Norwegian University of Life Sciences

Master's Thesis 2022 60 ECTS

Department of Animal and Aquaculture Sciences

Faculty of Biosciences

Novel Functional Feed Based on Bioactive Compounds from Spruce tree (*Picea abies*) and their Beneficial Health Effects on Distal Intestine of Atlantic Salmon

Dawit Teklay Okbayohanese

Master of Science in Aquaculture

Novel Functional Feed Based on Bioactive Compounds from Spruce tree (*Picea abies*) and their Beneficial Health Effects on Distal Intestine of Atlantic salmon

Master in Aquaculture

Dawit Teklay Okbayohanese

Supervisors

Dr. Byron Morales-Lange

Dr. Sergio Rocha

Prof. Margareth Øverland

Department of Animal and Aquaculture Sciences

Faculty of Biosciences

Ås

June 2022

Acknowledgements

First, I would like to express my sincere gratitude to my supervisors **Dr. Byron Morales-Lange**, **Dr. Sergio Rocha**, and **Prof. Margareth Øverland** for their patience, guidance, support, and valuable comments on this thesis paper. With all the workload you have, I was really impressed by the quick feedbacks and comments I got every time I needed. It was due to your constructive criticisms that this thesis has reached its completion.

My sincere thanks to laboratory supervisors, especially **Christine** for here guidance and help during laboratory work. I would also like to acknowledge **Ricardo** and his colleagues for their help during the fish trials and sampling. I am thankful to all the members working with Foods of Norway team for the wonderful atmosphere and willingness to help when the need arises. I would like to thank my classmates and friends who have been supportive throughout my studies.

My deepest gratitude goes to my beloved family for the moral support and the amazing chances they gave me to continue my studies. **Dad,** you are gone but your belief in me has made this journey possible and your patience and sacrifice has continued to be an inspiration throughout my life. **Mom**, thanks for your prayers and consistent support in all aspects of my life. My sincere thanks to my brothers and sisters for your moral support and encouragements.

Above all I would like to thank, to a very special person, my lovely wife Selam, for her love and constant support, and for all the late nights. **Selam**, I am extremely grateful for your help and patience, and for the proper care you have taking for our beloved children Siet and Selihom**. Siet, and Selihom,** you are my inspiration to work hard. Words cannot express my gratitude to you, my kids, for your patience and tolerance.

Finally, I thank my **God**, my good Father, for letting me through all the difficulties.

Table of Content

Abbreviations

cDNA	Complementary DNA synthesis
CYPs	Cytochromes P450
DI	Distal intestine
D1	Diet 1
D2	Diet 2
D3	Diet 3
D4	Diet 4
D5	Diet 5
D6	Diet 6
D7	Diet 7
D8	Diet 8
EFα1	Elongation factor alpha 1
FM	Fish meal
FO	Fish oil
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal tract
HMR	Hydroxymatairesinol
IFNγ	Interferon gamma
IL-1β	Interleukin 1 beta
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
ln	Natural logarithm
MALTs	Mucosa associated lymphoid tissues
NKEF	Natural killer enhancing factor
NO	Nitric oxide
NSE1	Norway spruce extract 1
NSE2	Norway spruce extract 2
PBD	Plant based diet
Prx2	Peroxiredoxin 2
RIN	RNA integrity number
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SEM	Standard error of the mean
SOD	Superoxide dismutase
TGFb	Transforming growth factor beta

Abstract

Aquaculture is an important industry that has the potential to stimulate economical growth, create jobs and assisting in poverty alleviation. However, the rapid growth and intensification of this sector has raised concerns about the environmental impact and sustainability of fish farming. Multi-stressor conditions such as infectious diseases, algal blooms, and water quality are among the most serious challenges that causes loss of production to the industry. To face these problems, approved antibiotics and chemotherapeutants are used as a treatment methods. Nevertheless, they are more often neither effective nor environment-friendly. They negatively affect the immune system of aquatic animals and are also a global health threat due to emergence of anti-biotic-resistant bacteria. Hence, it is advised to use other prevention approaches such as feed additives in novel functional diets. Bio-active compounds from plants, as immunostimulants and dietary supplements, are currently arisen interest due to their beneficial health effects. The objective of this study was to evaluate the effects of two extracts from Norway spruce (*Picea abies*) woody biomass (Norway spruce extract 1: NSE1 and Norway spruce extract 2: NSE2), as bio-active compounds in functional diets, on gut health of Atlantic salmon (*Salmo salar*). For this, 640 Atlantic salmon pre-smolts (28.4 g ± 0.05) were randomly distributed into 16 fiberglass tanks (300 L capacity each). Fish were fed with one of eight diets for four weeks: a commercial-like plant-based control diet with no growth promoting components (D1), a diet with 0.2% of a commercial β-glucan (MacroGard®) (D2), and 0.02% (D3), 0.1% (D4) and 0.2% (D5) of NSE1 and 0.02% (D6), 0.1% (D7) and 0.2% (D8) of NSE2. While there was no significant difference in growth performance among all diets, a modulation in the gene expression of immune-related biomarkers were detected in the distal intestine of Atlantic salmon. D2 showed an increase in the expression of *Prx2* and *IL-1b*, compared D1. In addition, D5 and D6 also showed an up-regulation of *IL-1b*, compared to D1, which suggests an activation of the pro-inflammatory immune response. Interestingly, D8 group showed down-regulation of *IFNγ*, *IL-8* and *iNOS*, which may be related to the control or regulation of the immune response and the maintenance of homeostasis in the distal intestine. Based on these findings, both extracts from Norway spruce were shown to be strong candidates in functional aquafeeds, which could be used in different stages of salmon farming to achieve a more robust and resilient salmon, considering the multi-stressor conditions that fish face every day.

1. Introduction

1.1. Global Aquaculture

World population is projected to grow to 8.5 billion by 2030 and fisheries and aquaculture sectors are expected to meet the increasing fish and seafood demands and play important role in global food security (OECD/FAO, 2021). Aquaculture has been the main driver of the increase in fish production in the last three decades (FAO, 2021), where fed aquaculture production has outpaced that of wild fishery (FAO, 2020). This industry is forecast to account for 52% of all fish production by 2030, giving it the potential to stimulate economic growth, create jobs and assisting in poverty alleviation. From 2000 until 2012, it has grown globally from 41.7 million tonnes to 90.4 million tonnes, with 6.7% average yearly production growth since then (Tacon & Metian, 2015). In 2018, it was estimated that global fish production has reached about 179 million tonnes, overall capture fisheries were 96.4 million tonnes while aquaculture accounted 82.1 million tonnes and about 88% of this total production was used for direct human consumption, with the rest intended to non-food uses like fishmeal and fish oil (FAO, 2020; FAO, 2021).

In the past few years, the consumption of fish and aquaculture products in Europa has increased considerably and in 2018, reaching 24.36 kg per capita (Lucas et al., 2021). The household expenditure for fish and aquaculture products in 2019 was approximately equal to 56.6 billion euros (Lucas et al., 2021). Because capture fisheries are remaining stagnant, the aquaculture production has been increasingly important protein source for the growing demand of the world´s population (Reverter et al., 2014).

The Asia-Pacific region, which can be considered as the 'backbone' of aquaculture, is the main contributor to the aquaculture sector globally. China, as one of this region, is the major producer and the main exporter of fish and fish products since 2002 and stands out as the dominant nation in world's aquaculture production (De Silva & Yuan, 2022). On the other hand, US is the largest seafood importer, where 50% of this is from aquaculture production (Garlock et al., 2020). Norway is among the top ten countries with the largest total aquaculture production, where the production of farmed salmon has increased enormously.

Despite the large number and diversity of species being cultured, the aquaculture production by volume is dominated by a small group of species at the national, regional, and global levels. 27 species and species groups accounted 90 percent of the total finfish production in 2018 (FAO, 2020). Though Atlantic salmon (*Salmo Salar*) farming is not one of the dominant cultured species in the aquaculture production, it is one of the most important economic activities in Norway, for example, 304 million Atlantic salmon smolts were transferred to the Norwegian coastal waters in 2018 (Oliveira et al., 2021).

1.2. Norwegian Salmon Industry

Norway is world's largest producer of Atlantic salmon and the second main world's fish exporter after China (FAO, 2021). Norway is the leading aquaculture producer in Europe (Kiron, 2012), and it is considered as the best example of success in developed countries by being among the top ten producing nations. Thus it has shown the world that the production of aquaculture is possible still with high labor costs and strict regulatory standards (Garlock et al., 2020). Technological and favorable environmental conditions and good governance structure are the main drivers that have led Norway to a magnificent increase in salmon production by 1200 times (Cadillo-Benalcazar et al., 2020). Norway produced 53% percent of the world's Atlantic salmon production in 2015 and this accounted yearly value of 6.1 billion EUR (Olaussen, 2018; Oliveira et al., 2021) and in 2019, Norway accounted for 66% of the total global production of farmed Atlantic salmon (Abualtaher & Bar, 2020). In general, aquaculture production in Norway is projected to increase from the current annual 1.2 million tons to 5 million tons by 2050 (Solberg et al., 2021). After petroleum and petroleum related input factors, the Norwegian seafood industry is the third largest export industry in Norway. It is considered that sea food industry will be one of industries that can take over when petroleum activities have to scale down over the next decades (Hersoug, 2015).

As a result of concern about global environmental and social responsibilities, the Norwegian aquaculture industries focus has shifted towards sustainable production and growth since 2005 (Olaussen, 2018). Hence, aquaculture sector in Norway is strictly regulated, and each farm is obligated to keep detailed records. Consequently, Norway has the most detailed database registration in the world (Maroni, 2000). Moreover, regarding

food quality standards and the licensing process for medicines and pesticides, Norway follows EU regulations with a zero-detection limit for permitted levels of medicines and pesticides in aquaculture products at harvesting (Maroni, 2000).

1.3. Aquaculture Challenges

The increasing demands for fish as a source of animal protein and the new technological advances are the main drivers for the rapid expansion of the aquaculture sector. Consequently, the intensification of aquaculture has magnified stressors for fish and thus heightening the risk of infectious disease which is the most series constraint that causes economic loss (it accounts almost 50% of production loss) to the industry (Assefa & Abunna, 2018; Reverter et al., 2014) . Factors such as poor water quality, algal blooms, poor nutritional status, overcrowding, periodic handling, temperature changes and salinity, contribute to physiological changes in fish such as stress or immunosuppression and thus increase the susceptibility to disease (Oliveira et al., 2021; Reverter et al., 2014). Stress reduces fish appetite and growth, causes degeneration of the intestinal mucosa and perturbs its barrier function and uptake mechanisms and thus, increase susceptibility to diseases by influencing on the population level of the fish intestinal microbiota (Olsen et al., 2002).

In 2018, the yearly Atlantic salmon mortality in the Norwegian aquaculture was 27.8% (Oliveira et al., 2021). In Norway, Salmon louse (*Lepeophtheirus salmonis*) infections have the greatest economic impact of all parasites affecting aquaculture. The rapid resistance development to treatments like chemotherapeutants make it difficult to control sea lice infestations (Overton et al., 2019). Some of the best approaches in controlling infectious diseases are vaccines, water disinfection and dietary supplements such as prebiotics or various immunostimulants. In general, aquaculture advise to focus on preventing diseases outbreak rather than treatment (Assefa & Abunna, 2018).

1.4. Feed Additives and Functional Ingredients in Aquafeeds

Aquaculture practicing has changed from solely dependent on fishmeal (FM) and fish oil (FO) as a fish feed to alternative feed plant-ingredients like soybean meal, corn, wheat, rapeseed meal and sunflower. Consequently, there is a significant reduction in use of FM and FO in aquafeed formulation (Jannathulla et al., 2019; Kok et al., 2020). Alternative novel feed ingredients need to be cost effective, available in sufficient quantities year-round, nutritious, free from contaminants and other undesirable compounds like antinutrients, and able to support the desired nutritional traits of seafood such as highly unsaturated omega-3 fatty acids (HUFAs) (Jannathulla et al., 2019). In general, significant growth enhancement, immunity and diseases survival were found on the use of plant-enriched diets in fish feed (Reverter et al., 2021).

There is no industrial food production that is totally sustainable. As an example, the production of soybean is not truly sustainable because of deforestation and the use of non-renewable phosphorous sources (Ytrestøyl et al., 2015). Other concerns regarding environmental sustainability, conflict with the demand for direct human consumption, and the presence of anti-nutrient factors, especially saponins and trypsin inhibitor that challenge function and health of the gut of fish have been raising with the use of soybean as fish feed (Bjørgen et al., 2020; Jannathulla et al., 2019; Ytrestøyl et al., 2015). These scenarios as well as the preference towards focusing on locally available ingredients to reduce the expense of the transport cost, have forced aquaculture nutritionists to develop sustainable novel feed ingredients based on non-food resources like insect-meals, macro- and microalgae and single-cell micro-organisms like bacteria, fungi and yeast. These novel ingredients are considered as most promising protein sources due to their renewable and fast-growing characteristics (Jannathulla et al., 2019).

Microbial ingredients, particularly yeasts, have the ability to convert low-value non-food biomass from forestry and agricultural industry into high-value feed resources, thus they can serve as a high-quality protein source for aquaculture fish production, though the production cost is too high at present (Solberg et al., 2021; Øverland et al., 2013). Yeasts can improve the immune and antioxidative responses, enhance growth and survival rate of fish (Bharathi et al., 2019). The crude protein content of yeast dry matter is high which usually varies between 40% and 55%, and in general yeast amino-acid composition is

comparable with fish meal, except for the low methionine contents (Øverland & Skrede, 2017). Study by Øverland group (Øverland et al., 2013) showed that 40% of Atlantic salmon protein feed meal can be replaced by yeast proteins. Additionally, yeasts have less environmental impacts due to their limited dependence on arable land and water use (Couture et al., 2019; Øverland et al., 2013). Yeast cell wall components, like prebiotic β-glucans and Mannan oligosaccharide (MOS), have shown to influence the immunostimulant effect in fish by promoting gut health and growth, immune response and protection against infectious pathogens, (Talpur et al., 2014). Other important components in fish feed are plant derived bio-active compounds as a functional feed supplements that are believed to have immunostimulant effects.

Researchers have shown interest in polyphenols, plant derived compounds as a functional feed supplements because they can induce physiological functions and health performance of fish over the normal feed additives (Bharathi et al., 2019). Functional feeds refer to ingredients which are supplemented in small quantities to the feed to enhance the health benefits of the organism beyond the basic requirements of the fish for normal growth and health (Martin & Król, 2017). Polyphenol possess a variety of biological properties such as antioxidant, anti-inflammatory, antibacterial, anti-protozoal, and antiviral activities (Marcotullio et al., 2018), and they play a vital role in the prevention and cure of various diseases. In addition, the use of approved antibiotics and chemotherapeutants as a treatment methods in aquatic animals are more often neither effective nor environment-friendly (Kiron, 2012). They negatively affect the immune system of aquatic animals and are also a global health threat due to emergence of anti-biotic-resistant bacteria (Reverter et al., 2021), thus it is preferable to avoid treating fish diseases with antibacterial drugs. Therefore, unless the use of antibiotics and chemotherapeutics in some circumstances are a must, aquaculture are advised to use other prophylactic approaches such as functional feed additives (Assefa & Abunna, 2018). Hence, plant extracts for diseases control in aquaculture has arisen interest as an alternative to antibiotics and chemical treatments (Reverter et al., 2014).

1.5. Plant Extracted Bioactive Compounds as a Functional Feeds

Plant derivatives like phytobiotic or phytogenic compounds are heterogeneous feed additives originated from different part of the plants and their addition to the feed improves the growth and health performance of the fish (Bharathi et al., 2019). Bioactive compounds derived from by-product of woody biomass (low commercial importance) are promising sources of functional food ingredients and food additives, (Jablonsky et al., 2017). Some beneficial effects of these phenolic substances have been observed. A previous study, for instance, have shown that such compounds can lower the risk of cancer or cardiovascular diseases in higher vertebrates due to their anti-oxidant properties (Jablonsky et al., 2017).

Compounds derived from plant phenols attract intense intentions as dietary antioxidant supplementation and food preservation (Halliwell et al., 1995). Antioxidants are well known in controlling stress conditions and prevention of oxidation reactions, which cause tissue damage in living organisms. In humans, antioxidants protect the body against damage by reactive oxygen species (ROS), thus delay or prevent oxidation, as example, they inhibit lipid peroxidation and in addition, they might raise the levels of endogenous antioxidant defences (Halliwell et al., 1995). According to (Kähkönen et al., 1999) a wide variety of antioxidants, especially phenolic antioxidants, are abundant in knots, wood chips and barks of spruce tree (*Picea abies*). Though, extracts from woody biomass have antioxidant properties, the knot extracts from several trees have strong antioxidant effects (Pietarinen et al., 2006). In the knot wood of spruce tree, 30% of polyphenols can be extracted (Pietarinen et al., 2006).

In Norway, forests occupy about 38% of the country's total surface area and 47% of this is Norway spruce (Hu et al., 2018). Norway spruce knot wood is a rich source of lignans and other bioactive compounds (Mansikkala et al., 2020). Lignans, complex polyphenolic compounds, are strong antioxidants, and they are used as wound healing and anti-fungal effects, as well as hormonal activity in trees as a natural defence mechanism (Korkina, 2007; Mansikkala et al., 2020). The high molecular weight of lignans provide rigidity to the cell wall structure of plants and these properties make lignans important in improving pellet quality and durability (Colombo et al., 2020). In China, lignan bio-active compounds from *Taxus yunnanensis* have been used in traditional medicine for treatment of cancer,

diabetic disease and kidney problems (Tezuka et al., 2011). Hydroxymatairesinol (HMR) are plant dominant lignans and they are abundantly (17%) found in Norway spruce (Mansikkala et al., 2020). The antioxidant and antitumor properties of HMR were tested *in vitro* study in rats, dogs, and humans, and in all cases significant inhibitory effect on tumor growth and effective antioxidant result were observed (Kangas et al., 2002). The use of extracted HMR from *Picea abies* as a dietary supplement in a feed of a high fat diet mice improved body weight, and fat and sugar metabolism (Biasiotto et al., 2018). In another *in vitro* study, addition of extracted bio-active lignan compounds on Cytochromes P450 (CYPs) showed strong inhibition effect (Tezuka et al., 2011). CYPs are enzymes that oxidize metabolism of drugs in human liver microsomes, hence, reduce drugs bioavailability and influences the residence time of toxic chemicals in the body (Tezuka et al., 2011). Furthermore, it was also shown that lignan extracted compounds can act as a potent antiproliferative effects on human breast cancer cell studies (Hafezi et al., 2020). Though, there are not so many studies that have been performed on fish, however, hydrolyzed lignin improved feed quality and fish growth performance in Atlantic salmon (Colombo et al., 2020).

Natural polyphenols which act as important substances in the field of nutrition, health and medicine are also found in softwood barks (Jablonsky et al., 2017). Bark extractives can be classified as hydrophilic and lipophilic components and the content of the extractable components obtained from the bark of individual tree species differs significantly (Jablonsky et al., 2017). The lipophilic components are mainly fats, waxes, terpenoids and higher aliphatic alcohols, and they can be extracted by non-polar solvents like diethyl ether and dichloromethane (Jablonsky et al., 2017). Hydrophilic components are aromatic compounds and can be extracted by water or polar solvents such as acetone and ethanol (Jablonsky et al., 2017).

In addition, stilbenes are polyphenolic compounds present in the bark parts of several plant families. The potential beneficial health effects of stilbene phenolic compounds are attracting a lot of interests. As an example, resveratrol is the most extensively studied stilbene due to its biological properties including its anti-inflammatory and anti-cancer effects (Maru et al., 2014). Stilbenes are considered as phytoalexins, compounds that are synthesized in plant tissues in response to disease, injury, or abiotic stress such as UV irradiation (Maru et al., 2014). It has been also shown that stilbenes can protect plants

against oxidative stress, inhibit the growth of competing plants and deter herbivorous (Hammerbacher et al., 2011).Though, the biosynthesis of stilbene is well known in plants like grape, peanut and pine, but very little is known about the biosynthesis and ecological role of stilbenes in spruce (Hammerbacher et al., 2011). Astringin and isorhapontin are the most abundant stilbenes presented in the softwood bark of spruce tree (Hammerbacher et al., 2011). These bio-active compounds have nutraceutical potential and capable of producing protective effects against chronic and degenerative diseases (Jablonsky et al., 2019).

In Norway in 2020, more than 50 million out of 300 million smolts transferred to sea died prior to harvest (Sommerset et al., 2021), which is a significant economic loss for producers. Therefore, the antioxidant properties of the bio-active compounds may enhance fish health and consequently fish welfare and production profits. Furthermore, it is environmentally and economically sustainable to use biomass waste from forest or agricultural industry as fish feed ingredients. Hence, continues efforts are being made in extracting and purifying of these substances.

1.6. Gut Health in Atlantic salmon

The gastrointestinal tract (GIT) is an organ with multiple functions in immunity and it is the main site where environmental microorganisms and antigens interact with the host (Montalto et al., 2009). The gut microbiota in GIT is essential for intestinal development, homeostasis and protection against pathogenic challenges, and play a fundamental role in the maturation of the host's innate and adaptive immune responses (Koch et al., 2021; Montalto et al., 2009). The gut mucosa is rich in immune cells such as B and T lymphocytes, macrophages and granulocytes, and can trigger local responses (Press & Evensen, 1999). Gut-associated lymphoid tissue (GALT) is one among the other three main mucosa-associated lymphoid tissues (MALTs) (skin-associated lymphoid tissue, gill-associated lymphoid tissue and nasopharynx-associated lymphoid tissue) in teleost that are constituted of both innate and adaptive immune cells and molecules that work together to maintain homeostasis at the mucosa (Salinas, 2015). GALT is the critical protective immune system in the GIT, and it helps in defending against pathogenic stimuli that penetrate the luminal mechanical barrier, as example, many of the chemokines secreted

in GALT participate in the regulation of intestinal immune responses (Caballero, 2005; Neu, 2008).

Distal intestine of Atlantic salmon is considered to play an important role in immune-related gut functions and functional ingredients are claimed to have beneficial effects within the intestine (Wang et al., 2020). Functional feed additives could directly stimulate the innate immune system or enhancing the growth of gut microbiota (Dawood et al., 2018). These additives boost the natural defence of a fish by stimulating the immune system or increasing immune resistance, promote the proliferation of gut microflora and enhance the specific or nonspecific defense mechanisms (Assefa & Abunna, 2018).

Gut health problems like intestinal inflammation and lipid malabsorption, and consequently fish susceptibility to various diseases, are observed in association with increased use of plant-based salmon feed (Li et al., 2019). Studies have shown that high dietary inclusion of plant-based protein sources such as soybean meal and pea protein concentrate in diets for farmed Atlantic salmon cause proliferative or inflammatory conditions in the intestinal mucosa. These antinutrients challenge function and health of the gut of fish (Agboola et al., 2021; Gajardo et al., 2017; Penn et al., 2011).

Considering the above background, the present study aimed to investigate the potential beneficial health effects of two locally available Norway spruce extracts (NSE1 and NSE2) and increase the knowledge of these compounds as novel functional feed ingredients, and to characterize modulatory properties in the gut of Atlantic salmon, which, as MALT, is a key organ in coordinating the immune response, and may help to achieve improved overall fish health.

2. Hypothesis

Dietary inclusion of bio-active compounds from Norway spruce woody biomass (NSE1 and NSE2) in novel functional aquafeeds, modulate gut health through a differentiated expression of immune-related genes, and thereby improving immune function and robustness of Atlantic salmon.

3. Objectives

3.1. General objective

To characterize the immunomodulatory effects of NSE1 and NSE2 from Norway spruce woody biomass as bio-active compounds in functional feeds on gut health of Atlantic salmon.

3.2. Specific objective

- To develop functional feeds for Atlantic salmon based on a well-balanced feed formulation and inclusion of NSE1 and NSE2 from Norway spruce woody biomass as bio-active compounds.
- To assess the effects of the functional feed on gene expression of immune-related biomarkers in the hindgut of Atlantic salmon.
- To correlate the gene expression of the target biomarkers in the hindgut of Atlantic salmon fed a plant-based commercial diet (control diet) with different inclusion levels of NSE1 and NSE2.

4. Materials and Methods

4.1. Diets

In this study, eight diets were used (Table 1), these were a plant-based commercial diet (control diet) and seven experimental diets with different inclusion of bioactive compounds (MacroGard®, NSE1 and NSE2). The diets were produced at the Centre for Feed Technology (Norwegian University of Life Sciences) in Ås, Norway.

Table 1: Description of the diets used in the study.

Diet number	Diet code	Description (g/kg)	Inclusion (%)
Diet 1	D1	Plant-based diet (PBD), control	0%
Diet 2	D2	PBD + MacroGard (2g/kg)	0.2 %
Diet 3	D3	PBD + NSE1 (0.2g/kg)	0.02 %
Diet 4	D4	PBD + NSE1(1g/kg)	0.1 %
Diet 5	D5	PBD + NSE1 (2g/kg)	0.2 %
Diet 6	D6	PBD + NSE2 (0.2g/kg)	0.02 %
Diet 7	D7	PBD + NSE2 (1g/kg)	0.1 %
Diet 8	D8	PBD + NSE2 (2g/kg)	0.2 %

The control diet was used as the basis for the production of all other diets (Table 2). This diet was formulated to meet or exceed the nutrient requirements for Atlantic salmon (NRC, 2011). The feed pellets were produced using P35A pasta machine. Each feed ingredient was weighed and mixed thoroughly with a professional small-scale commercial paddle mixer for four minutes, prior to the adding of water, gelatin, and fish oil. Gelatin was dissolved in 1500 ml of cold water and heated until 55℃. The solution was added to the dry ingredients and stirred in spiral mixer (Prismafood Solutions, Italy).

Cold pelleting was carried out using pasta machine (ITALGI P35A, Italy) to produce pellets of 2.5mm of size. Approximately 600ml of warm water was added for each diet to obtain the optimal consistency. After pellet formation, they were dried at 60℃until the moisture content below 10%. The feeds were then transferred into labelled bags and stored at 4℃ until used.

Table 2: Formulation of plant-based commercial diet (PBD).

Component	**g kg^{-1}**	**Amino acids**	**g kg^{-1}**
Fishmeal[a]	260	Ala	21.7
Soy protein concentrate[b]	245	Arg	27.6
Wheat gluten[c]	100	Asp	36.9
Pre-gelatinized potato starch	118	Cys	4.6
Gelatin	60	Glu	83.3
Fish Oil	160	Gly	28.6
Monocalcium phosphate	15	His	9.9
Vitamin/Mineral premixed[d]	5.0	Ile	16.1
L-lysine	4.0	Leu	28.7
DL-Methionine	1.5	Lys	28.6
Choline chloride	1.0	Met	9.4
Yttrium oxide	0.1	Phe	18.3
Calculated nutrient content		Pro	30.3
Dry matter	932.2	Ser	18.3
Crude protein	483.3	Thr	14.9
Crude lipids	216.4	Tyr	11.4
GE (MJ kg^{-1})	17.7	Val	16.9
Total P	9.9		
Calcium	14.5	**SUM**	405.9

[a]LT fishmeal, Norsildmel, Egersund, Norway; [b]Triple A AS, Honsyld, Denmark; [c]Amilina AB, Panevezys, Lithuania; [d]Vit/min premix, NMBU Fish Premix Basic, Trouw Nutrition, LA Putten, The Netherlands. Per kg of feed**:** Vitamin A 2500 IU; Vitamin D2 1500 IU; Vitamin E (all-rac-alpha-tocopheryl acetate) 200 IU; Vitamin K3 (Menadione nicotinamide bisulfite) 10.05 mg; Vitamin B1 (Thiamine mononitrate) 15.0 mg; Vitamin B2 (Riboflavin) 25.0 mg; Calcium D-pantothenate 40.0 mg; Niacinamide 75.05 mg; Vitamine B6 (pyridoxine hydrochloride) 15.0 mg; Folic acid 5.0 mg; Vitamin B12 (cyanocobalamin) 25.0 mg; Vitamin C 125.0 mg; Biotin 275.0 mg; Calcium iodate, anhydrous, Iodine 30.0 mg; Manganese (II) oxide, Manganese 15.0 mg; Zinc oxide, Zinc 105.0 mg. Carrier: Calcium carbonate.

4.2. Experimental design and fish trails

The fish experiment was conducted at the Center for Fish Research at the Norwegian University of Life Sciences (NMBU, Ås, Norway). The experimental procedures were performed according to the Norwegian Animal Research Authority's established guidelines (Directive 2010/637EU) and Norway (FOR-2015-06-18-761).

Atlantic salmon eggs were obtained from AquaGen AS from Sjøanlegg, Norway and hatched at 5 °C. All fish were fed the same commercial salmon feed and kept at 24 hours light and average temperature of 14 °C prior to the start of the experiment.

Fish experiment was conducted for four weeks (from 25 October 2021 to 25 November 2021). A total of 640 parr with average weight of 28.4 g ± 0.05, were randomly distributed into 16 fiberglass tanks (300 L capacity each). Each tank was stocked with 40 fish and each dietary treatment was fed to duplicate tanks (Figure 1).

Fish were kept at 24 hours light and water quality parameters were checked and recorded daily. The average temperature was kept at 14 °C and flow rate was adjusted to maintain the oxygen saturation above 75% and dissolved oxygen concentration above 7.0 mg L^{-1}. Feed was weighed and placed on an automatic feeder each day and fish were fed once a day for a period of six hours (from 6:00 AM to 12:00 PM). Uneaten feed was collected on a screen for each tank and recovered within one hour after feeding and weighed as described by Helland et al. (1996). From that, the amount of feed supplied to the fish for the next day was adjusted per tank. Each tank was checked daily for abnormal behavior or mortality.

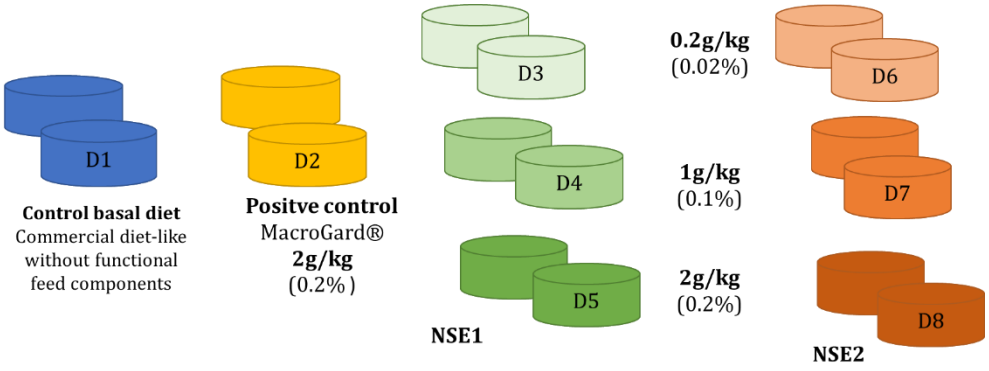

Figure 1. Experimental design of the experiment for Atlantic salmon with 40 fish per tank (duplicate tanks per diet). Feeding period: 4 weeks.

4.3. Sampling fish tissue and measurement of physical parameters

Six fish from each tank were randomly sampled, sedated with metacaine (MS-333; 15 mg L^{-1}) and euthanized with a sharp blow to the head. Fish weight and length was measured and recorded (total of 96 fish for all 16 tanks). Then, fish was opened with sterile scalpel and the intestine was removed and cleared of any adipose tissue. Thereafter, distal intestine (DI) was open longitudinally and one section (0.5-1.00 cm) was collected and preserved in RNAlater®, stored overnight at 4°C, and then kept at -80°C until RNA isolation.

The remaining fish in the tanks (34 fish per tank) were sedated with the same method described above and weighed in bulk.

4.4. RNA isolation in distal intestinal samples

Distal intestine samples (eight per dietary group from duplicated tanks) were thawed on ice for about one hour. Between 20-40 mg of each sample was taken into 2ml tube together with Qiazol® (900 µL). Then, the samples were disrupted and homogenized (twice) using metal beads in a TissueLyser II (Qiagen) for 2 minutes at 20 Hz. Each sample was carefully pipetted into a new Eppendorf tube and incubated at room temperature for 5 minutes. After, 180 µL chloroform was added to each sample and shook for 20 seconds. Then, the samples were centrifuged at 13800 x g for 15 minutes (4°C). Thereafter, 450 µL of the upper phase (containing RNA and water) of each sample was transferred to a new Eppendorf tube. Subsequently, 450 µL isopropanol was added to each sample and, after mixing, samples were incubated at room temperature for 10 minutes. The samples were then centrifuged again at 13800 x g for 15 minutes (4°C) and the supernatants were removed without touching the pellets. Samples were centrifuged for 3 min and the excess liquid were removed. Later, 70 % ETOH (200 µL) was added, and all samples were centrifuged at 13800 x g for 10 minutes (4°C). Supernatants were removed and again centrifuged for 3 minutes. Any excess liquid was removed. Tubes were laid down on side with open lid to air dry for 10 minutes to dry the RNA pellet. Following drying, the RNA pellets were dissolved in 40 µL RNAse free H_2O, incubated at 55°C for 10 minutes, and then kept on ice.

The concentration and purity of RNA samples were measured by NanoDrop TM 8000 spectrophotometer (Nanodrop Technologies), and the RNA integrity was checked on a Bioanalyzer (Agilent 2100). Finally, 64 RNA samples for 64 fish were stored at -80 °C until further analysis.

4.5. Complementary DNA synthesis

The complementary DNA synthesis (cDNA) was performed using AfinityScript qPCR cDNA Synthesis Kit, according to the kit manufacturer's protocol. Briefly, RNA samples were diluted to a final concentration of 500ng mL^{-1}. A mix with all the kit components was made enough to all reactions (Table 3). Subsequently, 14 µL of the mixed reagents and 6 µL from RNA sample were loaded into an individual reaction tube. Finally, to allow cDNA synthesis, samples went through a thermal cycle (Table 4) on GeneAmp PCR system 9700 (ThermoFisher).

Table 3. Component and volume of the components for cDNA synthesis per each reaction.

Component	**Volume (µL)**
First strand master mix	10
Random primers	3
AffinityScript Reverse transcriptase	1
RNA template	6
Total volume	20

Table 4. Thermo Cycle for cDNA synthesis.

Step	**Temperature (°C)**	**Time (m)**
Allowing primer Annealing	25	5
Allowing cDNA synthesis	42	45
Terminating cDNA synthesis reaction	95	5

Synthesized cDNA was diluted to 1:10, and then, the samples were stored at -20 °C until forward analysis.

4.6. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR of selected genes (Table 5) was subsequently performed on cDNA samples (8 per dietary group from duplicated tanks) by using LightCycler® 480 SYBR Green I Master and following supplier recommendations. Briefly, each reaction was composed by 10 µL of SYBR Green, 1.5 µL of PCR primers (10nM each), 5.5 µL water and 3 µL of diluted cDNA, a total of 20 µL (table 6). A mix was prepared enough to all the samples and additional controls.

Reactions were performed in duplicates in a white multiplate 96 well PCR plates (Bio-Rad white Multiwell Plate 96). For each primer pair two wells with no template were included (no template control), where cDNA template was replaced by 3 µL of PCR grade water. The plate was then sealed, briefly centrifuged, and then loaded in CFX96 Touch Well Real-Time PCR machine to perform RT-qPCR with 40 cycles (Table 7). A melting curve was added to provide the accurate identification of single amplified cDNA sample. Gene expression was normalized to a housekeeping elongation factor 1 alpha.

Table 5. Genes evaluated by qPCR

Gene	Forward Primer	Reverse primer	Melting Temp (°C)
EFα1 (Elongation factor 1 alpha)	GCAGTGGCAGTGTGATTTCG	GTAGATCAGATGGCCGGTGG	61
IL-1β (Interleukin 1 beta)	AGGACAAGGACCTGCTCAACT	CCGACTCCAACTCCAACACTA	64
IL-8 (Interleukin-8)	ATTGAGACGGAAAGCAGACG	CGCTGACATCCAGACAAATCT	63
TGFb (Transforming growth factor beta)	AGTTGCCTTGTGATTGTGGGA	CTCTTCAGTAGTGGTTTGTCG	63
iNOS (Inducible nitric oxide synthase)	AGGTGCTGAATGTGTTGCAC	GTATTCTCCTGCCTGGGTGA	64
SOD (Superoxide dismutase)	CCACGTCCATGCCTTTGG	TCAGCTGCTGCAGTCACGTT	64
IFNγ (Interferon gamma)	GTGAGCGGAGGGTGTGGATG	CAGGAAGTAGTGTTTCTGGGTC	64
Prx2 (Peroxiredoxin 2)	TCACTGACAAACACGGAGAAG	CTGCTGCTTGGAGAAGAAGT	64

Table 6. Components for preparation of PCR Mix for one 20µL reaction

Component per reaction	Volume (µL)
LightCycler® 480 SYBR Green I Master	10
Primers (10nm) (Forward + Reverse)	1.5
DNA Template	3
Water, PCR grade	5.5
Total	20

Table 7. Thermo cycle for RT-qPCR

Step	Duration	Temperature (°C)
Activation of DNA-polymerase	7 min	95
Amplification of the target DNA (40x)	10 sec	95
Primer Annealing (40x)	15 sec	variable
Elongation (40x)	15 sec	72
Melting curve	5 sec -	95
	1 min	65
	variable	97
Cooling	10 sec	40

4.7 Data analysis

Microsoft Excel 2013 was used for graphical presentation and calculation of means and standard error of the mean (SEM). Moreover, after Shapiro's test, log transformation (ln) in all qPCR data was performed and multiple t tests was 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 when p-value < 0.05.

Correlation coefficients among diets were calculated by Corrplot (v.0.92) in R (Wei & Simko, 2021) . The correlation was considered significant when p value < 0.01 (degrees of freedom=5).

5. Results

5.1 Health status and physical parameters

During the fish trial, no mortality was recorded, and no significant differences in length and final body weight were observed between the dietary groups (Figure 2).

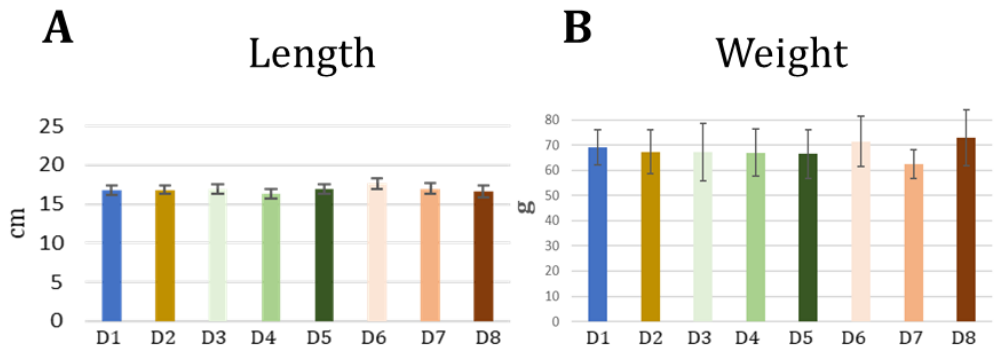

Figure 2. Physical parameters. Length (A). Final body weight (B). Control: D1; MacroGard: D2; Diet 3: D3; Diet 4: D4; Diet 5: D5; Diet 6: D6; Diet 7: D7; Diet 8: D8. Each bar=6 fish.

5.2 Gene expression of immune-related biomarkers

The group fed MacroGard (D2) showed an up-regulation of *IL-1β* ($p<0.005$) and *Prx2* ($p<0.020$), compared to Control diet (D1) (Figures 3/4 A and E). No significant differences were detected within the other genes.

The inclusion of compounds from spruce tree showed that the highest inclusion level of NSE1 D5 was able to induce a modulation of immune-related genes (Figure 3 A), with a significant up-regulation of *IL-1β* ($p<0.001$) compared to control diet D1. Similarly, Diet 6 also showed this same trend (Figure 4 A), with an increase in *IL-1β* expression ($p<0.041$).

In the group fed Diet 8, a different trend in the expression of immune-related genes was observed. In this group, the down-regulation in the expression of *IFNγ* ($p<0.007$), IL-8 ($p<0.001$) and *iNOS* ($p<0.010$) were detected compared to control diet (D1).

Regarding to the other genes (*TGFβ*, *Prx2* and *SOD*), no significant differences were detected between the different diets with spruce compounds at all inclusion levels.

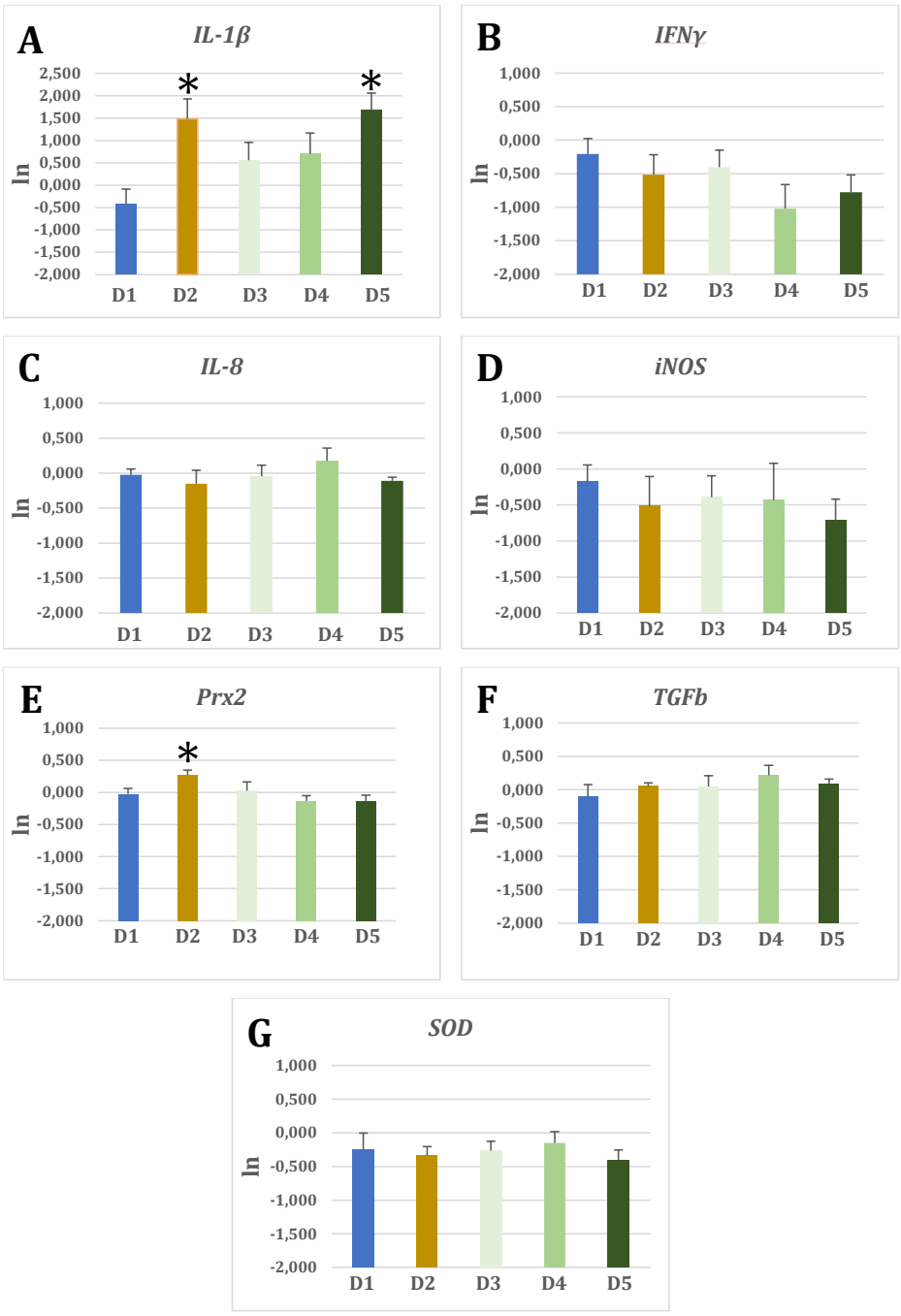

Figure 3. Gene expression (by qPCR) of immune-related biomarkers (A: Interleukin 1 beta, B: Interferon gamma, C: Interleukin 8, D: Inducible nitric oxide synthase, E: Peroxiredoxin 2, F: Transforming growth factor beta G: Superoxide dismutase) in DI from Atlantic salmon fed diets with three different inclusion levels of NSE1 (D3, D4, and D5). D1: Control diet. D2: control diet with MacroGard. Each bar= 6 fish. *: significant difference, compared to the control diet (p value <0.05).

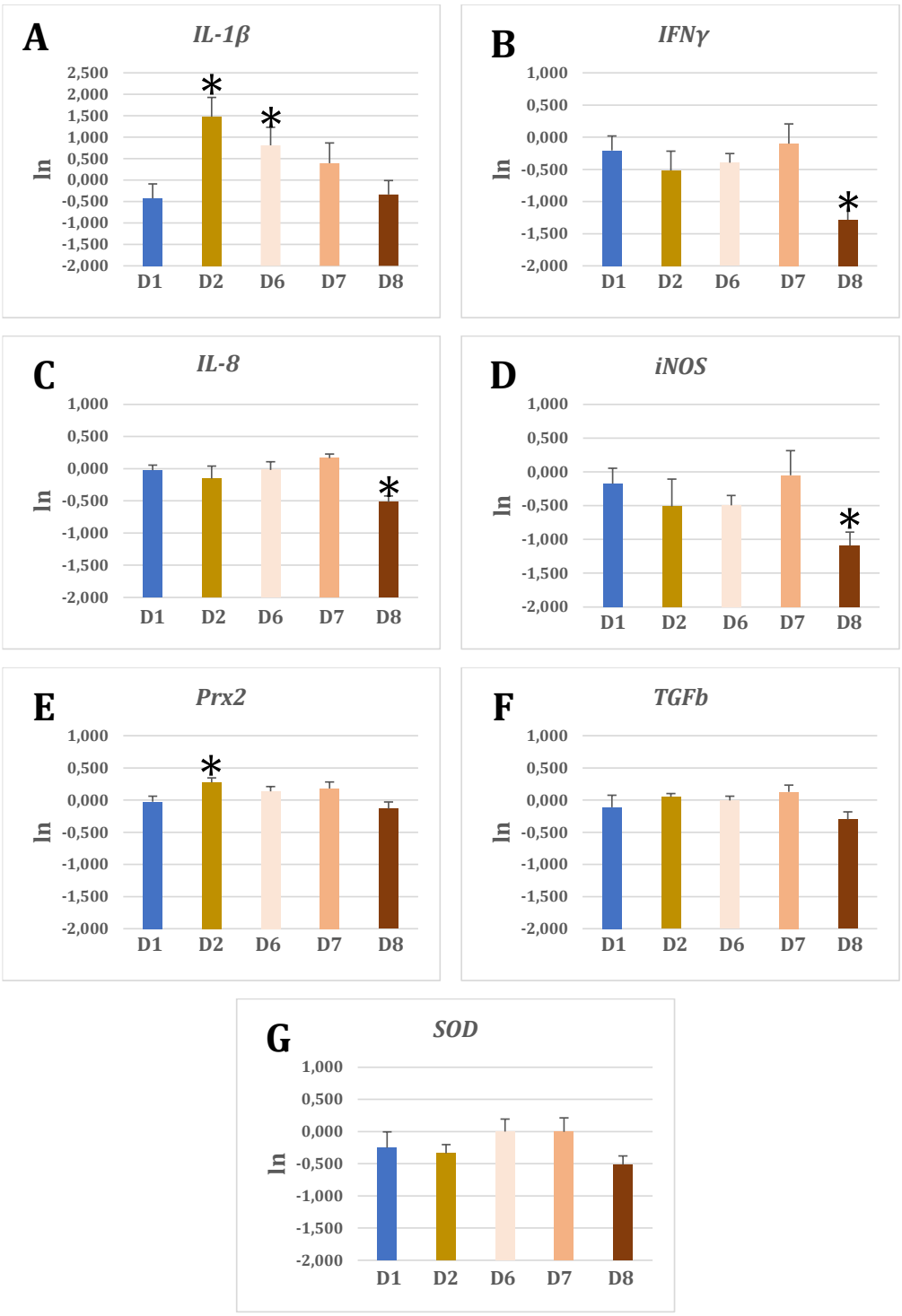

Figure 4. Gene expression (by qPCR) of immune-related biomarkers (A: Interleukin 1 beta, B: Interferon gamma, C: Interleukin 8, D: Inducible nitric oxide synthase, E: Peroxiredoxin 2, F: Transforming growth factor beta, G: Superoxide dismutase) in DI from Atlantic salmon fed diets with three different inclusion levels of NSE2 (D6, D7 and D8). D1: control diet. D2: Control diet with MacroGard. Each bar= 6 fish. *: significant difference, compared to the control diet (p value <0.05).

5.3. Correlations

Statistical analysis showed that NSE1 D3 and NSE2 D7 diets were positively correlated with almost all other diets except with control diet (D1: -0.38 and -0.26), and NSE2 D8 (D8: 0.72 and 0.76). Moreover, both MacroGard (D2), NSE1 D5 and NSE2 D6 were correlated with the other diets, except with control (D1: -0.52, -0.57 and -0.49, respectively), NSE1 D4 (D4: 0.79, 0.86 and 0.84, respectively) and NSE2 D8 (D8: 0.61, 0.59 and 0.72, respectively).

Interestingly, neither the control diet nor diet 8 showed significant correlations. In addition, no significant negative correlations were detected.

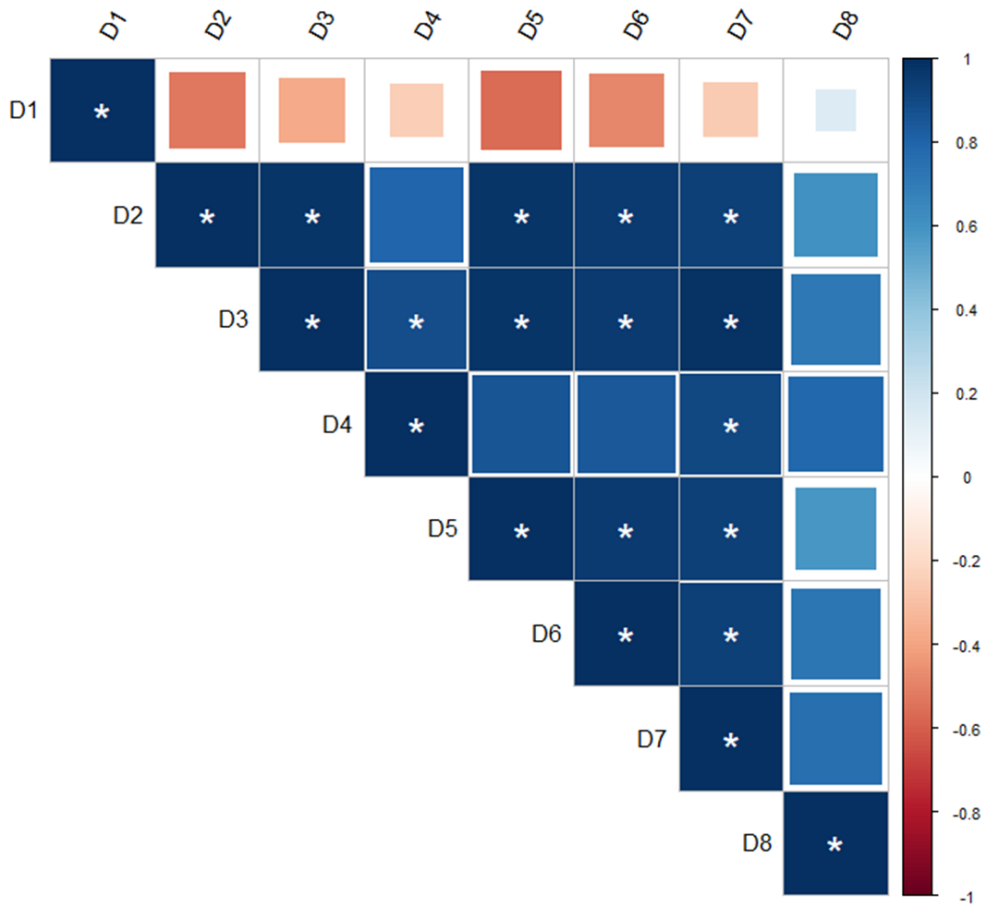

Figure 5. Correlation analysis among dietary groups using the gene expression data from target immune-related biomarkers. The prefix 'D' refers for Diet. All the parameters that are significantly correlated (p-value < 0.01) are denoted by '*'. Degrees of freedom = 5. In blue: Positive correlation. In red: Negative correlation.

6. Discussion

Aquaculture is an important economic industry helping to solve the growing demand of the world´s population for more high-quality proteins. Finfish aquaculture industry has progressively caught up with capture fisheries and is expected to overtake it in the near future. Furthermore, advances in fish farming techniques and the nutritional and health promoting value of fish products will probably drive an even higher acceleration of farmed fish production in the future (FAO, 2020; FAO, 2021)

However, the rapid growth of this sector has raised concerns about the environmental impact and sustainability of fish farming. As an example, one argument against the sustainability of aquaculture is the dependence of fish feed industry on fish meal and fish oil, and the effects on the environment and wild fish stocks. Furthermore, with the increasing use of plant ingredients in fish feeds, deforestation concerns and the need for phosphorus, an essential nutrient, is increasing. Thus, it is believed that phosphorus will become a limited resource for food production soon (Ytrestøyl et al., 2015). Besides, the intensification of salmon farming has been facing many constraints and challenges, which cause large economic losses. Among these, multi-stressor conditions such as handling, infectious diseases, algal blooms, and water quality, are the most serious challenges that causes huge loss of production (Assefa & Abunna, 2018; Kiron, 2012; Sommerset et al., 2021).

Research efforts have increased during the last 20 years to understand the link between fish immune response, nutrition and resistance to diseases (Buchmann & Secombes, 2022; Kiron, 2012). Teleost fish have both innate and adaptive responses, which operates in a coordinated interaction between them and the environment. Among important immune organs, DI is a crucial site for the modulation of the host's response. Distal intestine, as a MALT (Cesta, 2006; Jung-Schroers et al., 2018) has a key role in the modulation of both local and systemic physiological responses by regulating pro- and anti-inflammatory cytokines, effector molecules, and coordinating antigen-presenting cells (Morales-Lange et al., 2022). Bioactive compounds in functional feeds (e.g., α-glucan, β-glucan, α-mannan, and nucleic acids) have the potential to improve fish health and welfare, acting as immuno-modulatory compounds (Kiron, 2012; Talpur et al., 2014).

These ingredients can enhance or suppress the immune response, reducing stress, protecting from pathogens, and increasing the resistance of fish to infectious diseases.

β-glucans, which are non-digestible carbohydrates that as a feed additive can act as a prebiotics, enhance the innate immune responses and, consequently, improving gut and overall fish health and growth performance. Hence, it is common to use them as an immunomodulator in the aquaculture industry (Dawood et al., 2018; Koch et al., 2021). In the current study, a dietary group had a 0.2% inclusion level of a commercially available β-glucan (MacroGard®), which showed a significant up-regulation of IL-1β and Prx2 compared with the control. This differential gene expression could be an indication of the activation of immune cells related to innate responses in distal intestine, as reported previously (Schmitt et al., 2015). Moreover, in an experiment with Nile tilapia (*Oreochromis niloticus*) fed a diet containing β-glucan, significantly higher activity of intestinal lysozymes was observed, thus indicating improved humoral innate immune responses and the tilapia's resistance to bacterial diseases (*Aeromonas sobria* and *Streptococcus agalactiae*) (Koch et al., 2021). In another study, a significant expression of IL-1β was observed in intestinal epithelial cells of rainbow trout in response to β-glucan -supplemented diets (Schmitt et al., 2015).

IL-1β is a pro-inflammatory cytokine with a key role in the production of other pro-inflammatory cytokines (e.g., IL-8 and IL-6) and in the activation of lymphocytes and phagocytic cells, which can increase fish resistance to bacterial infection (Sakai et al., 2021). In rainbow trout, an increase in the number of phagocytes that migrate into the peritoneal cavity were observed with the increasing expression of IL-1β (Zou & Secombes, 2016). Moreover, IL-1β also play a key role on the production of anti-inflammatory cytokines such as IL-10 and TGF-β (Buchmann & Secombes, 2022; Khansari et al., 2017), contributing then the immune homeostasis.

In fish, further studies have confirmed that an increase in the expression and release of Prx2 was observed after the increase of IL-1β in a study of viral infection (Valero et al., 2015). Prx2, also known as natural killer enhancing factor (NKEF), is an antioxidant enzyme from the family peroxiredoxins and it is important for the control of the cellular redox potential and facilitate tissue repair after damage. It can also act as modulators of inflammation in pathogen infection as observed in European sea bass (*Dicentrarchus*

labrax), where viral loads of nervous necrosis virus in the brain were decreased after intramuscular injection of Prx2 (Valero et al., 2015).

In this study, the impact of bio-active compounds NSE1 and NSE2 (from Norway spruce tree) on the gene expression of immune biomarkers in distal intestine were evaluated. Interestingly, both higher inclusion of NSE1 and lower inclusion of NSE2 induced upregulation of IL-1β (compared to control diet), which, similar to MacroGard®, suggests the activation of immune cells and the activation of inflammatory processes.

Contrary to what was described above, the high inclusion of NSE2 induced a downregulation of several immune-related biomarkers (IL-8, IFNγ and iNOS) compared to control diet. Similar phenomenon was observed in grass carp where the up-regulation of *IL-1β, IL-8* and *iNOS,* induced by Lipopolysaccharide, were inhibited by TGF-β (which have immunosuppressive functions) (Zou & Secombes, 2016). However, there was no significant difference in the expression of TGF-β in this study. Hence, the justification for the down regulation of IFNγ, IL-8 and iNOS in this research is not clear. Nevertheless, by damping the inflammatory response, NSE2 can contribute for the immune regulation and homeostasis in the gut, since long-period inflammation may cause over-stimulation or exhaustion of the immune system, tissue damage and at last, immunosuppression (Koch et al., 2021)

Both IL-8 and IFNγ are pro-inflammatory cytokines. IL-8, also known as CXCL-8, is a good indicator of the occurrence of inflammatory processes, and it is known for its importance in the regulation of inflammatory responses during bacterial and parasitic infections (Carriero et al., 2020). IL-8 is a chemokine that exerts a chemoattractant effect on neutrophils and mononuclear leukocytes and induces neutrophil infiltration, therefore, it is highly involved in the initiation of the inflammatory process (Carriero et al., 2020).

IFNγ is a powerful pro-inflammatory cytokine involved in bactericidal activity and contributes to cell-mediated immunity by activating macrophages against pathogens during both innate and adaptive immune responses, and it is also able to increase production of nitric oxide (Buchmann & Secombes, 2022; Xu et al., 2012; Zou & Secombes, 2016). Chronic granulomatous disease (genetic disorder), which is chronic hereditary immune disorder can be treated by IFNγ (Samuel, 2001). However, studies in higher

vertebrates have also shown that higher expression of IFN- can lead to immunopathology (Xu et al., 2012).

Inducible nitric oxide synthase (iNOS) is one of three key isoforms that is expressed by a variety of cells like T cells, macrophages, and dendritic cells. After induction by cytokines or other stimuli, it can regulate the differentiation and function of immune cells (Panettieri et al., 2019; Xue et al., 2018). iNOS produces nitric oxide (NO), which is a reactive species mediator that protects the host against various pathogens, including bacteria, parasites, and viruses. NO is generally considered detrimental due to the accumulation of unstable reactive nitrogen species (RNS), including peroxynitrite, which is known as oxidative stressor and is harmful to the cells. Increased RNS can also be removed by antioxidant enzymes such as Prx2 (Morales-Lange et al., 2018).

Altogether, NSE1 and NSE2 proven to induce different responses in salmon, as well as different responses with different inclusion level. Not only the extraction and fractioning process of woody compounds, industrial handling can also influence the bioactive properties of Norway spruce extracts (Jyske et al., 2022).

It has already been described that differences in the proportion and level of bio-active compounds could differentially modulate how sensible Atlantic salmon is to the compounds and how they change their physiological response at the intestinal level (Morales-Lange et al., 2022).

NSE may have application in the salmon farming industry to prevent or reduce disease outbreaks and/or improve function and health, particularly during challenging periods. Salmon farming takes place in both freshwater and seawater during different stages like egg stage, fry or parr stage, smolt stage (that is ready for seawater transport) and on growing stage in the seawater. Mortality in Atlantic salmon occurs in all the production cycle in the freshwater stage, during sea transfer, as well as in on growing stage at sea. Infectious diseases outbreak is high, for example, when Atlantic salmon switch between fresh and saltwater. At that point, stress levels are high and fish are more vulnerable to a variety of factors associated with the aquaculture environment and management procedures, such as exposer to new environment, high stocking densities, handling and delousing lice treatments (Iversen et al., 2005; Martin & Król, 2017). Under such stressor conditions, activation of the immune response would be helpful, thus NSE1 can stimulate

the immune system, enhancing the host's protection against invasion of pathogens which then can reduce mortality.

However, an aggressive immune response over long periods of time is not only harmful to a potential pathogen, it can also be harmful to the host. Therefore, NSE2 could serve as a functional feed ingredient, which may enable to reduce or control over-inflammatory processes in Atlantic salmon smolts.

Lastly, this study was conducted in a controlled laboratory environment, where the fish were healthy, not exposed to pathogens or contaminants, constantly monitored, good water quality, and deprived of general stressful conditions. However, in future studies, which consider the dietary inclusion of NSEs, fish should be exposed to multiple stress conditions under unfavorable environmental conditions combined with relevant pathogens challenges for Atlantic salmon, to evaluate the efficacy of these compounds in promoting a more robust fish.

7. Conclusion

Atlantic salmon fed bio-active compounds (NSE1 and NSE2) extracted from Norway spruce have shown to modulate immune-related biomarkers in the distal intestine. While the use of NSE1 as a functional ingredient in novel feeds induced a modulation of IL-1β, suggesting the activation of the pro-inflammatory response, NSE2 showed a pattern of down-regulation associated with IL-8, iNOS and IFNγ, which may be related to the control of the immune response and the maintenance of homeostasis in the distal intestine.

These results allow establishing the bases for the evaluation of NSEs, which could be used in future investigations that seek to expand the knowledge related to the immunomodulatory properties of novel ingredients from woody biomass in functional feeds, improving productive aspects of salmon farming, and allowing Atlantic salmon to cope with multi-stressor conditions.

8. References

Abualtaher, M. & Bar, E. S. (2020). Food-Loss Control at the Macronutrient Level: Protein Inventory for the Norwegian Farmed Salmon Production System. *Foods*, 9 (8): 1095.

Agboola, J. O., Schiavone, M., Øverland, M., Morales-Lange, B., Lagos, L., Arntzen, M. Ø., Lapeña, D., Eijsink, V. G., Horn, S. J. & Mydland, L. T. (2021). Impact of down-stream processing on functional properties of yeasts and the implications on gut health of Atlantic salmon (Salmo salar). *Scientific reports*, 11 (1): 1-14.

Assefa, A. & Abunna, F. (2018). Maintenance of fish health in aquaculture: review of epidemiological approaches for prevention and control of infectious disease of fish. *Veterinary medicine international*, 2018.

Bharathi, S., Antony, C., Cbt, R., Arumugam, U., Ahilan, B. & Aanand, S. (2019). Functional feed additives used in fish feeds. *International Journal of Fisheries and Aquatic Studies*, 7 (3): 44-52.

Biasiotto, G., Zanella, I., Predolini, F., Archetti, I., Cadei, M., Monti, E., Luzzani, M., Pacchetti, B., Mozzoni, P. & Andreoli, R. (2018). 7-Hydroxymatairesinol improves body weight, fat and sugar metabolism in C57BJ/6 mice on a high-fat diet. *British Journal of Nutrition*, 120 (7): 751-762.

Bjørgen, H., Li, Y., Kortner, T. M., Krogdahl, Å. & Koppang, E. O. (2020). Anatomy, immunology, digestive physiology and microbiota of the salmonid intestine: Knowns and unknowns under the impact of an expanding industrialized production. *Fish & Shellfish Immunology*, 107: 172-186.

Buchmann, K. & Secombes, C. J. (2022). Principles of Fish Immunology.

Caballero, B. (2005). *Encyclopedia of human nutrition*: Elsevier.

Cadillo-Benalcazar, J. J., Giampietro, M., Bukkens, S. G. & Strand, R. (2020). Multi-scale integrated evaluation of the sustainability of large-scale use of alternative feeds in salmon aquaculture. *Journal of Cleaner Production*, 248: 119210.

Carriero, M. M., Henrique-Silva, F., Meira, C. M., Gato, I. M. Q., Caetano, A. R., Lobo, F. P., Alves, A. L., Varela, E. S. & Maia, A. A. M. (2020). Molecular characterization and gene expression analysis of the pro-inflammatory cytokines IL-1β and IL-8 in the South American fish Piaractus mesopotamicus challenged with Aeromonas dhakensis. *Genetics and Molecular Biology*, 43.

Cesta, M. F. (2006). Normal structure, function, and histology of mucosa-associated lymphoid tissue. *Toxicologic pathology*, 34 (5): 599-608.

Colombo, S. M., Zhang, Z., Wong, D. F. & Yuan, Z. (2020). Hydrolysis lignin as a multifunctional additive in Atlantic salmon feed improves fish growth performance and pellet quality and shifts gut microbiome. *Aquaculture Nutrition*, 26 (4): 1353-1368.

Couture, J. L., Geyer, R., Hansen, J. Ø., Kuczenski, B., Øverland, M., Palazzo, J., Sahlmann, C. & Lenihan, H. (2019). Environmental benefits of novel nonhuman food inputs to salmon feeds. *Environmental science & technology*, 53 (4): 1967-1975.

Dawood, M. A., Koshio, S. & Esteban, M. Á. (2018). Beneficial roles of feed additives as immunostimulants in aquaculture: a review. *Reviews in Aquaculture*, 10 (4): 950-974.

De Silva, S. & Yuan, D. (2022). *Regional review on status and trends in aquaculture development in Asia and the Pacific–2020*: Food & Agriculture Org.

FAO. (2020). The State of World Fisheries and Aquaculture 2020.Sustainability in action. (Rome). doi: https://doi.org/10.4060/ca9229en.

FAO. (2021). FAO. 2021. The impact of COVID-19 on fisheries and aquaculture food systems, possible responses: Information paper, November 2020. Rome. . doi: https://doi.org/10.4060/cb2537en.

Gajardo, K., Jaramillo-Torres, A., Kortner, T. M., Merrifield, D. L., Tinsley, J., Bakke, A. M. & Krogdahl, Å. (2017). Alternative protein sources in the diet modulate microbiota and functionality in the distal intestine of Atlantic salmon (Salmo salar). *Applied and environmental microbiology*, 83 (5): e02615-16.

Garlock, T., Asche, F., Anderson, J., Bjørndal, T., Kumar, G., Lorenzen, K., Ropicki, A., Smith, M. D. & Tveterås, R. (2020). A global blue revolution: aquaculture growth across regions, species, and countries. *Reviews in Fisheries Science & Aquaculture*, 28 (1): 107-116.

Hafezi, K., Hemmati, A. A., Abbaszadeh, H., Valizadeh, A. & Makvandi, M. (2020). Anticancer activity and molecular mechanisms of α-conidendrin, a polyphenolic compound present in Taxus yunnanensis, on human breast cancer cell lines. *Phytotherapy Research*, 34 (6): 1397-1408.

Halliwell, B., Aeschbach, R., Löliger, J. & Aruoma, O. (1995). The characterization of antioxidants. *Food and Chemical Toxicology*, 33 (7): 601-617.

Hammerbacher, A., Ralph, S. G., Bohlmann, J., Fenning, T. M., Gershenzon, J. & Schmidt, A. (2011). Biosynthesis of the major tetrahydroxystilbenes in spruce, astringin and isorhapontin, proceeds via resveratrol and is enhanced by fungal infection. *Plant physiology*, 157 (2): 876-890.

Helland, S., Grisdale-Helland, B. & Nerland, S. (1996). A simple method for the measurement of daily feed intake of groups of fish in tanks. *Aquaculture*, 139 (1-2): 157-163.

Hersoug, B. (2015). The greening of Norwegian salmon production. *Maritime Studies*, 14 (1): 1-19.

Hu, X., Iordan, C. M. & Cherubini, F. (2018). Estimating future wood outtakes in the Norwegian forestry sector under the shared socioeconomic pathways. *Global Environmental Change*, 50: 15-24.

Iversen, M., Finstad, B., McKinley, R. S., Eliassen, R. A., Carlsen, K. T. & Evjen, T. (2005). Stress responses in Atlantic salmon (Salmo salar L.) smolts during commercial well boat transports, and effects on survival after transfer to sea. *Aquaculture*, 243 (1-4): 373-382.

Jablonsky, M., Nosalova, J., Sladkova, A., Haz, A., Kreps, F., Valka, J., Miertus, S., Frecer, V., Ondrejovic, M. & Sima, J. (2017). Valorisation of softwood bark through extraction of utilizable chemicals. A review. *Biotechnology advances*, 35 (6): 726-750.

Jablonsky, M., Haz, A., Sladkova, A., Strizincova, P., Skulcova, A., Majova, V. & Jablonsky, J. (2019). Nutraceuticals as phenolic bioactive compounds analysis of softwood bark and their possibilities of industry applications. *Journal of Hygienic Engineering and Design*, 26: 93-99.

Jannathulla, R., Rajaram, V., Kalanjiam, R., Ambasankar, K., Muralidhar, M. & Dayal, J. S. (2019). Fishmeal availability in the scenarios of climate change: Inevitability of fishmeal replacement in aquafeeds and approaches for the utilization of plant protein sources. *Aquaculture Research*, 50 (12): 3493-3506.

Jung-Schroers, V., Adamek, M., Harris, S., Syakuri, H., Jung, A., Irnazarow, I. & Steinhagen, D. (2018). Response of the intestinal mucosal barrier of carp (Cyprinus carpio) to a bacterial challenge by Aeromonas hydrophila intubation after feeding with β-1, 3/1, 6-glucan. *Journal of Fish Diseases*, 41 (7): 1077-1092.

Jyske, T., Brännström, H., Halmemies, E., Laakso, T., Kilpeläinen, P., Hyvönen, J., Kärkkäinen, K. & Saranpää, P. (2022). Stilbenoids of Norway spruce bark: does the variability caused by raw-material processing offset the biological variability? *Biomass Conversion and Biorefinery*: 1-15.

Kangas, L., Saarinen, N., Mutanen, M., Ahotupa, M., Hirsinummi, R., Unkila, M., Perälä, M., Soininen, P., Laatikainen, R. & Korte, H. (2002). Antioxidant and antitumor effects of hydroxymatairesinol (HM-3000, HMR), a lignan isolated from the knots of spruce. *European Journal of Cancer Prevention*: S48-S57.

Khansari, A. R., Parra, D., Reyes-López, F. E. & Tort, L. (2017). Cytokine modulation by stress hormones and antagonist specific hormonal inhibition in rainbow trout (Oncorhynchus mykiss) and gilthead sea bream (Sparus aurata) head kidney primary cell culture. *General and comparative endocrinology*, 250: 122-135.

Kiron, V. (2012). Fish immune system and its nutritional modulation for preventive health care. *Animal Feed Science and Technology*, 173 (1-2): 111-133.

Koch, J. F. A., de Oliveira, C. A. F. & Zanuzzo, F. S. (2021). Dietary β-glucan (MacroGard®) improves innate immune responses and disease resistance in Nile tilapia regardless of the administration period. *Fish & Shellfish Immunology*, 112: 56-63.

Kok, B., Malcorps, W., Tlusty, M. F., Eltholth, M. M., Auchterlonie, N. A., Little, D. C., Harmsen, R., Newton, R. W. & Davies, S. J. (2020). Fish as feed: Using economic allocation to quantify the Fish In: Fish Out ratio of major fed aquaculture species. *Aquaculture*, 528: 735474.

Korkina, L. (2007). Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health. *Cellular and molecular biology*, 53 (1): 15-25.

Kähkönen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J.-P., Pihlaja, K., Kujala, T. S. & Heinonen, M. (1999). Antioxidant activity of plant extracts containing phenolic compounds. *Journal of agricultural and food chemistry*, 47 (10): 3954-3962.

Li, Y., Kortner, T. M., Chikwati, E. M., Munang'andu, H. M., Lock, E.-J. & Krogdahl, Å. (2019). Gut health and vaccination response in pre-smolt Atlantic salmon (Salmo salar) fed black soldier fly (Hermetia illucens) larvae meal. *Fish & shellfish immunology*, 86: 1106-1113.

Lucas, S., Soler, L.-G. & Revoredo-Giha, C. (2021). Trend analysis of sustainability claims: The European fisheries and aquaculture markets case. *Food policy*, 104. doi: 10.1016/j.foodpol.2021.102141.

Mansikkala, T., Patanen, M., Kärkönen, A., Korpinen, R., Pranovich, A., Ohigashi, T., Swaraj, S., Seitsonen, J., Ruokolainen, J. & Huttula, M. (2020). Lignans in Knotwood of Norway Spruce: Localisation with Soft X-ray Microscopy and Scanning Transmission Electron Microscopy with Energy Dispersive X-ray Spectroscopy. *Molecules*, 25 (13): 2997.

Marcotullio, M. C., Curini, M. & Becerra, J. X. (2018). An Ethnopharmacological, Phytochemical and Pharmacological Review on Lignans from Mexican Bursera spp. *Molecules*, 23 (8): 1976.

Maroni, K. (2000). Monitoring and regulation of marine aquaculture in Norway. *Journal of Applied Ichthyology*, 16 (4-5): 192-195.

Martin, S. A. & Król, E. (2017). Nutrigenomics and immune function in fish: new insights from omics technologies. *Developmental & Comparative Immunology*, 75: 86-98.

Maru, G., Kumar, G., Ghantasala, S. & Tajpara, P. (2014). Polyphenol-Mediated In Vivo Cellular Responses during Carcinogenesis. *Polyphenols in Human Health and Disease*: 1141-1179.

Montalto, M., D'onofrio, F., Gallo, A., Cazzato, A. & Gasbarrini, G. (2009). Intestinal microbiota and its functions. *Digestive and Liver Disease Supplements*, 3 (2): 30-34.

Morales-Lange, B., González-Aravena, M., Font, A., Guzmán, F. & Mercado, L. (2018). Detection of peroxiredoxin-like protein in Antarctic sea urchin (Sterechinus neumayeri) under heat stress and induced with pathogen-associated molecular pattern from Vibrio anguillarum. *Polar Biology*, 41 (10): 2065-2073.

Morales-Lange, B., Djordjevic, B., Gaudhaman, A., Press, C. M., Olson, J., Mydland, L. T., Mercado, L., Imarai, M., Castex, M. & Øverland, M. (2022). Dietary Inclusion of Hydrolyzed Debaryomyces hansenii Yeasts Modulates Physiological Responses in Plasma and Immune Organs of Atlantic salmon (Salmo salar) Parr Exposed to Acute Hypoxia Stress. *Frontiers in physiology*: 429.

Neu, J. (2008). *Neonatology: Questions and Controversies Series: Gastroenterology and Nutrition*: Elsevier Health Sciences.

NRC. (2011). *Nutrient requirements of fish and shrimp*: The National Academies Press Washington DC, USA.

OECD/FAO. (2021). OECD-FAO Agricultural Outlook 2021-2030, OECD Publishing, Paris,. doi: https://doi.org/10.1787/19428846-en.

Olaussen, J. O. (2018). Environmental problems and regulation in the aquaculture industry. Insights from Norway. *Marine Policy*, 98: 158-163.

Oliveira, V. H., Dean, K. R., Qviller, L., Kirkeby, C. & Bang Jensen, B. (2021). Factors associated with baseline mortality in Norwegian Atlantic salmon farming. *Scientific Reports*, 11 (1): 1-14.

Olsen, R. E., Sundell, K., Hansen, T., Hemre, G.-I., Myklebust, R., Mayhew, T. M. & Ringø, E. (2002). Acute stress alters the intestinal lining of Atlantic salmon, Salmo salar L.: An electron microscopical study. *Fish physiology and biochemistry*, 26 (3): 211-221.

Overton, K., Dempster, T., Oppedal, F., Kristiansen, T. S., Gismervik, K. & Stien, L. H. (2019). Salmon lice treatments and salmon mortality in Norwegian aquaculture: a review. *Reviews in Aquaculture*, 11 (4): 1398-1417.

Panettieri, R. A., Schaafsma, D., Amrani, Y., Koziol-White, C., Ostrom, R. & Tliba, O. (2019). Non-genomic effects of glucocorticoids: an updated view. *Trends in pharmacological sciences*, 40 (1): 38-49.

Penn, M. H., Bendiksen, E. Å., Campbell, P. & Krogdahl, Å. (2011). High level of dietary pea protein concentrate induces enteropathy in Atlantic salmon (Salmo salar L.). *Aquaculture*, 310 (3-4): 267-273.

Pietarinen, S. P., Willför, S. M., Ahotupa, M. O., Hemming, J. E. & Holmbom, B. R. (2006). Knotwood and bark extracts: strong antioxidants from waste materials. *Journal of wood science*, 52 (5): 436-444.

Press, C. M. & Evensen, Ø. (1999). The morphology of the immune system in teleost fishes. *Fish & shellfish immunology*, 9 (4): 309-318.

Reverter, M., Bontemps, N., Lecchini, D., Banaigs, B. & Sasal, P. (2014). Use of plant extracts in fish aquaculture as an alternative to chemotherapy: current status and future perspectives. *Aquaculture*, 433: 50-61.

Reverter, M., Tapissier-Bontemps, N., Sarter, S., Sasal, P. & Caruso, D. (2021). Moving towards more sustainable aquaculture practices: a meta-analysis on the potential of plant-enriched diets to improve fish growth, immunity and disease resistance. *Reviews in Aquaculture*, 13 (1): 537-555.

Sakai, M., Hikima, J.-i. & Kono, T. (2021). Fish cytokines: Current research and applications. *Fisheries Science*, 87 (1): 1-9.

Salinas, I. (2015). The mucosal immune system of teleost fish. *Biology*, 4 (3): 525-539.

Samuel, C. E. (2001). Antiviral actions of interferons. *Clinical microbiology reviews*, 14 (4): 778-809.

Schmitt, P., Wacyk, J., Morales-Lange, B., Rojas, V., Guzmán, F., Dixon, B. & Mercado, L. (2015). Immunomodulatory effect of cathelicidins in response to a β-glucan in intestinal epithelial cells from rainbow trout. *Developmental & Comparative Immunology*, 51 (1): 160-169.

Solberg, B., Moiseyev, A., Hansen, J. Ø., Horn, S. J. & Øverland, M. (2021). Wood for food: Economic impacts of sustainable use of forest biomass for salmon feed production in Norway. *Forest Policy and Economics*, 122: 102337.

Sommerset, I., Bang Jensen, B., Bornø, G., Haukaas, A. & Brun, E. (2021). *The health situation in Norwegian aquaculture 2020. Norwegian Veterinary Insitute*.

Tacon, A. G. J. & Metian, M. (2015). Feed Matters: Satisfying the Feed Demand of Aquaculture. *Reviews in fisheries science & aquaculture*, 23 (1): 1-10. doi: 10.1080/23308249.2014.987209.

Talpur, A. D., Munir, M. B., Mary, A. & Hashim, R. (2014). Dietary probiotics and prebiotics improved food acceptability, growth performance, haematology and immunological parameters and disease resistance against Aeromonas hydrophila in snakehead (Channa striata) fingerlings. *Aquaculture*, 426: 14-20.

Tezuka, Y., Morikawa, K., Li, F., Auw, L., Awale, S., Nobukawa, T. & Kadota, S. (2011). Cytochrome P450 3A4 inhibitory constituents of the wood of Taxus yunnanensis. *Journal of natural products*, 74 (1): 102-105.

Valero, Y., Martínez-Morcillo, F. J., Esteban, M. Á., Chaves-Pozo, E. & Cuesta, A. (2015). Fish peroxiredoxins and their role in immunity. *Biology*, 4 (4): 860-880.

Wang, J., Kortner, T. M., Chikwati, E. M., Li, Y., Jaramillo-Torres, A., Jakobsen, J. V., Ravndal, J., Brevik, Ø. J., Einen, O. & Krogdahl, Å. (2020). Gut immune functions and health in Atlantic salmon (Salmo salar) from late freshwater stage until one year in seawater and effects of functional ingredients: A case study from a commercial sized research site in the Arctic region. *Fish & Shellfish Immunology*, 106: 1106-1119.

Wei, T. & Simko, V. (2021). package "corrplot": Visualization of a Correlation Matrix; 2017. *Version 0.84*.

Xu, C., Guo, T.-C., Mutoloki, S., Haugland, Ø. & Evensen, Ø. (2012). Gene expression studies of host response to Salmonid alphavirus subtype 3 experimental infections in Atlantic salmon. *Veterinary research*, 43 (1): 1-10.

Xue, Q., Yan, Y., Zhang, R. & Xiong, H. (2018). Regulation of iNOS on immune cells and its role in diseases. *International journal of molecular sciences*, 19 (12): 3805.

Ytrestøyl, T., Aas, T. S. & Åsgård, T. (2015). Utilisation of feed resources in production of Atlantic salmon (Salmo salar) in Norway. *Aquaculture*, 448: 365-374.

Zou, J. & Secombes, C. J. (2016). The function of fish cytokines. *Biology*, 5 (2): 23.

Øverland, M., Karlsson, A., Mydland, L. T., Romarheim, O. H. & Skrede, A. (2013). Evaluation of Candida utilis, Kluyveromyces marxianus and Saccharomyces cerevisiae yeasts as protein sources in diets for Atlantic salmon (Salmo salar). *Aquaculture*, 402: 1-7.

Øverland, M. & Skrede, A. (2017). Yeast derived from lignocellulosic biomass as a sustainable feed resource for use in aquaculture. *Journal of the Science of Food and Agriculture*, 97 (3): 733-742.

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

Postboks 5003
NO-1432 Ås
Norway