wall component is a polysaccharide named chitin, however, it exists in a concentration much less than glucans and mannans. The effect of chitin on fish is still inconsistent and needs further investigation (Ringo et al., 2012; Shurson, 2018). Although, Atlantic salmon fed chitin from prawn shell have been shown to have reduced growth and nutrient utilization at an inclusion level above 1% (Karlsen et al., 2017). Yet, chitin can be used as an immunostimulant when supplemented in feed for many fish species (Ringo et al., 2012).

The function of β-glucan in teleost fish seems to be similar to humans but a lot of knowledge of its pathway is yet to be discovered. Yet, in Atlantic salmon macrophages has evidence shown a presence of β-glucan receptors and in other fish species also found in neutrophils and leukocytes (Petit et al., 2019). It is although well known that β-glucan can enhance immune responses in Atlantic salmon (Bridle et al., 2005; Robertsen et al., 1990). Several studies have shown the positive impact that MOS and β-glucans can have on fish, it has also been varied results and many studies also indicate no effects on the growth rate, feed intake and immune response (Agboola et al., 2021). The variation in results can depend on inclusion level, molecular structure of β-glucan and MOS, culture condition, time of feeding, used fish species, growth stage, and health status of the fish (Torrecillas et al., 2014).

Currently, the main yeast strains used in aquaculture studies, either for β-glucan extraction or extraction of other cell wall components, is *Saccharomyces cerevisiae* yeast. This yeast specie has specially been used to its extent due to its health stimulating effects in various fish species. However, non-*Saccharomyces* species have also been emerging and several studies has demonstrated that *Candida* yeast, especially *Cyberlindnera jadinii* could be included in the diet of Atlantic salmon (Øverland et al., 2013). *Candida utilis* yeast could also be a candidate for Atlantic salmon feed, since it has shown the potential to improve health properties and facilitate smoltification of Atlantic salmon during SWT (Sahlmann et al., 2019). The yeast specie *Debaryomyces hansenii* have been recently proven as potent functional ingredient for aquafeeds (Angulo et al., 2020; Morales-Lange et al., 2022).

D. hansenii is a non-pathogenic ubiquitous yeast that can tolerate high salinity and capable of living in marine environment and in the fish gut (Navarrete & Tovar-Ramírez, 2014). It has significant enzymatic potential such as proteas (Bolumar et al., 2008), lipase (Takac & Sengel, 2010), α-galactosidase (Viana et al., 2006; Viana et al., 2007; Viana et al., 2009; Viana et al., 2011), and superoxide dismutase (SOD) (Garcia-Gonzalez et al., 2009). The function of these enzymes can for example be reduction of raffinose oligosaccharides (anti-nutritional factor often found in soybean products) by α-galactosidase (Viana et al., 2007) and SOD is an antioxidant (Garcia-Gonzalez et al., 2009). *D. hansenii* has important roles in immunomodulation for several different fish species (Angulo et al., 2020). Inclusion level of 0.1% *D. hansenii* in the diets for Atlantic salmon showed the ability to counteract consequences of acute hypoxia stress (Morales-Lange et al., 2022). Further on, it has proven increased survival and gastrointestinal enzymatic activity for sea bass (*Dicentrarchus labrax)* larvae (Tovar et al., 2002), improved weight gain for leopard grouper (*Mycteroperca rosacea*) (Reyes-

Becerril et al., 2008b; Reyes-Becerril et al., 2011) and upregulated immune-related gene expression for gilthead seabream (*Sparus aurata*) fed *D. hansenii* (Reyes-Becerril et al., 2008a; Reyes-Becerril et al., 2012).

1.2.2 Seaweed

Seaweed is marine algae that exist in three main groups, red (*Rhodophyta*), green (*Chlorophyta*), and brown (*Phaeophyta*) macroalgae (Abdel-Latif et al., 2022). They get their energy through photosynthesis, converting carbon dioxide into sugars and oxygen with the help of sunlight (Morais et al., 2020). Seaweed has unique health and nutritional benefits for several fish species (Abdel-Latif et al., 2022), especially known for its richness in polysaccharides, some vitamins, and minerals (Holdt & Kraan, 2011). Seaweed can be a natural alternative to soybean with both economic and nutritional advantages for fish (Borquez et al., 2011; Morais et al., 2020). However, seaweed is most often used in animal feeds for the functional properties of its polysaccharides (Kumar & Kaladharan, 2007).

The nutritional composition of seaweed varies with species but also depends on the season and the area of production with varying external factors such as temperature, nutrient concentration in water, and light intensity (Connan et al., 2004; Dawczynski et al., 2007; Khan et al., 2007; Marinho-Soriano et al., 2006). Brown seaweeds have a more diversified and richer content of bioactive molecules (Misurcova, 2011) and therefore are an interesting animal feed despite their low protein content (Gupta & Abu-Ghannam, 2011). Seaweed does have a high mineral content due to the absorption of inorganic substances from the environment and thereby often containing 10-20 times more minerals compared to land plants (Gaillard et al., 2018). The lipid content of brown seaweed is usually around 1-5%, with predominantly polyunsaturated fatty acids like 20:5 n-3 (EPA) (Hamed et al., 2015; Misurcova, 2011). Brown seaweed also contains some of the most important vitamins like C, B, and E (Misurcova, 2011). Furthermore, brown seaweed are rich in polysaccharides (Misurcova, 2011) such as fucoidans (Zyyagintseva et al., 2003).

Fucoidan is a complex sulphated polysaccharide rich in fucose that commonly exists in the cell walls of brown seaweed (Senthilkumar et al., 2013). The bioactive potential in fucoidans is due to their sulphate groups (Mir et al., 2018). Fucoidan can be extracted from different brown seaweeds by using dilute acid or alkali, hot water (Rioux et al., 2007), or aqueous organic solvents (Albuquerque et al., 2004). However, since the cell walls consist of complex polymers (Wijesinghe & Jeon, 2012a) it can be necessary to use microwave-assisted extraction (Rodriguez-Jasso et al., 2011) or enzyme-assisted extraction (Wijesinghe & Jeon, 2012b).

Fucoidan extracted from brown seaweeds has potentially several different biological functions including immunomodulatory, anti-inflammatory, anti-viral, anti-bacterial, cardio-protection, and growth promoting effects (Saeed et al., 2021). It can regulate inflammation-related gene expression (Saeed et al., 2021), and reduce the pro-inflammatory cytokines (Iraha et al., 2013). Research on Atlantic salmon shows that *Saccharina latissima* has the potential to be included in the diet for smolt with a supplement level below 10%. With supplement levels of 3% and

10%, the fish growth performance was significantly better than the control group, and seaweed supplements could also increase the protein efficiency ratio and the food conversion ratio (FCR) (Kamunde et al., 2019). Research has also been done on other fish species, suggesting that fucoidan can have an important role in boosting the immunity of Nile Tilapia (*Orechromis niloticus*) (Isnansetyo et al., 2016), modulating the gut microbiota of zebrafish (*Danio rerio*) (Ikeda-Ohtsubo et al., 2020) and enhancing growth in barramundi (*Lates calcarifer*) (Tuller et al., 2014).

1.3 Immunonutrition

Immunonutition is defined as the activation of the immune system by interventions with specific nutrients (Calder, 2003). These components would be recognised as MAMPs by PRRs and upon recognition activate the innate immune system. PRR are a group of receptor types which contain receptors such as RIG-1 like receptors (Zou et al., 2010), C-type lectin receptors (CLRs) and Toll-like receptors (among other), which has a central role in the early innate response and have a key function in the communication between the innate and adaptive immune system (Rivera et al., 2016). The main immune tissues of Atlantic salmon are: the head kidney, thymus, spleen, trunk kidney, gut-associated lymphoid tissue (GALT), salmonid bursa, gills with the interbranchial lymphoid tissue (ILT), and the olfactory organ with nasopharynx-associated lymphoid tissue (NALT) (Bjorgen & Koppang, 2021; Yu et al., 2020). Nevertheless, the liver is an organ that also has immune functions. Upon pathogen challenge does the liver generate an inflammatory response which results in secretion of acute phase proteins into the circulation. The liver coregulate both metabolism and immunity, which makes it an essential organ to understand in immunonutritional manipulation (Taylor et al., 2022).

1.4 Liver

The liver is an organ located anteriorly in the abdominal cavity in teleost fish (Kryvi & Poppe, 2021). The liver has an essential role in metabolism and detoxification (Chiang, 2014), as well as being an important immunological organ (Heymann & Tacke, 2016). This organ is involved with several different biochemical functions related to carbohydrates, amino acid, and fatty acid metabolism (Figure 3). The liver maintains the blood glucose levels depending on the need of the animal through groups of processes called, glycolysis, glycogenesis, glycogenolysis and gluconeogenesis (Alamri, 2018). After digested meal glucose will be absorbed from the intestine into the circulation, resulting in elevated blood glucose levels. Further on glucose will be transported into the main cells of the liver, hepatocytes, via liver glucose transporter 2 (Alamri, 2018). Glycolysis is an aerobic series of ten reactions converting glucose into pyruvate. Pyruvate further gets converted with oxidative decarboxylation which results in acetyl CoA, a major fuel of the tricarboxylic acid (TCA) cycle that provides the main source of energy to cells (Ferrier, 2014). Glycogenesis transforms excess glucose into glycogen, a process that is stimulated by insulin. Glycogenolysis transforms glycogen into glucose and it occurs

when the insulin/glucagon ratio decreases and the blood glucose level gets normal two to three hours after a meal (Chiang, 2014). During a prolonged fast or starvation, the glycogen stores will be depleted, and glucose will be formed from noncarbohydrate products like lactate in a process called gluconeogenesis (Ferrier, 2014). It is activated by glucagon and inhibited by insulin (Chiang, 2014).

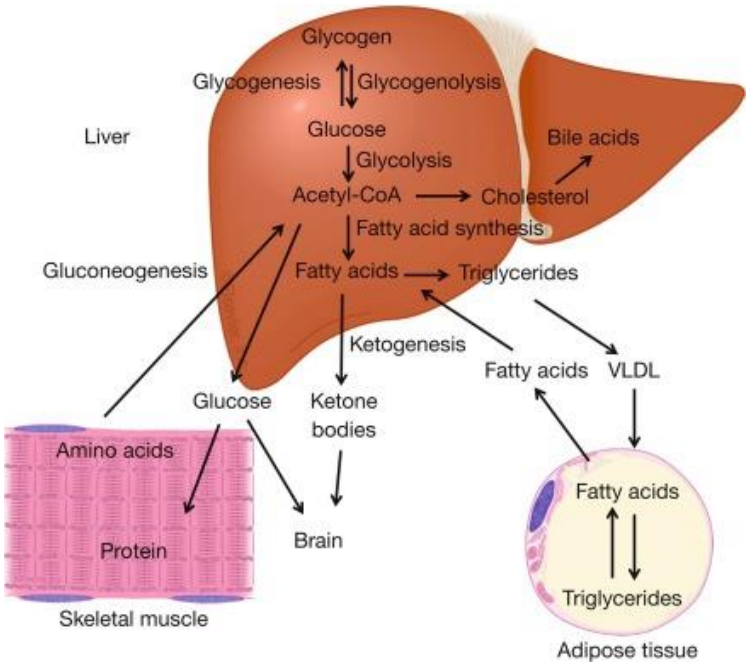

Figure 3. Overview of the major metabolic functions of the liver (Chiang, 2014)

Free amino acids can be catabolized by removal of the alpha-amino groups which will form ammonia that will partly be excreted in the urine but mostly used in the synthesis of urea. In the liver, the urea cycle occurs and urea is produced to get rid of toxic ammonia that will be transported via the blood and to the kidneys for the excretion in the urine (Ferrier, 2014). Free fatty acids (FFAs) can be oxidized by the liver and used for energy, membrane synthesis, and storage in the form of triglycerides (TG). However, fatty acid can also be synthesized from glucose in a process called de novo lipogenesis. Upon increased glucose and insulin levels, excess glucose will go through a multi-enzymatic process that transforms glucose into fatty acids. FFAs will normally undergo α, β or ω oxidation to generate energy, however, if there are excess FFAs they will be stored in the form of TG. TGs are assembled with Apoprotein B and they will be secreted from the liver into the blood circulation with very-low density lipoproteins (VLDL) (Alamri, 2018). VLDL transports TG for storage to adipose tissues or muscles and other tissues for energy metabolism. The liver can also convert excess acetyl-CoA from FFAs to ketone bodies which are the energy source for the brain and muscles during fasting (Chiang, 2014).

Cholesterol is partly absorbed from the diet, or it is produced by the body. The liver is the main organ that uses cholesterol to synthesize bile acids. In order to transport and absorb dietary fats and lipid-soluble vitamins are bile acids essential. Bile acids are also important when disposing

of toxic metabolites, xenobiotics, and drugs (Alamri, 2018). The liver plays a major part in drug and xenobiotics detoxification, with the aim to produce water-soluble compounds which can be excretable compounds in the bile. The hepatic drug detoxification includes two phases, the first phase being oxidation, reduction, and hydrolysis reactions which are mediated by cytochrome p450. Cytochrome p450 is a family consisting of a group of enzymes that uses iron to perform oxidation and reduction reactions to enhance water solubility. The second phase is conjugative reactions (Vaja & Rana, 2020).

1.4.1 Immunity in liver

A local immune environment for defense and tolerance is constructed by GALT which is connected to the liver via blood and bile (Wu et al., 2016). This makes the liver an important immunological organ with high exposure to circulating antigens and endotoxins from the gut microbiota. The gut-liver immunity is well known in mammals, however for fish still being largely unknown (Wu et al., 2016). The liver is mainly enriched with innate immune cells (Heymann & Tacke, 2016), containing both Kupffer cells and endothelial cells. Kupffer cells are macrophages that could phagocytize large particles like bacteria and damaged cells. Kupffer cells can also produce cytokines, a process activated when they phagocytize microbes. Upon activation of cytokines, more leukocytes will be recruited. The endothelial cells have endocytic capacity functioning by taking up foreign macromolecules through receptor-mediated endocytosis (Sjaastad et al., 2016). The liver also produces the majority of the complement proteins (Thorgersen et al., 2019). While the complement system consists of a series of plasma proteins that react with one another whereby they ultimately directly kill pathogens. The complement system can be activated in three different pathways, classical pathway, alternative pathway, and mannan-binding pathway also called lectin pathway (Kania & Buchmann, 2022). Additionally, liver do produce antimicrobial peptides which possess antimicrobial activities against pathogens like bacteria (Zhang et al., 2019). Examples of antimicrobial peptides produced in the liver is liver-enriched antimicrobial peptide – 2 (Varimo et al., 2022).

The bile acid synthesized by the liver has a major role in the communication between the fish gut and the liver. It can provide immune proteins such as cytokines and antibodies to the intestine mucus open inflammation as well as during homeostasis (Sipka & Bruckner, 2014). Furthermore, the liver has an impact on the fish ability in handling SW transfer and smoltification (Shwe et al., 2022).

The immune system does require a large amount of energy and the liver is required to produce a high level of acute phase response proteins to neutralize invading pathogens. Optimal feed management for salmonids is essential to increase their capability to fight infection effectively (Trichet, 2010). According to Martin et al. (2010) will the feeding regime prior to a challenge significantly affects the transcriptional responses to infection in fish. Starved fish with reduced energy reserves does have a high energy demand on protein synthesis for acute phase proteins, implementing that dietary control of fish could be important if an immune response is anticipated (Martin et al., 2010). Furthermore, the replacement of fish meal and fish oil with terrestrial alternative ingredients does affect the liver transcriptome of Atlantic salmon. Feed

formulation does not only have important liver transcriptome impacts on metabolism but also immune-related transcripts (Caballero-Solares et al., 2018). Similar findings also indicate that Atlantic salmon given ingredients of terrestrial origin modulated metabolism, inflammation, growth-related mechanisms, and oxidative stress in the liver (Caballero-Solares et al., 2020).

Functional feeds can also have significant effects on biological processes in the liver. Results from a study by Tacchi et al. (2011) indicated that functional feed containing health premix which contained nucleotides, MOS, fructooligosaccharides, vitamin C and E, decreased aspects of fish metabolism, enhancing growth and other performance indicators of the fish. The same functional feeds also down-regulated several genes involved in the immune system of the fish, which could result in greater energy resources used for growth. This finding suggested that fish can more efficiently use energy while at the same time maintaining the ability to respond immunologically if needed (Tacchi et al., 2011).

1.5 Hypothesis

Novel functional ingredients containing *Debaryomyces hansenii* yeast or fucoidan extracted from brown algae *Saccharina latissima*, can improve fish growth performance and modulate hepatic osmoregulation, immune and energy at gene expression level in Atlantic salmon during SWT.

1.6 Objectives of the study

General objective

To characterize the effect of functional feeds containing *Debaryomyces hansenii* or fucoidan on growth performance and liver osmoregulatory, immune and energy gene expression responses of Atlantic salmon during SWT.

Specific objectives

1. To evaluate growth performance in Atlantic salmon fed *Debaryomyces hansenii* or fucoidan during SWT.
2. To evaluate the effect of *Debaryomyces hansenii* or fucoidan on the expression of specific genes involved in liver osmoregulatory, immune and energy responses of Atlantic salmon during SWT.
3. To evaluate the correlation of the growth performance with the expression of specific genes in liver of Atlantic salmon fed *Debaryomyces hansenii* or fucoidan during SWT.

2. Materials and methods

This thesis is part of a larger project called "Resilient salmon" where *D. hansenii* and fucoidan were evaluated in three different nutritional programming used during SWT. However, this thesis only includes one of the nutritional programming which is denoted as feeding regime 3 (R3).

2.1 Experimental design

The experiment consisted of two periods, FW and SW. The experimental part in FW period was conducted at the fish laboratory at NMBU in Ås, Norway and lasted for 7 weeks. The experimental part in SW period was conducted at Norsk institutt for vannforsking (NIVA) Solbergstrand, Norway and lasted for 6 weeks. The experiment was executed according to the guidelines of Norwegian Animal Research Authority. The general experimental design for R3 and Control group can be seen in Figure 4.

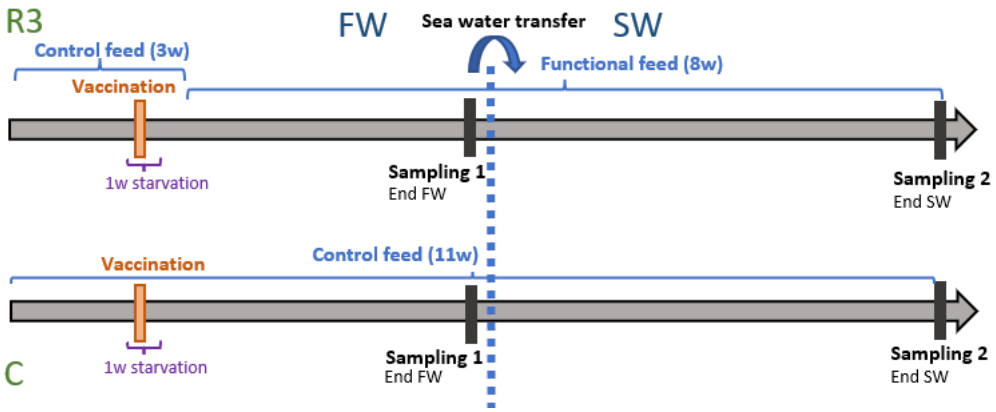

Figure 4. An overview of the experimental setup for regime 3 (R3) and control group (C), including when the vaccines and samplings where conducted. It also gives information regarding the feeding regime and seawater transfer.

The eggs for Atlantic salmon used in this experiment originated from Gain family group GEN-innOva R GAIN from Aquagen AS; Trondheim, Norway. Prior to the experiment, the fish was fed a commercial salmon feed at fish laboratory at NMBU. When fish reached a size of approximately 25 g they were all distributed into 18 tanks (45 fish per tank), each feeding regime given in duplicate tanks. Control group was given the control diet throughout the whole experiment while R3 was fed control diet until vaccination, followed by functional feed fed for 4 weeks in FW and 4 weeks in SW. After 18 days from the start of the experiment the fish of approximately 35 g of weight was PIT-tagged and vaccinated with ALPHA JECT micro-6 (Pharmaq, Overhalla, Norway).

During FW phase the fish was exposed to an 8-hour light and 16-hour dark regime for 5 weeks followed by 24 hours light regime for 2 weeks. The average water temperature in FW was around 14°C and the water flow was regulated to approximately 8 min^{-1} with oxygen level kept above 85%. The fish laboratory at NMBU was run with a recirculatory aquaculture system (RAS).

After 7 weeks in FW a total of 33-35 fish, at approximately 90 g of weight, were packed into transparent bags (151L water) with added oxygen and anaesthesia (Aquis-S; 5 mg L^{-1}) and transferred to SW. Each bag was allocated to a single tank and kept unopen for 20-30 min time to facilitate a slow water temperature transition. The fish was thereafter exposed to 9.4 ppt salinity that gradually increased until full salinity after 10 days. A light regime with 24h light was used, and the temperature of the water was approximately 8°C. The water flow was regulated to be around 3.25 L min^{-1} and oxygen level above 85%. Seawater was supplied to the experimental room from Oslofjord and pumped up from a depth of 60 m. The intake water was passed through a UV filter and drum filter.

In both stages, FW and SW, the fish were fed by belt feeders 6 hours a day until satiation, approximately given 110% of their expected feed intake. Uneaten feed was collected once a day and the water quality parameters were measured regularly.

At the end of the FW and SW phase, 6 fish per tank (12 fish per diet) were randomly sampled, anesthetized (80 mg L^{-1} MS222), individual weight and length were recorded. Thereafter, approximately 1mL blood was withdrawn from caudal vein using 2 mL heparinised syringes. The blood samples were kept on ice until plasma was separated by centrifugation at 7000 x g for 3 min. Plasma samples were aliquoted and placed in sterile Eppendorf tubes to be further stored at -80 °C. After withdrawing the blood, fish were dissected and eviscerated. For gene and protein expression distal intestine, spleen, liver, head kidney, and gills of approximately 100 mm^3 size were placed in cryotubes containing 1,6 mL RNAlater, placed at 4 °C for 24 h and stored at -80 °C until further analysis.

2.2 Fish diets

The diets were formulated together with BIOMAR (Table 1), and extruded feed was produced at the feed technology center (Fôrtek) at NMBU. In total, there were three diets produced:

- C: Commercial like control diet
- D2: Control diet + 0.2% *Debaryomyces hansenii* yeast
- D3: Control diet + 0.2% extract of fucoidan from *Saccharina latissima*

Tabel 1. The formulation of the control diet used in this study.

Ingredients	**%**	**Amino acid composition**	**Kg^{-1}**
Fish meal NA LT[a]	15	Alanine	16.59
Fish meal NA[b]	15	Arginine	18.98
Soy protein concentrate>62%[c]	16	Aspartic acid	25.22
Wheat gluten[d]	17.78	Cystine	4.73
Wheat milling quality[e]	11.76	Glutamic acid	90.85
Fish oil[f]	5.031	Glycine	15.50
Rapeseed oil[g]	11.738	Histidine	12.22
Premix[h]	7.215	Isoleucine	16.39
Chemical composition	**%**	Leucine	29.39
Dry matter	95.07	Lysine	28.83
Crude protein	49.41	Methionine	8.53
Crude fat	19.0	Phenylalanine	15.24
Starch	9.5	Proline	26.82
Ash	8.7	Serine	15.30
Energy (MJ/kg)	22.2	Tryptophan	15.67
Calcium	1.2	Tyrosine	5.75
Sodium	0.8	Valine	14.50
Magnesium	0.2	**SUM AA**	**360.52**
Potassium	1.0		
Phosphorus	1.7		

[a]Fish meal NA LT: Pelagia/FF Skagen, Norway/Denmark. [b]Fish meal NA: FF Skagen, Denmark. [c]Soy protein concentrate: Nordsildmel Innoviation, Brazil. [d]Wheat gluten: Cargill NV, Belgium. [e]Wheat milling quality: Hedegaard, Denmark. [f]Fish oil: ED&F MAN, Mexico. [g]Rapeseed oil, crude: Vilomix, Denmark. [h]Premix: Aliphos, Bulgaria.

The characterization of *D. hansenii* can be seen in Table 2.

Table 2. Characterization of hydrolyzed D. hansenii-based product

	D. hansenii
Cell size (µm)	3.5 ± 0.6
Cell wall thickness (nm)	119.2 ± 24.8
α-glucans (% w/w)	6.6
β-glucans (% w/w)	19.3
Mannans (% w/w)	14.8
Mannans length (nm)	105
Ratio β-glucans/Mannans	1.3
Origin	Dairy

% w/w, expressed as % of dry matter

Extract rich in fucoidan was made from the brown algae *S. latissima*. The seaweed was firstly grinded and further on, extracted using acid. The extract was also stepwise filtrated and analysed for its bioactivity, before being used as a component in the feed.

2.3 Fish growth performance

Uneaten feed was collected daily to estimate feed intake, feed conversion ratio (FCR), and specific growth rate (SGR). A recovery test was also conducted at the end of FW and SW phase, to ensure correct calculations of uneaten and eaten feed. The recovery values for each diet were measured for each tank according to Helland et al. (1996). The different diets were analyzed

for chemical composition at Labtek NMBU, including analysis of dry matter (DM) and content of ash, protein, starch, raw fat, energy, and several minerals (calcium, natrium, magnesium, potassium and phosphor).

Specific growth rate (SGR) is presented in this thesis including or excluding starvation days. Following the standard routine for the vaccination procedure, fish was starved two days prior and three days post-vaccination, in order to reduce stress and risk of inflammation in the intestine (Pharmaq, 2022).

2.3.1 Calculations

Feed intake expressed in gram DM per fish was calculated as followed:

$$Feed\ intake = \text{T}otal\ feed * Feed\ DM - \frac{(uneaten\ feed * uneaten\ feed\ DM)}{Recovery\ value}$$

Feed conversion ratio (FCR) was calculated to evaluate feed utilization, calculated by individual average feed intake and weight increase:

$$FCR = \frac{Feed\ intake}{Weight\ increase}$$

Specific growth rate (SGR), being the increase in body weight per day expressed in percentage, was calculated to estimate the growth performance of the fish:

$$SGR = \frac{\ln(FBW) - \ln(IBW)}{\Delta \text{t}} * 100\ \%$$

Relative body weight (RBW) was calculated as follows:

$$RWG = \frac{(FBW - IBW)}{IBW} * 100\ \%$$

2.4 Gene expression

The gene expression quantification of the liver samples was done by real time – quantitative polymerase chain reaction (RT – qPCR).

2.4.1 RNA extraction

Total RNA was extracted using RNeasy Plus Mini Handbook with slight modifications for the tissue. Briefly, one stainless 5mm steel bead was added into each 2.0 mL Eppendorf tube. A

piece of liver tissue (approximately between 13 mg-68 mg) was placed in the tube together with 900 µL QIAzol Lysis Reagent. The sample was placed evenly distributed in TissueLyser (Retsch, Basel, Schweiz) for 2 min at a speed of 20Hz. The tubes were rearranged in opposite directions and operated in TissueLyser for another 2 minutes at 20Hz. The lysates were carefully pipetted into a new 2.0 mL Eppendorf tube and 180 µL chloroform was added to separate the molecules. The new mixture was shaken by hand for 15 s and later rested at room temperature for 2-3 min. The sample was placed in Heraeus Fresco 21 centrifuge (Thermo Fisher scientific, Wilmington, USA) at 4°C for 15 min at a speed of 12,000 x g. The upper aqueous phase containing the RNA was separated and taken out and placed inside an Eppendorf 2.0 mL tube, approximately 450 µL. An equal volume of 70% ethanol was added, and it was shaken slowly back and forth a couple of times. 700 µL of the sample were transferred to a RNeasy spin column placed in a 2 mL collection tube. The samples were placed into the centrifuge at a speed of 8,000 x g for 15 s. All centrifugal from here on were done at room temperature. The RNA that was left inside the filter was kept for further processes while the flow-through was discarded. The remaining sample in the tube was taken out and centrifuged according to the same procedure.

Buffer RW1 was added at a volume of 700 µL followed by centrifugal for 15s at a speed of 8,000 x g. Then, 500 µL buffer RPE was added and afterward centrifuged again for 15s at a speed of 8,000 x g. Buffer RPE was added at a volume of 500 µL and centrifuged for 2min at a speed of 8,000 x g. The filter part was placed inside a new collection tube and centrifuged for 1min at full speed. Flow-through was always discarded after each centrifugal during the steps mentioned above. The filter part was placed inside another collection tube and 30 µL RNase-free water was added. The sample was centrifuged for 1min at the speed of 8000 x g. The concentration of total RNA was determined using NanoDrop TM 8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Total RNA was stored at -80°C until further analyses.

2.4.2 cDNA synthesis

All samples were normalized to the concentration of 500 ng μL^{-1} prior to cDNA Synthesis Master Mix. The anneal primer to template RNA was made by pipetting together 1µL Milli-Q water, 0.5 µL dNTP mix, 0.5 µL oligo d(T)20 primer, and 11 µL template RNA per RNA sample. The mix was placed in a plate and vortexed, spinned and placed in GeneAMP PCR System 9700 (Applied Biosystems, Massachusetts, USA) at 65°C for 5 min and then incubated on ice for a minimum one minute. The reverse transcription (RT) reaction mix was created by pipetting 1.6 µL Milli-Q water, 4 µL 5x SSIV buffer, 1 µL 100nM DTT, 0.2 µL RNaseOUT recombinant RNase inhibitor and 0.2 µL superscript IV reverse transcriptase (200 U/µL) together per sample. The RT reaction mix was vortexed, spinned and lastly evenly distributed into the plate. The plate was placed in GeneAMP PCR System 9700 at 50-55°C for 10 min followed by 80°C for 10 min. Until further analysis were cDNA samples stored at -20°C.

2.4.3 RT – qPCR

A set of genes involved in liver osmotic regulation, growth responses, energy responses, and immune responses were analyzed by RT-qPCR, LightCycler 480 System (Roche, Basal, Schweiz). A total of 11 genes were analyzed including the reference gene (EFAα) that met the qualifications of transcriptional stability in the liver. The sequence for all primers used in this thesis can be seen in Table 3. The qPCR reactions were conducted with 7.5 µL mastermix and 2.5 µL cDNA previously diluted 1:10. The mastermix consisted of 1.5 µL Milli-Q water, 0.75 µL gene specific primers, 5 µL SybrGreen times the amount of samples it was being used for. The qPCR conditions were 95°C for 17 min, approximately the annealing temperature for each primer for 15 min, 72°C for 20 min times 39 cycles and lastly 95°C for 5 min. At the end of the program, a melting curve analysis was performed to confirm the absence of unspecific products or primer dimers.

Table 3. Primer sequence for each specific gene, given as forward and reversed, accession number and annealing temperature.

Gene name	Genes	Fwd/ Rev	Primer sequence	Annealing temp. (°C)
Annexin A1	Anxa1	Fwd	ATGGTGAACCAGGAACTTGC	63.6
		Rev	ATAGCCTTTGCCACATCCAC	
Aquaporin-8ab	AQP8ab	Fwd	GTTGGCATAGTTCTCCTTTGATG	62.5
		Rev	TTTCAACCCTCCCTTCACC	
Complement 3	C3	Fwd	TACGCTGCCTGGGTCCAAA	64.7
		Rev	ACAGCACTACAGAGCACGTT	
Cytochrome c oxidase subunit 1	COX1	Fwd	CTGGTGAGGAGGAGATAGCC	63.1
		Rev	GTAGAACTCCAGCGCATCAA	
Elongation factor alpha 1	EFAα	Fwd	TGACTTCGGCGGCAACA	65.0
		Rev	GCCATAGCCCGTTGGTTTACT	
Hepcidin	Hepcidin	Fwd	GAAGGCCTTTAGTGTTGCAGTGGT	67.9
		Rev	GTTGATGTTCCCCAACTGGACTGT	
Insulin like growth factor 1	IGF-1	Fwd	TGACTTCGGCGGCAACA	65.0
		Rev	GCCATAGCCCGTTGGTTTACT	
Interleukin 10	IL-10	Fwd	ATGAGGCTAATGACGAGCTGGAGA	67.8
		Rev	GGTGTAGAATGCCTTCGTCCAACA	
Leptin	Leptin	Fwd	GGATGATATCACGCTGCCCA	65.1
		Rev	CACTGGACCCACACTCAGAC	
Nuclear factor of activated T-cells 5b1	NFAT5b1	Fwd	CAACAGCAACAGCAAATTCAG	61.7
		Rev	GTTTCTGTTGTTGTTGTTGCTG	
Nuclear factor of activated T-cells 5b2	NFAT5b2	Fwd	AGCAACTACAGCAACAGCAACT	66.0
		Rev	GCTGCTGTTTCTGTTGTTGCCT	

2.5 Data analysis

GraphPad Prism 8.0.2 was used for calculations regarding growth performance and gene expressions, as well as graphical presentation and calculation of means and standard deviation (SD) or standard error of the mean (SEM). The gene expressions were calculated according to

the $2^{-\Delta\Delta_{CT}}$ method (Livak & Schmittgen, 2001; Rao et al., 2013). Furthermore, to ensure normality and homogeneity of the data, Shapiro's test, log transformation in all qPCR data was performed and outliers were removed (Teixeira et al., 2022). Multiple t tests were used (GraphPad Prism 8.0.2) to compare the gene expression of the different biomarkers between the control diet and the other experimental diets. Differences were considered significant at $p < 0.05$. Correlation coefficients among diets were calculated by Corrplot (v.0.92) in R (Wei & Simko, 2021). The correlation was considered significant at $p < 0.05$, a trend for all measured parameters was considered at $p < 0.1$.

3. Results

During the experimental period there were no mortalities observed, and the fish appeared to be in good health in both FW and SW phase.

3.1 Growth performance

The growth performance data for FW phase are presented in Table 5 and Figure 5. The final average weight and the average weight increase were significantly different for *D. hansenii* (D2) and Fucoidan (D3) compared to Control (C). The feed intake was significantly different between C and D3. In addition, SGR was significantly different for D2 and D3, compared to C. RWG was significantly different for D2 and D3 compared to C. The initial weight was significantly higher for D2 compared to D3. No significant difference was observed regarding FCR, but tendencies show that FCR is reduced for D2 and D3 compared to C.

Table 5. Fish weight at the start and end of the fresh water (FW) phase (g/fish), weight increase (g/fish), feed intake (g/fish), Feed convertion ratio (FCR), Specific growth rate (SGR) including or excluding starvation days and relative weight gain (RWG) for three dietary treatments in the FW phase.[a]

Fresh water	**Control (C)**	***D. hansenii* (D2)**	**Fucoidan (D3)**	**p-value (C – D2)**	**p-value (C – D3)**	**p-value (D2-D3)**
Initial weight (g)	31.58 ± 0.13	31.63 ± 0.02	31.36 ± 0.03	0.636	0.138	0.010*
Final weight (g)	57.92 ± 0.86[a]	66.22 ± 0.73[b]	68.52 ± 1.18[b]	0.009*	0.009*	0.144
Weight increase (g)	26.34 ± 0.74[a]	34.59 ± 0.74[b]	37.16 ± 1.21[b]	0.008*	0.009*	0.125
Feed intake (g DM)	22.05 ± 0.41[a]	25.39 ± 1.10[ab]	27.14 ± 0.10[b]	0.057	0.004*	0.154
FCR	0.84 ± 0.04	0.73 ± 0.02	0.73 ± 0.02	0.089	0.089	0.999
SGR	1.19 ± 0.02[a]	1.45 ± 0.02[b]	1.53 ± 0.04[b]	0.007*	0.007*	0.091
RWG	83.41 ± 2.01[a]	109.34 ± 2.40[b]	118.51 ± 3.99[b]	0.007*	0.008*	0.108

[a]Data are presented as means ±SD per fish per dietary treatment. Means with different superscript letters in a row are significantly different ($p<0.5$).

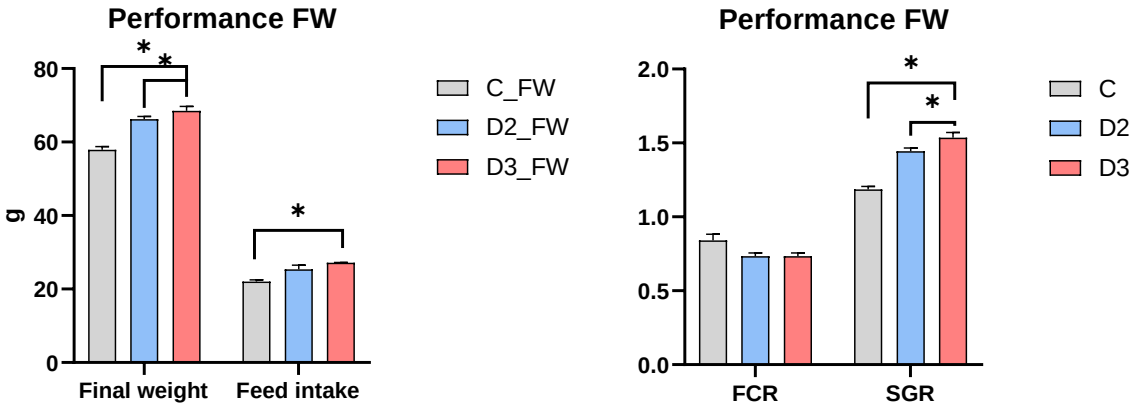

Figure 5. Fish performance including final weight, feed intake, feed conversion ratio (FCR) and specific growth rate (SGR) for three dietary treatments in the fresh water (FW) phase. C: control diet. D2: D. hansenii diet. D3: Fucoidan diet. Data are presented as means ± SD per fish per dietary treatment. Significant differences are denoted with '' and was considered significant at p<0.05.*

The growth performance data for SW phase are presented in Table 6 and Figure 6. The only significant difference detected during SW phase was the weight increase being significantly higher in D2 group compared to D3. There were tendencies that fish from D2 and D3 group performed better than C.

Table 6. Fish weight at the start and end of the seawater (SW) phase (g/fish), weight increase (g/fish), feed intake (g/fish), Feed convertion ratio (FCR), Specific growth rate (SGR) including or excluding starvation days and relative weight gain (RWG) for three dietary treatments in the SW phase.[a]

Seawater	**Control (C)**	***D. hansenii* (D2)**	**Fucoidan (D3)**	**p-value (C-D2)**	**p-value (C-D3)**	**p-value (D2-D3)**
Initial weight (g)	65.83 ± 0.96	66.86 ± 0.40	68.86 ± 1.21	0.303	0.110	0.157
Final weight (g)	81.09 ± 5.49	87.06 ± 0.20	87.20 ± 0.73	0.264	0.259	0.810
Weight increase (g)	15.26 ± 6.45^{ab}	20.20 ± 0.20^{a}	18.34 ± 0.48^{b}	0.392	0.570	0.038*
Feed intake (g DM)	11.10 ± 2.20	12.89 ± 0.75	12.16 ± 0.55	0.389	0.575	0.383
FCR	0.77 ± 0.18	0.64 ± 0.04	0.66 ± 0.05	0.433	0.522	0.642
SGR	0.72 ± 0.28	0.91 ± 0.01	0.81 ± 0.03	0.442	0.676	0.072
RWG	23.25 ± 10.14	30.22 ± 0.48	26.65 ± 1.17	0.434	0.684	0.058

[a]Data are presented as means ±SD per fish per dietary treatment. Means with different superscript letters in a row are significantly different (p<0.5).

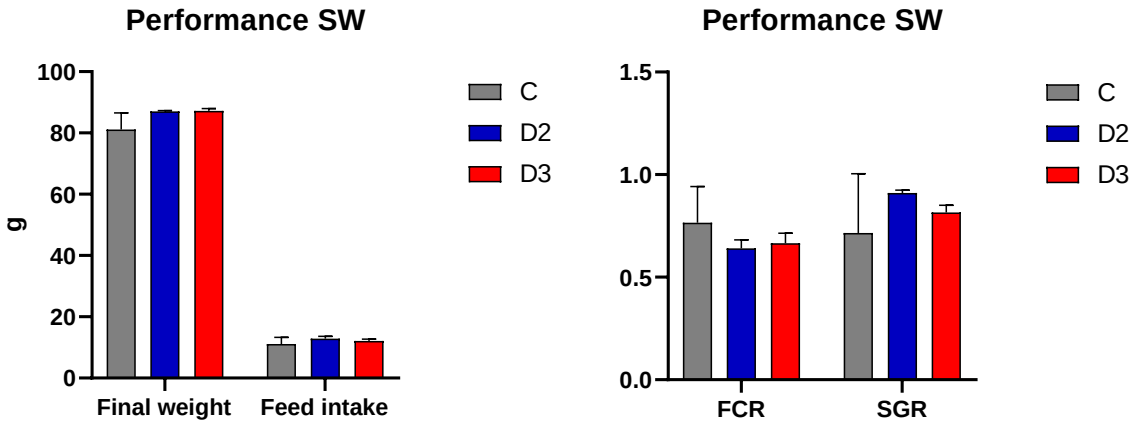

Figure 6. Fish performance including final weight, feed intake, feed conversion ratio (FCR), specific growth rate (SGR) for three dietary treatments in the seawater (SW) phase. C: control diet. D2: D. hansenii diet. D3: Fucoidan diet. Data are presented as means ± SD per fish per dietary treatment.

3.2 Gene expression

The gene expression biomarkers involved in energy and growth regulation showed different pattern of expression for different dietary groups within and between different phases (Figure 7). No significant differences were denoted for *COX1* between dietary groups in FW and SW. Nevertheless, *COX1* was up-regulated for D3 in SW compared to FW ($p<0.022$), and the same tendencies was shown for D2 ($p<0.084$).

For *IGF-1* within FW phase, significant down-regulation for D3 was shown compared to C ($p<0.007$) and D2 ($p<0.0009$). No further significant differences were denoted for *IGF-1*. *Leptin* was significantly up-regulated for D3 compared to D2 ($p<0.047$) in FW. No significant differences were denoted in SW. All diets showed a significant up-regulation of *leptin* in SW compared to FW, C ($p<0.0012$), D2 ($p<0.0007$) and D3 ($p<0.022$).

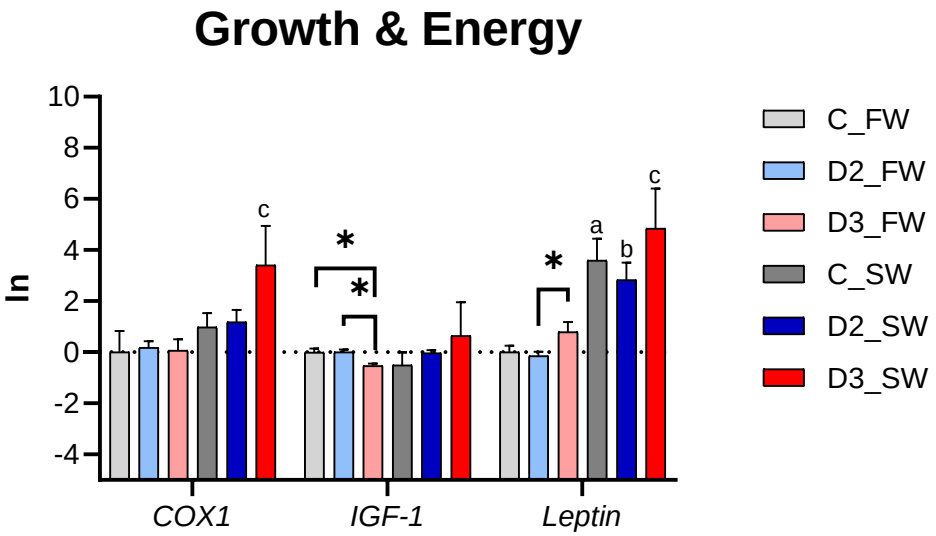

*Figure 7: Gene expression (by qPCR) of energy and growth related biomarkers (COX1: Cytochrome c oxidase subunit 1, IGF-1: Insulin like growth factor 1, Leptin) in liver from Atlantic salmon fed functional diets with either D. hansenii or fucoidan. C: Control diet. D2: D. hansenii diet. D3: Fucoidan diet. FW: Fresh water. SW: Seawater. *: significant difference within the same phase. a: significant difference from C in FW phase. b: significant different from D2 in FW phase. c: significant different from D3 in FW phase. P<0.05 was considered significant.*

Regarding the expression of genes involved in osmotic regulations (Figure 8), *NFAT5b1* biomarker shows an up-regulation in D3 compared to C (p<0.045) in SW. The same gene showed a significant difference in the gene expression between FW and SW period for D2 (p<0.011) and D3 (p<0.028).

Furthermore, *NFAT5b2* has shown significant up-regulation for C in SW compared to FW (p<0.037), and the same tendency was shown for D3 group (p<0.097). *AQP8ab* did not show any significant regulation of gene expression between dietary groups or phases. Although, AQP8ab showed tendencies of being up-regulated in C (p<0.07) and D3 (p<0.085) in SW compared to FW.

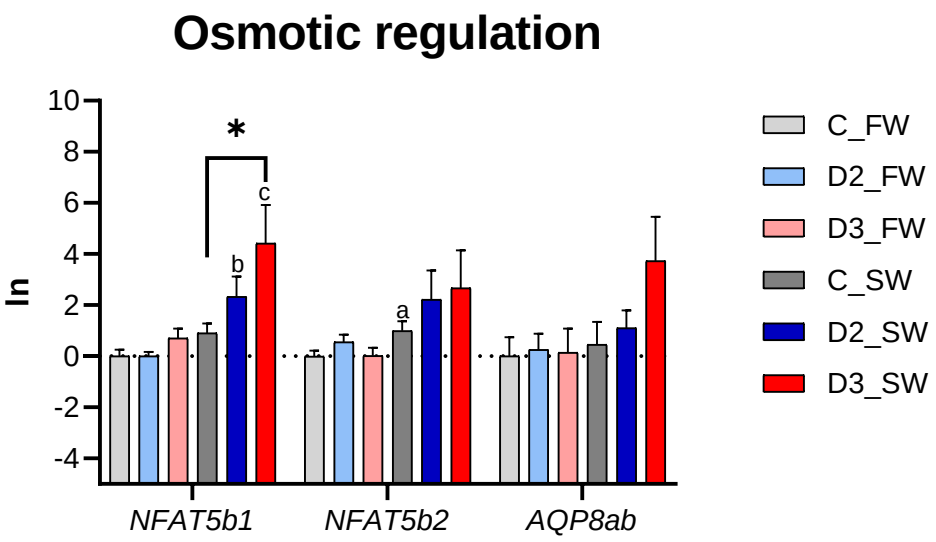

*Figure 8. Gene expression (by qPCR) of osmotic regulation related biomarkers (NFAT5b1: Nuclear factor of activated T-cells 5b1, NFAT5b2: Nuclear factor of activated T-cells 5b2, AQP8ab: Aquaporin-8ab) in liver from Atlantic salmon fed functional diets with either D. hansenii or fucoidan. C: Control diet. D2: D. hansenii. D3: Fucoidan. FW: Fresh water. SW: Seawater. *: significant difference within the same phase. a: significant difference from C in FW phase. b: significant different from D2 in FW phase. c: significant different from D3 in FW phase. P<0.05 was considered significant.*

Immune-related genes showed different pattern of expression for different dietary groups within and between different phases (Figure 9). For *C3* no significant differences was denoted between dietary groups in FW. However, in SW phase was *C3* significantly up-regulated for D3 compared to C (p<0.042).

Hepcidin was significantly up-regulated for D2 compared to D3 in FW (p<0.004), no further significant regulation was denoted. For *IL-10* was no significant regulation between dietary groups or water phases denoted. Although, tendencies showed that *IL-10* was up-regulated for D3 compared to C in SW (p<0.962).

Anxa1 was significantly up-regulated in D2 compared to C in FW (p<0.034), no significant regulation was denoted in SW. Although, C was significantly up-regulated in SW compared to FW (p<0.025), and the same tendencies was shown for D2 (p<0.07) and D3 group (p<0.11).

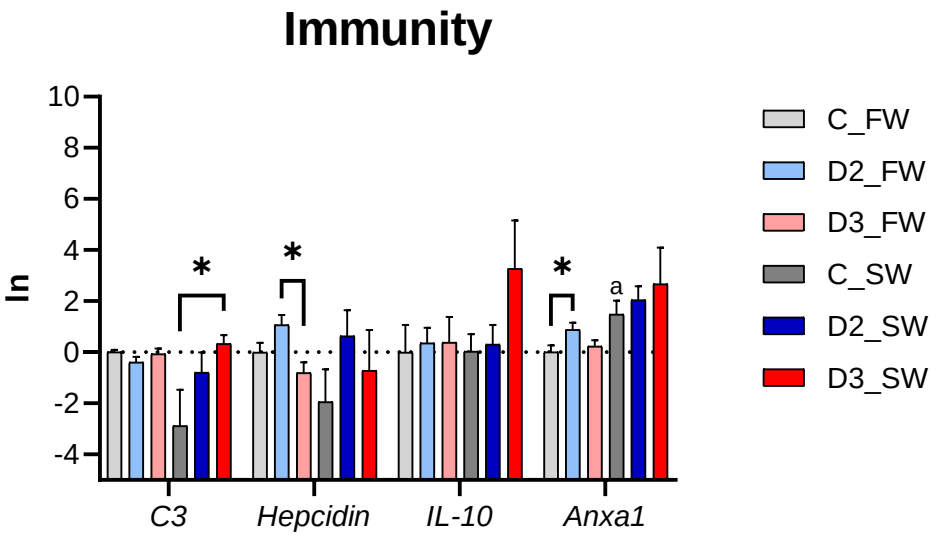

*Figure 9. Gene expression (by qPCR) of immunity related biomarkers (C3: Complement 3, Hepcidin, IL-10: Interleukin 10, Anxa1: Annexin A1) in liver from Atlantic salmon fed functional diets with either D. hansenii or fucoidan. C: Control diet. D2: D. hansenii. D3: Fucoidan. FW: Fresh water. SW: Seawater. *: significant difference within the same phase. a: significant difference from C in FW phase. b: significant different from D2 in FW phase. c: significant different from D3 in FW phase. P<0.05 was considered significant.*

3.3 Correlation

In this study, correlation was used to analyse and to visualize the relationship among gene expression data, among growth performance data, and correlation among the growth performance data and the gene expression of genes related to growth and energy (Figure 10). Correlation A regarding the relationship among gene expressions data showed weak correlation in FW. In SW phase C group was positively correlated to both experimental diets. The analysis between FW and SW demonstrated positive correlation only for D3. Correlation B regarding the growth performance showed significant positive correlation between each dietary group in both FW and SW phase. Correlation C regarding the growth performance include the genes related to energy and growth (COX1, IGF-1 and Leptin). Again, significant positive correlation between each dietary group in both FW and SW phase was shown. C and D2 showed a significant positive correlation also between the water phases.

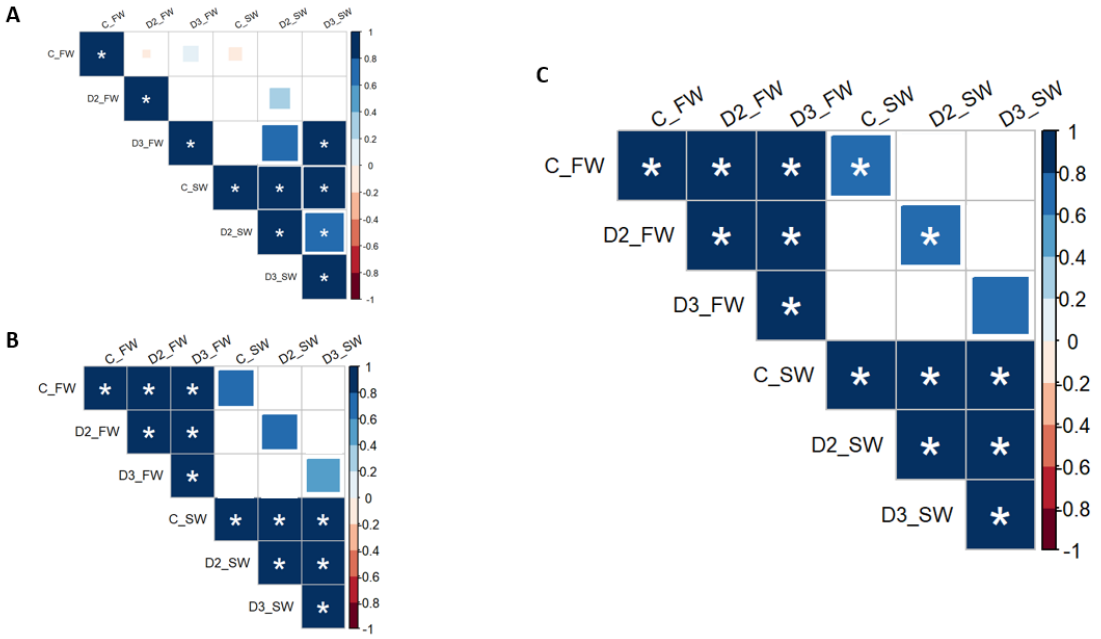

Figure 10. A correlation analysis among the different parameters. A: Correlation including all gene expressions. B: Correlation including the growth performance. C: Correlation including the growth performance and the gene expression of genes related to growth and energy. C_FW: Control diet in fresh water phase. D2_ FW: D. hansenii in fresh water phase. D3_FW: Fucoidan in fresh water phase. C_SW: Control diet in seawater phase. D2_ SW: D. hansenii in seawater phase. D3_SW: Fucoidan in seawater phase. All the parameters that are significantly correlated (p-value<0.05) are denoted by ''.*

4. Discussion

A critical period in Atlantic salmon farming is the SWT, where a major part of the mortalities and economical losses occur during the first months in the sea (Aunsmo et al., 2008). An increased number of infectious diseases can be seen after SWT (Johansson et al., 2016). A good smolt quality is crucial for optimal adaptation to the environment. A solution that could help to prevent disease outbreaks in a more cost-effective way than vaccines and antibiotics, is through nutritional intervention. Specific functional feed components which have the potential to promote the health status and growth performance of the fish (Holdt & Kraan, 2011) can be a part of the solution to these challenges.

In this thesis, the immunostimulating properties of 0.2% inclusion level of *Debaryomyces hansenii* yeast and extract of fucoidan from brown algae *Sacccharina latissima* were evaluated. IMM acts directly on the immune system of the fish (Sakai, 1999), modulating their response and effectively increasing the disease resistance and the immunocompetence of the fish (Dawood et al., 2018). Enhancing the immune system can positively affect the fish's potential to resist pathogens. However, it is of high importance that the immune system does not get over stimulated as that could have a negative impact on other physiological processes such as growth (Tacchi et al., 2011). The aim is not to suppress the immune system, but to obtain a balanced immune stimulation where beneficial effects are enhanced and adverse effects of the immune system are decreased (Munang'andu et al., 2020). Furthermore, IMM should be provided to the fish during a time that the host has the best opportunity to resist the disease or during particularly stressful situations, such as during the SWT (Tacchi et al., 2011). In this study, IMM was given orally in the feed, 4-weeks prior and 4-weeks post SWT, with the aim to enhance the fish's capacity of handling stress associated with SWT. When evaluating IMM, it would be ideal to have a challenge model with alive pathogens (Galeotti, 2007) although this is not always feasible. In this experiment, the fish were exposed to natural pathogen challenge during SWT, where an increased number of infectious diseases often can be seen (Johansson et al., 2016). Therefore, SWT was the model used to evaluate the immunostimulants effect.

β-glucans and MOS components of *D. hansenii* and extract of fucoidan, are all natural inactivated microbes that can stimulate the immune system (Dawood et al., 2018). Both MOS and fucoidan has previously shown to have a beneficial effect on growth performance (Refstie et al., 2010; Saeed et al., 2021). Refstie et al. (2010) showed that a supplement level of 2% MOS derived from the cell wall of baker´s yeast had a positive effect on the growth performance of Atlantic salmon. Reyes-Becerril et al. (2011) did also show that an inclusion level of 1.1% of *D. hansenii* enhanced the growth performance of leopard grouper. Supplementing diets with 3% and 10% *S. latissima* has shown to enhance the growth performance of Atlantic salmon (Kamunde et al., 2019). In giant tiger prawn (*Penaeus monodon),* improved growth with an inclusion level of 0.1%-0.3% dietary fucoidan has been shown (Sivagnanavelmurugan et al., 2014).

The results from this thesis are in accordance with the study of Refstie et al. (2010) and Reyes-Becerril et al. (2011) which used MOS to promote growth performance. Although these studies used inclusion levels of 2% and 1.1% respectively, in this experiment enhanced growth performance was shown with a lower inclusion level of yeast. This finding could be a cost-efficient way to produce feed with IMM for the fish feed industry, with a smaller impact on the total price for the fish farmer. The mode of action of IMM as growth promoters are still not completely understood. For example, Refstie et al. (2010) showed that MOS in a diet with a soybean meal inclusion level of 14% could eliminate enteritis in the intestine of Atlantic salmon and improve the diarrheic condition. This in turn coincided with more efficient utilization of nutrients and improved growth performance. MOS is also known to bind and block glycoprotein receptors on pathogens, forcing them to pass through the gut and preventing host colonization. Thereby, MOS could also improve gut health (Refstie et al 2010). In this thesis, we speculate that the inclusion of yeast in the diet stimulated and maintained favorable intestinal environment and, thus, improved growth performance. Regarding fucoidan, the result from this thesis is also in accordance with Kamunde et al. (2019) whom supplemented diets with 3 or 10% *S. Latissima* and Sivagnanavelmurugan et al. (2014) whom supplemented diets with 0.1-0.3% fucoidan to enhance the growth performance. The improved growth performance of fish might be associated with increased digestive enzymatic activities (Cui et al., 2020), and fucoidan might have prevented the activities of myostatin protein, which subsequently increase the muscle fiber area (Ramazanov et al., 2003). Furthermore, increased secretion of digestive enzymes due to sulphated polysaccharides might have contributed to improved feed utilization and thereby growth rate (Ozorio et al., 2015).

During this experiment, C group underwent one extra sampling compared to D2 and D3 groups that was not relevant to this thesis work, but it might have had adversely affected growth performance. The impact is, however, expected to be low as the sampling was performed by experienced staff with minimal disturbance of the fish. Additionally, the initial weight for D3 was lower than the D2 group which could have an impact on the growth performance. Thus, if the initial weights were equal, the growth performance could have been even better in D3 group.

The growth performance from SW phase showed larger variation among tanks as indicated by the increased standard deviation compared to FW phase. An explanation for this could be a larger variance in the microenvironment among the different tanks. This could have been due to differences in the water flow in the tanks, which could in turn also influenced the oxygen levels. Although, the oxygen level was measured daily and nothing out of the ordinary was observed. The largest standard deviation was observed in the C group, where the duplicate tanks were positioned at opposite side of the wall in the fish laboratory facility, while the other experimental groups were positioned on the same side of the wall (Figure 11). This could potentially contribute to the larger variation seen in C group compared to the other groups. Nevertheless, D2 showed a higher weight increase compared to D3. A tendency could still be seen that both D2 and D3 did have an enhanced growth performance in SW phase, strengthening the theory that an inclusion level of 0.2% could have a beneficial role in promoting growth performance. Although both experimental diets performed similarly, it was observed that D2 performed slightly better in SW period. It would have been interesting to follow the fish for a

longer period to investigate whether fish fed D2 would increase their growth performance even more and overtake D3 in terms of final weight. It would also be interesting to follow the fish for a longer period to understand prolonged effect of feeding with functional diets containing yeast and fucoidan on fish growth performance and health.

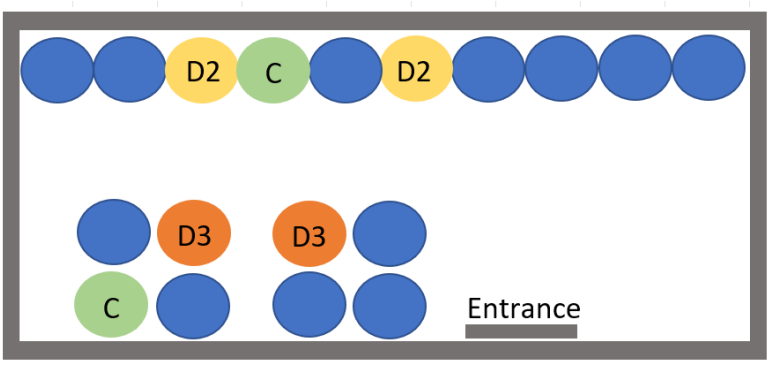

Figure 11. The tank positions in seawater facility. C: Control group. D2: D. hansenii group. D3: Fucoidan group.

The effect of the diets was also evaluated on a transcriptional level in the liver which is an organ with an essential role in metabolism (Chiang, 2014) and could be a crucial organ to understand in regards to immunonutritional manipulations. The liver is connected to the GALT with blood and bile (Wu et al., 2016), thus, the digested feed has direct effect on the liver metabolic changes. The hepatic transcriptomic results showed significant modulation due to different IMM inclusion and due to different production phases. COX1 is a gene related to energy, which is responsible for the production of prostaglandins associated with normal physiological processes (Kim, 2014). In previous studies, COX1 appeared unaffected by stress unlike other subgroups such as COX2, most likely due to its constitutive function in performing maintenance and homeostatic processes (Olsen et al., 2012). The results from this thesis, in accordance with Olsen et al. (2012) could indicate that the up-regulation of *COX1* for D2 and D3 group would make the fish more prepared to regain homeostasis in SW phase.

Liver is one of the primary producers of IGF-1, which is part of an endocrine system that plays a major role in controlling somatic growth in teleost fish like Atlantic salmon. Temperature and nutrition seem to be the primarily regulation of IGF-1 (Hevroy et al., 2015; Triantaphyllopoulos et al., 2019), as well as growth hormone (Triantaphyllopoulos et al., 2019). The plasma levels of IGF-1 generally correlate with feed intake, nutrient uptake, and growth performance (Beckman, 2011). Fish fed D3 in FW phase showed the best growth performance, and it would be expected to have the highest expression of *IGF-1*, however, in the present study the a down-regulation of this gene was observed. No obvious reasons for this can be explained. The liver is the organ that produces IGF-1, but muscle tissue would also exhibit high expression of IGF-1 during increased growth. A possible explanation might be that a higher circulating level of IGF-1 was secreted into the plasma from muscle tissue, that in turn could have induced down-regulation of IGF-1 expression in the liver, which would regulate its expression depending on the plasma levels. Furthermore, to understand the broader picture it would have been interesting to analyse IGF-1 expression levels in both, plasma and muscle, and to measure the growth hormone levels in all these tissues. Although, it is very important to mention that the changed

transcriptomic levels may not always necessarily correspond to the changes on the protein level, thus the future studies should also include the levels of protein production.

The leptin gene is related to growth and the regulation of body weight. The leptin gene is an important factor in energy homeostasis in mammals (Blanco & Soengas, 2021), but its role in teleost´s is not completely defined, yet increased levels of *leptin* are thought to reduce growth in Atlantic salmon by affecting the feed intake (Murashita et al., 2011). D3 had an up-regulation which was not expected since that dietary group have had the highest feed intake in FW phase. It is assumed that the leptin levels in plasma would have showed a different picture and it would therefore be relevant to also investigate the leptin levels in plasma, and thereby get a better understanding of the liver transcriptomic changes in FW. Furthermore, *leptin* was also up-regulated for all dietary groups during the second sampling post SWT, which could be explained by increased stress which often can be correlated with the decreased appetite. The increased leptin levels could also be explained by the two days of starvation before sampling. Starvation period and feed restriction are known to increase the hepatic leptin gene expression in Atlantic salmon (Trombley et al., 2014).

There are many more factors that could explain a growth performance such as how well the fish handles the osmotic regulation upon SWT. Poor smoltification and struggles with osmotic regulation often result in increased stress for the fish which in turn correlates with suppressed growth (Lai et al., 2021). All the genes that are involved in osmotic regulation did show tendencies of being up-regulated upon SWT. NFAT5b group plays an osmotic stress response role. An increased NFAT5b mRNA expression in salmon could potentially mitigate osmotic stress. Upon osmotic stress binding of NFAT5b will induce a downstream increase in the availability of local active thyroid hormone. This will subsequently switch on thyroid activated genes which promote SW adaptation in the gills (Lorgen et al., 2017). The results from the experiment would indicate that the fish fed D3 was better equipped for the osmotic regulation and that they had an enhanced response upon osmotic stress which could mitigate that challenge. The gene AQP8ab also relates to osmotic regulation, whose channels are located at the brush border of enterocytes and its physiological role is involved in transcellular water uptake upon SW exposure. Its expression is upregulated in response to SWT (Engelund et al., 2013), which correlated well with the results of the gene expression for C and D3 in this thesis. Which indicated that these fishes were better equipped for the SWT, especially D3 group which had the highest gene expression of *AQP8ab.* The results of the gene expressions suggested that fish fed D3 was better equipped to handle the SWT. As previously mentioned, this could also result in better growth performance due to less stress. The high growth performance of D3 could thereby partly be explained by the osmotic regulation that the fish fed D3 seemed to be better equipped for, compared to the other groups. Although, to strengthen this theory it would have been relevant to investigate the cortisol levels related to osmotic regulation. This would have been highly relevant since cortisol levels are a good indicator directly related to stress (Madison et al., 2015). The results are on the other hand contributing to an overall understanding of the liver role in the osmotic regulation functions since it is a research field not so well investigated so far. Yet, it would have been interesting to further investigate the transcriptional impact of

osmotic regulation related genes in other organs that are more commonly associated with osmoregulation such as the gills.

An increased number of infectious diseases can often be seen after SWT (Johansson et al., 2016) and the period of smoltification is a stressful situation for the fish which makes them more sensitive to handling stress (Barton & Iwama, 1991) and reducing their immune capacity (Maule et al., 1989). It has also been shown in the previous study that smoltification and SWT are associated with systematic repression of the immune transcriptome for Atlantic salmon (Johansson et al., 2016). As already mentioned above, the liver is exposed to many circulating antigens and endotoxins from the gut microbiota, and it is connected to the gut via blood and bile (Wu et al., 2016). The liver is enriched with mainly innate immune cells (Heymann & Tacke, 2016), but the immune system does require a large amount of energy in the liver due to the production of acute-phase proteins and it is therefore essential with good feed management for salmonids (Trichet, 2010). According to previous studies, the liver transcriptomic results showed that liver plays a major role in regulating immunological status in fish (Taylor et al., 2022).

It has previously been shown that β-glucan has the potential to enhance the immune responses in Atlantic salmon (Bridle et al., 2005; Robertsen et al., 1990) and MOS has also proven to have positive effects on the health of Atlantic salmon (Refstie et al., 2010). Seaweed with its bioactive component fucoidan also has been proved to have beneficial roles for the immune system, showing immunomodulatory and anti-inflammatory effects (Saeed et al., 2021). Previous studies have shown that yeast and seaweed with their bioactive components have the potential to promote the immune response in fish and the results of this experiment are in accordance with previous findings.

C3 is a central component of the complement system that plays a key role in innate immunity. It belongs to acute-phase proteins which means that the synthesis of C3 will increase immediately upon inflammatory stimuli (Lovoll et al., 2007). C3 is mainly produced in the liver and induced by proinflammatory cytokines (Bayne & Gerwick, 2001), making the liver the start of the C3 pathway. β-glucans have previously been known to activate the complement system (Akramiene et al., 2007), but the results of this study are not completely concomitant with previous findings. Many studies have also seen that the effectiveness of β-glucans depends on several factors such as molecular structure and size, which could explain why the results in this thesis were not completely correlated to previous studies (Rodrigues et al., 2020). Furthermore, it has been shown that fucoidan can inhibit complement activation in humans and interfere with the first steps of the classical pathway activation, although this pattern was to a lesser extent seen for C3 (Tissot et al., 2003). Similar to this has algal fucoidan previously been described as an anticomplementary molecule (Li et al., 2008). Yet in African catfish fed fucoidan showed enhanced humoral immunity, although C3 was not investigated in particular (El-Boshy et al., 2014). In the results of this experiment, *C3* was up-regulated in SW phase although it was kept at similar levels between different dietary groups in FW phase. For the other diets, it seemed like the SWT had a suppression effect on *C3*, yet our result indicated that this was not the case for the fish fed fucoidan (D3). In recent years, it has been noticeable, that C3 has versatile roles,

and it is involved in a variety of homeostatic processes as well as in immune surveillance (Ricklin et al., 2016). An up-regulation in D3 group could mean that the fish fed with fucoidan better handled pathogen load and challenges in SW and would have been faster to further develop immune response compared to C and D2 group. Although it is not wanted with uncontrolled activation of the complement system since it can be harmful to the host organism.

Hepcidin is another immune related gene that is a central iron-regulating hormone, involved in the sequestration of iron from serum to macrophages. Hepcidin is induced by pro-inflammatory cytokines that are activated by invading bacteria (Diaz et al., 2021). The hormone is predominantly produced in hepatocytes, the main cell type of the liver (Nemeth & Ganz, 2009). Parasites or other invading microorganisms' primary nutrient requirement is iron from their host. The host, such as Atlantic salmon, can limit the availability of iron and thereby nutritionally starve the invaders (Valenzuela-Muñoz & Gallardo-Escárate, 2017). Hepcidin is also considered an antimicrobial peptide that can entrap and destroy pathogens to keep them out of the host organism, it is related to systematic responses and most often highly expressed in serum (Valero et al., 2019). D2 did have an up-regulation in FW phase which meant that the immune response of *hepcidin* could have been enhanced. Yet, it is uncertain if that response would have been necessary for FW phase with fewer pathogenic challenges compared to SW phase. Previous study on Atlantic salmon infected with infectious salmon anaemia virus showed that fish that died early had a higher gene expression level of hepcidin (Jorgensen et al., 2008). Therefore, it could be risky with an uncontrolled enhanced expression of hepcidin. It was also detected (close to tendency with $p<0.161$) that in the group fed with C there was a suppressed expression level of *hepcidin* after SWT, while the higher expression levels in the group fed experimental diets (D2 and D3) might indicated that β-glucans and fucoidan have influenced those responses.

IL-10 is an anti-inflammatory cytokine that limits the host's immune response to pathogens which is important to prevent damage to the host and maintain normal tissue homeostasis. It is important to correct regulations of IL-10, to prevent enhanced immune response to infection which thereby could increase the risk for the development of many autoimmune diseases (Iyer & Cheng, 2012). D3 contained fucoidan which previously has been showed to decrease the levels of pro-inflammatory cytokines, acting as an anti-inflammatory agent (Saeed et al., 2021). Mice have shown increased mRNA levels of anti-inflammatory cytokines like IL-4 and IL-10 in the liver of infected mice after fucoidan treatment (Bai et al., 2020). The results from this thesis correlated with previous studies showing anti-inflammatory effects of fucoidan. It has also previously been reported that hepcidin can increase the level of IL-10 cytokines in leukocytes cells from trout (Alvarez et al., 2022), which potentially mean that hepcidin could have influenced IL-10 expression. Yet, D3 did not show an increased *hepcidin* expression compared to other diets and thereby doesn´t seem to have had the same effect in the liver of Atlantic salmon.

Anxa1 is thought to play an important immune regulatory role during inflammation. It exhibits important inhibitory actions on leukocytes (such as monocytes, neutrophils, and lymphoid cells) activation (Babbin et al., 2008). Anxa1 also function as a stress protein that respond to

environmental stresses such as heat, a response which is in order to protect cells from harmful effects (Rhee et al., 2000). As previously mentioned, SWT is a stressful situation and *Anxa1* was up-regulated for all dietary treatments (C, D2, and D3) 4 weeks post SWT, possibly as a response to environmental stressors. *Anxa1* was also up-regulated for D2 group in FW which potentially could be an anti-inflammatory response to the enhanced immune response of genes such as hepcidin. The analysis of these two anti-inflammatory genes (IL-10 and Anxa1) were important components to consider since they are very crucial for the overall homeostasis of inflammation and oxidative stress both during and before infection (Iddir et al., 2020). It would have been ideal if we have had included pro-inflammatory genes in these analyses as the balance between pro-inflammatory and anti-inflammatory mechanism would give us better overview of the immune response. Although both C3 and hepcidin are induced by pro-inflammatory cytokines and it is most likely that the pro-inflammatory genes would have a similar profile of expression as C3 and hepcidin.

Obtained results of the correlation analysis indicated that fucoidan had the most distinct pattern and behaved differently compared to the other two diets. This opens new questions that should be addressed in the future research, to evaluate fucoidan with a larger panel of biomarkers and in relation to multi-stressor conditions, combining SWT with other challenges. Furthermore, the results from this study suggested that functional ingredients used for 4 weeks prior and post SWT enhanced the immunity and thereby also improved the fish health. The immune system require a large amount of energy (Trichet, 2010) and therefore, balanced modulation of immune response is desired as uncontrolled or overreacted response could limit the energy resources used for growth. However, this was not seen in this experiment. Even with an enhanced immune response, the fish fed experimental diets performed very well, exceeding the growth performance of the control group. A combination of boosted immunity and an improved osmotic regulation for fish fed experimental diets have possibly eased the acclimatization to SW thus fish had an increased energy for growth (Ytrestøyl et al., 2020). The growth performance and feed intake are very well indicators of stress and welfare, where poor welfare often inhibits animal feed intake and growth (Barreto et al., 2022). The results from this study strongly support that the concept of functional aquafeeds can be used to extend beyond the satisfying basic nutritional requirements, to improve growth and feed utilization, but also to support the health and stress resistance of the animals.

5. Conclusion

This study described that functional feeds with 0.2% of *Debaryomyces hansenii* (D2) or fucoidan (D3) improved growth performance and induced transcriptional changes in liver during seawater transfer. In fresh water, dietary supplementation with fucoidan increased specific growth rate and feed intake, while *D. hansenii* (D2) enhanced specific growth rate. At the end of the experiment, fish fed functional feeds with fucoidan or *D. hansinii* performed better than those fed the control diet. Both functional feeds improved fish preparedness to regain homeostasis in seawater as indicated by the up-regulation of *COX1* after seawater transfer. Fish fed fucoidan also had increased osmoregulatory functions and were, thus, better adapted during the transfer. Both functional feeds also enhanced immune response and anti-inflammatory responses of the fish, which suggest immuno-homeostasis.

The result from this thesis raises new questions for future research. This includes to study more organs in the fish to get a broader picture of the response observed on a transcriptional level and to use other multi-omics approaches such as metabolomics and proteomics. In addition, it would be relevant to do a challenge trial with bacterial pathogens, which are emerging challenges today, such as *Moritella viscosa* or *Tenacibaculum spp.,* to evaluate the effect of the functional diets on disease resistance and robustness of Atlantic salmon. Further studies are still warranted to illuminate the underlying mechanism of how these bioactive components improve growth performance and modulate immune function of the fish.

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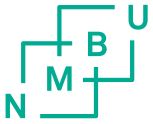

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