PhD in Aquatic Biosciences // No. 44 - 2022

Influence of feed ingredients and additives on mucosal health with focus on the intestine of Atlantic salmon (*Salmo salar*)

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A thesis for the degree of
Philosophiae Doctor (PhD)

PhD in Aquatic Biosciences no. 44 (2022)
Faculty of Biosciences and Aquaculture

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ISBN: 978-82-93165-43-9

Print: Trykkeriet NORD

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Preface

This dissertation is submitted in fulfilment of the requirements for the Degree of Philosophia Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University, Bodø, Norway. The original research presented in this thesis was part of the projects “Blodanalyser av laks som metode for vurdering av tarmhelse” funded by MABIT (project No. AF0082), and “Alger4laks” funded by the Research Council of Norway (project No. 260190), under the “MARINALGAE4aqua”, a COFASP ERA-NET project. Portions of the research in this thesis has also been supported by funds from Nord University.

The PhD project team consisted of the following members:

Solveig Lysfjord Sørensen, MSc, FBA, Nord University: PhD candidate

Mette Sørensen, Professor, FBA, Nord University: Main supervisor

Kiron Viswanath, Professor, FBA, Nord University: Co-supervisor

Solveig Lysfjord Sørensen

Bodø, 6th of April, 2022

Acknowledgements

This thesis would not have been completed without the many supportive and skilled individuals that have surrounded me over the past years.

First, I wish to acknowledge my supervisor Mette Sørensen and co-supervisor Kiron Viswanath, who found time in their busy schedules to guide me through experiments, lab work, and writing both papers and the thesis itself. Thank you for sharing from your vast experience, giving thorough feedback, and providing valuable input towards completing this work.

A big thank you to the helpful and supportive staff at the Mørkvedbukta Research Station for all their help with the feeding experiments and samplings. I am grateful to the technicians at FBA for their help with training, sampling and lab work. A special thank you to Dalia and Ghana for instruction and advice in histology and gene expression analysis, respectively.

Thank you to our many collaborators and co-authors from: (1) the Department of Pharmaceutical Technology and the Department of Microbiology and Immunology at University of Veterinary Medicine and Pharmacy in Košice, (2) the Department of Biosciences at University of Bergen (3) Quantidoc AS, (4) BioVivo Technologies AS, (5) SPAROS Lda., (6) Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR/CIMAR), (7) Instituto de Ciências Biomédicas de Abel Salazar (ICBAS) at University of Porto, and (8) Nord University.

I wish to express much gratitude to my dear friends and fellow PhDs who made everything from office work and lunch breaks to conferences, seminars, courses, and meetings more bearable and even fun (sometimes)! A special thanks to all my lovely climbing buddies, hiking and skiing buddies, and cabin buddies. <3

A big hug to my loving family, who supports and encourages me in everything I do. A special thanks to my mom, who always knew I could do anything. And lastly, I wish to thank my wonderful Halvor, who is always there for me.

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Declaration on the contributions in the publications shared with other PhD theses at the Faculty of Biosciences and Aquaculture

Parts of Paper III was also published in Yangyang Gong's PhD thesis 26/2018. The work addressing mucosal health in the paper is the contribution of Solveig Lysfjord Sørensen and therefore included in the current thesis.

The gene expression studies included Paper IV is the contribution of Solveig Lysfjord Sørensen and is included in the current thesis. The remaining sections will form parts of another thesis to be submitted to the Faculty.

Abstract

The continuing growth of intensive aquaculture requires increasing amounts of high quality fish feed. There is great interest in finding and developing new feed ingredients to produce safe, healthy and sustainable diets for Atlantic salmon aquaculture. Plant protein concentrates and plant lipids have replaced much of the former main ingredients fish meal and fish oil. Microalgae are promising novel protein and lipid sources for aquaculture diets, but disruption of cell walls by processing may be necessary to optimize their utilization. The mucosal surfaces of salmon, including the skin, gills, and gastrointestinal tract, are potentially affected by the fish diet. These mucosal surfaces have, in addition to their physiological functions, important barrier and immune functions that are vital for fish health and growth. Mucins and antimicrobial proteins (AMPs) are produced and excreted at mucosal surfaces to hinder, inactivate, or kill potentially harmful microbes. Information about Atlantic salmon mucosal health may be gathered by analysing the expression of these proteins. The main objectives of this thesis were to examine how different feed ingredients and additives affect the growth, feed utilization, nutrient digestibility and the mucosal health of intestine, skin and gills in farmed Atlantic salmon smolt, highlighting the importance of intestinal health. The thesis and included papers are based on four feeding experiments wherein farmed Atlantic salmon smolt were fed: (1) four diets containing fish- and/or plant-derived protein and lipid sources in different combination, plus one soybean meal diet, (2) four diets containing the microalgae *Nannochloropsis oceanica* and *Tetraselmis* sp., either whole or pre-processed, or a fish ingredient based control diet, (3) three diets containing pre-processed microalgae *N. oceanica* with or without two different additives, or a plant ingredient based control diet, (4) three diets based mainly on either fish-derived ingredients, soybean meal, or plant-derived ingredients, with or without coating of probiotic LAB (*Lactobacillus fermentum* and *L. plantarum*).

Our results showed that fish fed on all experimental diets had good growth. The apparent digestibility coefficients (ADCs) of protein, lipid, dry matter, and energy were in the range of 86.1-88.1%, 87.4-95.4%, 59.0-68.4%, and 73.1-83.8%, respectively, for salmon fed diets

containing plant derived protein or lipids. Fish fed diets containing 10% of the microalgae *N. oceanica* had ADCs of protein, lipid and dry matter in the range of 86.5-88.5%, 91.1-91.9% and 65.3-67.5%, respectively. The extrusion of microalgae improved salmon growth (*N. oceanica*) and antioxidant capacity in the liver (*Tetraselmis* sp.), and altered the availability of fatty acids (both microalgae) when microalgae were used in salmon diets at 30% inclusion levels. Concerning mucosal health and barrier function, a decreased *muc2* gene expression was observed in the distal intestine in conjugation with classic soybean meal induced enteritis (SBMIE) symptoms in salmon fed diets containing the ingredient, demonstrating that *muc2* can be used as a marker gene for intestinal health. Fish receiving diets containing mainly plant protein concentrates had inferior intestinal health compared to those fed marine protein, and mild enteritis-like symptoms were observed in the distal intestine. Intestinal health-related parameters of salmon were mostly unaffected by the microalgae-incorporated diets, though there were minor changes in some intestinal morphology parameters for fish fed whole-microalgae diets. Salmon fed probiotic LAB supplemented diets had improved mucosal barrier status in skin and gills, as they had an increased relative area or number of goblet cells. SBMIE-affected fish fed probiotics showed reduced severity of some symptoms compared to the control group. The results from expression of AMP and mucin genes in distal intestine, skin and gills of salmon showed that these can be applied to assess mucosal health. The AMP and mucin genes had a tissue-specific expression and some genes were modulated by feed ingredients and probiotics. In addition, the expression of mucin genes was inversely related to the number of goblet cells in the investigated mucosal tissues, which indicated a regulatory mechanism. The observations from the different studies described in this thesis highlight the impact of dietary components on the mucosal health of Atlantic salmon, and underline the importance of continuing to study this subject.

Abstract in Norwegian – Sammendrag på norsk

En fremtidig vekst av akvakulturnæringen krever stadig større mengder fiskefôr av høy kvalitet. Det er stor interesse for å utvikle nye fôringredienser for å kunne produsere trygt, sunt og bærekraftig fôr til oppdrett av atlantisk laks. Planteproteinkonsentrater og planteoljer har erstattet mesteparten av de tidligere hovedingrediensene fiskemel og fiskeolje. Mikroalger er lovende nye kilder til protein og lipider, men det kan være nødvendig å bryte ned celleveggen ved bruk av prosessering for å optimere utnyttelsen. Slimhinnene hos laks, som dekker overflatene på hud, gjeller, og mage-tarm-systemet, kan potensielt påvirkes av fôret. Disse slimhinnene har, i tillegg til deres fysiologiske funksjoner, flere viktige barriere- og immunfunksjoner som er essensielle for fiskens helse og vekst. Muciner og antimikrobielle proteiner produseres og skilles i slimhinnene for å hindre, inaktivere eller drepe potensielt skadelige mikroorganismer. Ved å studere genuttrykket til disse proteinene kan man innhente informasjon om slimhinnehelsen til atlantisk laks. Hovedformålene i denne avhandlingen var å undersøke hvordan ulike fôringredienser og tilsetningsstoffer påvirker vekst, fôrutnyttelse, fordøyelighet, og slimhinnehelse i tarm, hud og gjeller hos oppdrettet atlantisk laks, med ekstra fokus på tarmhelse. Avhandlingen og artiklene er basert på fire fôringsforsøk hvor oppdrettet smolt av atlantisk laks ble fôret: (1) fire fôr som inneholdt ulike kombinasjoner av protein og oljer fra både marine ingredienser og planteingredienser, i tillegg til ett fôr med soyamel, (2) fire fôr som inneholdt mikroalgene *Nannochloropsis oceanica* og *Tetraselmis* sp., enten som hele celler eller prosessert, eller et referansefôr basert på marine ingredienser, (3) tre fôr som inneholdt prosessert *N. oceanica*, med eller uten to ulike tilsetningsstoffer, eller et referansefôr basert på planteingredienser, (4) tre fôr som hovedsakelig inneholdt enten marine ingredienser, soyamel, eller planteingredienser, med eller uten supplementering av probiotiske melkesyrebakterier (*Lactobacillus fermentum* og *L. plantarum*).

Resultatene i avhandlingen viste at fisken hadde god vekst i alle fôringsforsøkene. Fordøyelighetskoeffisientene til protein, fett, tørrstoff og energi var i områdene 86.1-88.1%, 87.4-95.4%, 59.0-68.4%, og 73.1-83.8% for laks fôret med ulike planteingredienser. Laks

fôret med 10% *N. oceanica* hadde fordøyelighetskoeffisienter for protein, fett, og tørrstoff i områdene 86.5-88.5%, 91.1-91.9% and 65.3-67.5%. Ekstrudering av mikroalger forbedret laksens vekt (*N. oceanica*), økte antioksidantkapasiteten i laksens lever (*Tetraselmis* sp.), og påvirket tilgjengeligheten til fettsyrer (begge algene) da 30% mikroalger ble inkludert i laksefôr. Slimhinnehelse og barrierefunksjon var redusert og det ble observert en reduksjon av genuttrykket til *muc2* i distal tarm, i tillegg til klassiske symptomer på soyamelindusert enteritt (SBMIE), hos laks fôret med soyamel. Dette demonstrerer at *muc2* kan brukes som genmarkør for tarmhelse. Laks som ble fôret hovedsakelig med planteproteinkonsentrater hadde milde enterittlignende symptomer i distal tarm, og generelt dårligere tarmhelse sammenlignet med laks som fikk fôr med marint protein. De tarmhelserelaterte parameterne hos laks var stort sett upåvirket av mikroalgefôrene, men det var noen små endringer i tarmens morfologi hos laks fôret med hele mikroalgeceller. Laks som fikk fôr supplementert med probiotika hadde forbedret barrierestatus i hud og gjeller, med en økning i enten antallet slimproduserende celler eller deres relative areal. Laks med SBMIE som ble fôret med probiotika hadde redusert alvorlighetsgrad av noen symptomer sammenlignet med referansegruppen. Genuttrykkene til AMP- og mucingenene i distal tarm, hud, og gjeller hos laks viste at disse parameterne kan brukes til å vurdere slimhinnehelse. AMP- og mucingenene hadde et vevsspesifikt genuttrykk og noen av genene ble påvirket av fôringredienser og probiotika. I tillegg hadde genuttrykket av mucingener en invers korrelasjon med antallet slimproduserende celler i de undersøkte slimhinnene, noe som indikerte en reguleringsmekanisme. Observasjonene fra studiene som beskrives i denne avhandlingen fremhever påvirkningen fôrkomponenter har på slimhinnehelsen til atlantisk laks, og understreker viktigheten av å fortsette og studere dette temaet.

1. Introduction

1.1 Aquaculture can feed the world

A growing human population, increasing demand for fish for human consumption, and plateauing capture fisheries highlight the relevance of the aquaculture industry. Aquaculture had an annual growth rate of 4.6% through the years 2007-2018. While the growth rate is expected to decrease over time, The Food and Agriculture Organization of the United Nations (FAO) predicts the continued growth of the industry throughout the next decade. Aquaculture production intended for food overtook capture fisheries for human consumption in 2015. By 2030, the global aquaculture production is expected to also overtake the total capture fisheries (FAO, 2020).

In 2018, farmed Atlantic salmon (*Salmo salar*) accounted for 4.5% of the global finfish aquaculture production, valued at 2.44 million tonnes (FAO, 2020). The Norwegian salmon aquaculture industry contributed around half of the total amount; 1.28 million tonnes of Atlantic salmon was produced in 2018 (Fiskeridirektoratet, 2020). Norway's salmon production increased steadily through the '90s and '00s, before plateauing from 2012 and onwards (Figure 1), largely due to government restrictions on further growth because of issues linked to sea lice and diseases. Growth of the industry is currently only allowed with implementation of new production technology to stimulate sustainable development of the aquaculture sector. There is also increasing awareness of fish welfare and an important goal is to reduce mortality, especially during the sea phase. Future growth of the sector will also demand new sustainable feed ingredients.

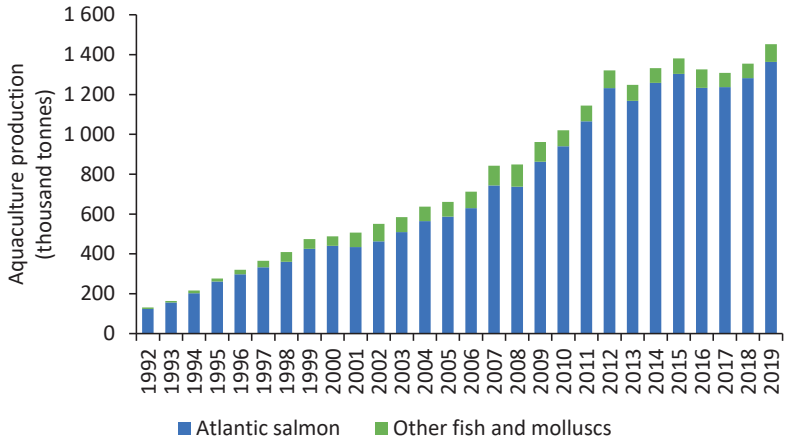

Figure 1. Norwegian Atlantic salmon production in relation to the country's total aquaculture production during the last decades. Data from Statistics Norway (2020).

1.2 Feed development in salmon aquaculture

The feed for salmon aquaculture has gone through various stages of development catering to the needs of the industry, both in terms of ingredients and manufacturing technology. The first generation of processed diets was comprised mainly of fish meal and fish oil, as these offer a favourable amino acid profile and high levels of n-3 polyunsaturated fatty acids (PUFAs), respectively. Some important PUFAs found in fish oil are eicosapentaenoic acid (EPA, 20:5(n-3)) and docosahexaenoic acid (DHA, 22:6(n−3)). However, fish meal and fish oil are derived from reduction fisheries or by-products from fish processed for human consumption, which are limited resources (Olsen and Hasan, 2012). Static supply of fish meal and fish oil, growing demand, increasing price, and sustainability issues were the key drivers in developing diets based on plant ingredients (New and Wijkström, 2002). The commercial diets currently used in farming of Atlantic salmon is the second generation high quality diet based on plant protein concentrates and plant oils substituting fish meal and fish oil (Ytrestøyl et al., 2015). In 2016 the Norwegian salmon industry used 1.63 million tonnes of feed ingredients, of which marine ingredients accounted for 25%, and plant ingredients constituted around 70% (Aas et al., 2019). The most common plant ingredients used in the current salmon diets are soybean protein concentrate, wheat gluten, corn gluten, fava

beans, and pea protein concentrate, which are protein sources; rapeseed and camelina oil, which are lipid sources; and wheat and pea starch, which are used as binders (Aas et al., 2019).

Compared to marine ingredients, plant ingredients have the following limitations: (1) imbalanced amino acid profile of plant protein, requiring supplementation of essential amino acids to the diets, (2) presence of residual antinutritional factors in plant protein concentrates, (3) lack of n-3 PUFAs (EPA and DHA) in plant oils and unfavourable balance between n-6/n-3 fatty acids, and (4) increasing awareness and criticism of feeding fish plant ingredients that can be consumed directly by humans (Beal et al., 2018, Olsen and Hasan, 2012, Aas et al., 2019).

To meet the need for more sustainable feed ingredients for the growing aquaculture industry, researchers are exploring the potential of third generation feed ingredients. Such ingredients are produced from or harvested at lower trophic levels and they do not directly compete with food for human consumption, which is in line with the demand for sustainability. Some examples are seaweed and microalgae, bacteria, yeast, and insects (yellow mealworm, black soldier fly, common house fly) (Sogari et al., 2019, Tibbetts, 2018, Solberg et al., 2021, Aas et al., 2006a, Kamunde et al., 2019). In the lowest trophic level we find the microbial ingredients, which are photosynthetic microalgae, bacteria and yeast. Microbial ingredients are also known as single-cell protein.

1.2.1 Microbial ingredients: Bacteria and yeast

Use of bacteria and yeast in feeding of fish has gained attention as they have high potential as feed ingredients, mainly as protein sources. Both bacteria and yeast can be produced in bioreactors using by-products from forestry and agriculture, where they convert non-food biomass into valuable protein. They can be grown almost anywhere, as the production is largely independent of factors like soil quality, water availability, and climate conditions (Øverland et al., 2010, Agboola et al., 2021).

The nutrient composition of bacterial meal varies with species, substrate, and post-fermentation processing. A bacterial meal produced from a culture consisting mainly of the methanotroph bacteria *Methylococcus capsulatus* has been investigated as a protein source in diets for a range of monogastric animals, including Atlantic salmon. The chemical composition of the bacterial meal was in the range of 67.0-73.4% protein, 8.0-10.7% lipid, 6.2-8.5% ash, and 9.9-11.1% nucleic acids (Øverland et al., 2010). When replacing fish meal with bacterial meal in diets for Atlantic salmon, it was found that the growth of the fish and digestibility of most nutrients decreased with increasing amount of bacterial meal. This has been suggested to be caused by bacterial cell wall components which the fish cannot digest. Another potential reason could be the recognition of immunogenic structures on the bacterial cell surface by intestinal receptors, leading to undesirable immune responses. These issues could be tackled by breaking the cell wall through processing and avoiding whole cells in the finished product (Øverland et al., 2010).

Yeasts are diverse single-celled eukaryotes, and use of yeast in fish diets for various species can be traced back to the 1970s. More recently, interest in yeasts has been revived because of their potential for sustainable production and application as probiotics. One of the main yeast species which has been studied as a fish feed ingredient is *Saccharomyces cerevisiae*. A review of several studies on this strain has calculated the average nutrient composition to be 50.1% protein, 1,8% lipid, 7.5% ash, 4.6% starch, and 4.8% nucleic acids (Agboola et al., 2021). As with bacteria and microalgae, suitability of the many different species of yeast as feed ingredients for Atlantic salmon depend on their nutrient composition and cell wall structure. For example, Øverland et al. (2013) found that replacing 40% of crude protein with three different yeast strains (*Candida utilis*, *Kluyveromyces marxianus* and *S. cerevisiae*) in diets for Atlantic salmon resulted in growth, digestibility and nutrient retention for two of the strains comparable to that of a fish meal diet, while the final strain had reduced performance. Vidakovic et al. (2020) concluded that two yeasts (*S. cerevisiae* and *Wickerhamomyces anomalus*) could replace up to 40% of fish meal (300 g/kg fish meal in base diet) in diets for rainbow trout (*Oncorhynchus mykiss*), while maintaining good growth, digestibility, and intestinal health.

1.2.2 Microbial ingredients: Microalgae

Microalgae are usually single-celled, photosynthetic organisms, though exceptions exist. They are primary producers in our ecosystems, and there is immense variation in the different species' tolerances for environmental factors such as temperature, pH, salinity, and light intensity. Microalgae are very attractive in terms of sustainability and environmentalism, since high growth can be achieved in photoreactors while assimilating CO_2 (Beal et al., 2018). They can also be produced in fermenters by converting organic material into new chemical compounds such as protein, lipid, carbohydrates and micronutrients. Ongoing research focuses on the optimisation of the growth conditions of microalgae to be used for several purposes, for example for production of biodiesel, omega-3 fatty acids, pigments, animal diets, or "superfood" for humans (Tibbetts, 2018, Alhattab et al., 2019). Additionally, the by-products from biodiesel production (defatted microalgae meal) have been studied as feed ingredients (Kiron et al., 2016, Gong et al., 2018).

Different strains of microalgae vary in their content of macronutrients such as protein, lipid, ash and carbohydrates and energy (Table 1). The lipid-rich strains are particularly interesting for the fish diet industry, especially those strains that can produce EPA and DHA. Compared to fish meal, microalgae have variable contents of protein, lipid and carbohydrate, while the ash and energy content is in the same range, depending on the species. There are many potential strains, but only a few are commercially produced and approved for use as feed ingredients. Two such microalgae are *Nannochloropsis* and *Tetraselmis.* Both of them are rich in PUFAs. *Nannochloropsis* can accumulate EPA at levels up to 28% of total fatty acids. *Tetraselmis* can produce α-linolenic acid (ALA) at around 10% of total fatty acids (Tibbetts, 2018). ALA is a precursor in biosynthesis of EPA and DHA, which occurs in many fish, including salmonids.

Depending on growth conditions and processing methods after harvesting, the microalgae *Nannochloropsis* and *Tetraselmis* may have lower protein content, but similar or higher level of lipid content, and higher carbohydrate content compared to fish meal (Table 1).

Table 1. Comparison of the proximate composition and energy content of selected microalgae and fish meal.

Ingredient	Ash (%)	Protein (%)	Lipid (%)	Carb. (%)	Energy (MJ kg^{-1})	References
Fish meal	5-20 15 15.9 8.8-10.3 10.2-21.5	52-72 60-72 67.9 69.6-71.7 59.0-72.0	5.8-10.4 6-10 9.7 8.4-11.9 7.6-10.1	3.7-16.3[a] - - 0.8-2.7[a] 0.6-1.0[b]	- - - -	(Cruz, 1997) (Cho and Kim, 2011) (Øverland et al., 2009) (Bragadóttir et al., 2004) (National Research Council, 2011)
Nanno-chloropsis sp.	7–23	18–48	2–68	8–36	19–27	(Tibbetts, 2018)
N. oceanica	31.8 ± 0.03 35.0	34.4 ± 0.09 34.3	6.8 ± 0.48 9.8	27.0 ± 0.6[c] 28.8[d]	17.18 ± 0.02 15.9	(Ferreira et al., 2021) (Valente et al., 2021)
N. gaditana	8.4 ± 0.2 8 ± 2 15.8	45.0 ± 0.6 44 ± 5 52 ± 9 41 38.5	29.3 ± 0.2 27 ± 4 27 ± 6 26 19.2	16.5 ± 0.2 21 ± 5 25 24.5	-	(Teuling et al., 2017b) (Valente et al., 2021)
Tetraselmis sp.	11–20 14 17	27–52 26 30	3–45 14 13	15–45 9 8	18–20	(Tibbetts, 2018) (Teuling et al., 2017b)
T. suecica	17.5	48.7	8.0	22.4[a]	-	(Valente et al., 2021)
T. impellucida	17.3 ± 0.1 15	34.7 ± 0.1 36	23.1 ± 0.7 19	17.9 ± 0.2 24	-	(Teuling et al., 2017b)
T. chuii	-	31	17	12	-	(Teuling et al., 2017b)

a: Nitrogen-Free Extract (NFE); b: Crude fiber; c: Calculated by estimation; d: Neutral detergent fiber. Values are on a dry matter basis. Differences in raw materials and methods of determination probably play a big part in the high variation.

One challenge in using microalgae in diets for carnivorous fish is the indigestible microalgae cell wall, which reduces bioavailability and prevents full utilization of nutrients and energy. About 10% of microalgal dry matter is composed of cell walls (Becker, 2007). There is great diversity in the composition of plant cell walls in general and in microalgal cell walls (Domozych et al., 2012, Scholz et al., 2014). The cell walls of the different species can be made up of a variety of compounds like proteins, lipids, pigments, tannins, lignin, and carbohydrates, especially polysaccharides, which include cellulose, chitin, pectins, fucans, algaenan, alginates and carrageenans (Scholz et al., 2014, Alhattab et al., 2019). A range of studies using different microalgae species over the last decade have largely concluded that the inclusion of microalgae in salmon diets should be kept below 10% to avoid decreased

digestibility and increased feed conversion ratio (Tibbetts, 2018, Teuling et al., 2017a, Sørensen et al., 2017, Kiron et al., 2016, Kiron et al., 2012, Sørensen et al., 2016).

The microalgae cell walls hinder extractability of chemical compounds and it is assumed that the digestive enzymes in fish gastrointestinal tract are unable to access the nutrients (Teuling et al., 2017a). Disruption of cell walls has therefore been a prioritized field of research, not only to improve the utilization of microalgae in fish diets, but also to extract oils and other valuable components such as pigments and PUFAs by the biodiesel and biotechnology industries. For the feed industry, disruption of microalgae cell walls is warranted to release the cell contents in order to improve either digestibility and nutrient availability, or product yield and extraction efficiency (Alhattab et al., 2019).

1.2.3 Disruption of microalgae cell walls to release cell content

Disruption of microalgae cell walls can be done by different methods. The various processing methods have been divided into four categories: chemical, biological, physical, and mechanical (Lee et al., 2017, Li et al., 2020a). Chemical processing requires use of chemicals, and include treatment with acid, oxidants, or surfactants. Ulloa et al. (2012) succeeded in disrupting the cells of *T. suecica* and increasing the yield of pigments using surfactants. Biological methods could be enzymatic lysis and algicidal treatment. Batista et al. (2020b) tested both physical disruption and use of enzymes to increase ADCs of protein, dry matter and energy when various microalgae diets (*N. oceanica*, *Chlorella vulgaris*, or *Tetraselmis* sp.) were fed to European seabass (*Dicentrarchus labrax*). The authors found that the efficiency of the tested cell wall disruption methods varied between the species of microalgae examined. Examples of physical methods include drying, microwaving, sonication, and pulsed electric fields (PEF) treatments. Becker (2007) compared the effect of different drying methods on the digestibility of a few microalgae and cyanobacteria. Kokkali et al. (2020) used PEF and solvents to extract antioxidants from *T. chuii* and *Phaeodactylum tricornutum*. Pataro et al. (2019) used PEF in combination with supercritical CO_2 to extract pigments from *N. oceanica*. Bead milling and high-pressure homogenization are mechanical types of cell disruption. Teuling et al. (2017a) disrupted the cells of three

microalgae (*C. vulgaris*, *Scenedesmus dimorphus* and *N. gaditana*) by bead milling, and found that roughly 50% of the algae cells were broken after 20-30 minutes of treatment, depending on the species. In a different study, Teuling et al. (2017b) found that the duration of bead milling required to completely disrupt the cells of the microalgae *Tetraselmis impellucida, N. gaditana*, and *S. dimorphus* were 30, 45, or 60 minutes, respectively.

To develop the next-generation feed ingredients one must have an understanding of the ability of the fish to assimilate the nutrients from the novel components. In this context, the fish gastrointestinal system should be examined through different approaches.

1.3 Gastrointestinal tract of salmon

1.3.1 Macromorphology

The gastrointestinal tract of Atlantic salmon (Figure 2) is similar to other carnivorous teleost fishes. Carnivorous fish are adapted to digest prey rich in protein and lipid, and they have low capacity to digest large amounts of carbohydrates (Grosell et al., 2011). The gastrointestinal tract is therefore relatively short. The short and straight oesophagus leads the food into the U-shaped stomach, where digestion starts. The oesophagus and stomach are both thick-walled with longitudinal primary folds (Løkka et al., 2013). In the stomach, gastric glands secrete hydrochloric acid and pepsinogen, which is converted to pepsin. Goblet cells are notably absent in the stomach, and hence, mucus is secreted by the epithelial cells in this part. An extra layer of smooth muscle (muscularis mucosa) contributes to the churning and blending of food (Løkka et al., 2013, Grosell et al., 2011). The stomach ends in a pyloric sphincter, which marks the start of the thin-walled intestine. Here, the epithelium consists of a single layer of columnar absorptive cells with an apical brush border, and scattered enteroendocrine cells, intraepithelial leucocytes, and mucus-secreting goblet cells (Grosell et al., 2011, Løkka et al., 2013). As the food enters the intestine, it is mixed with secretions from the liver, gall bladder and pancreatic tissue. These include bile acids that emulsify lipids, bicarbonate that neutralizes the acidic pH, and enzymes such as lipase, amylase and trypsin that digest lipids, carbohydrates, and peptides, respectively. Atlantic

salmon has, as a strict carnivore, limited capacity to produce and secrete amylase (Grosell et al., 2011). The proximal intestine branches off into numerous blind-end tubular appendages, which are called pyloric caeca, increasing the surface area for digestion and absorption of nutrients. The mucosa of the pyloric caeca appears identical to the main intestinal tract, except for a reduction in the number of goblet cells (Grosell et al., 2011, Løkka et al., 2013). Both these sections of the intestine have longitudinal folds. The transition to the mid intestine is marked by the lack of pyloric caeca, and the presence of irregular folds. The transition from mid to distal intestine is more pronounced, identifiable by an increase in diameter, darker colour, circular complex folds, and transverse blood vessels which can be seen externally as dark bands. The epithelium of the distal intestine is composed of columnar absorptive cells, leucocytes, and goblet cells as in the other intestinal regions (Løkka et al., 2013). Digestion and absorption of nutrients continues even here, as opposed to the mammalian colon (Grosell et al., 2011).

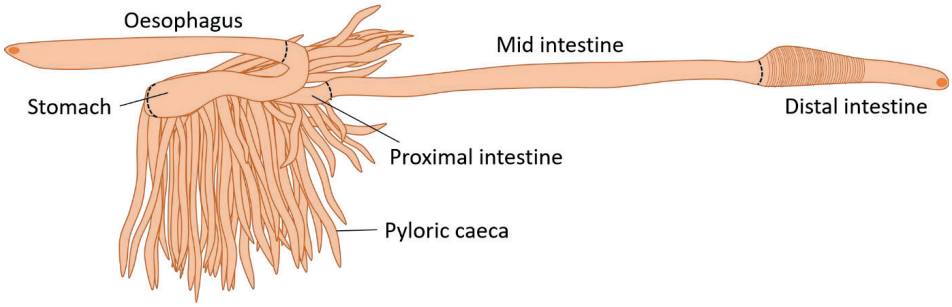

Figure 2. Illustration of the gastrointestinal tract of Atlantic salmon. Shown are the oesophagus, stomach, proximal intestine, pyloric caeca, mid intestine, and distal intestine.

1.3.2 Micromorphology

In general, the intestinal wall of vertebrates is divided into four layers, and, starting from the lumen, they are the mucosa, submucosa, muscularis, and serosa (Figure 3). The mucosa consists of the epithelium, and the connective tissues of the lamina propria, stratus compactum and stratum granulosum. If present, the muscularis mucosa is also part of this layer. The submucosa is an additional layer of deeper and looser connective tissue. The muscularis contains two layers of muscle: an inner circular and an outer longitudinal layer.

These are made up of smooth muscle, except in the oesophagus where they consist of skeletal muscle. Lastly, the serosa is a thin layer of connective tissue and a single-cell layer of mesothelial cells (Grosell et al., 2011, Løkka et al., 2013).

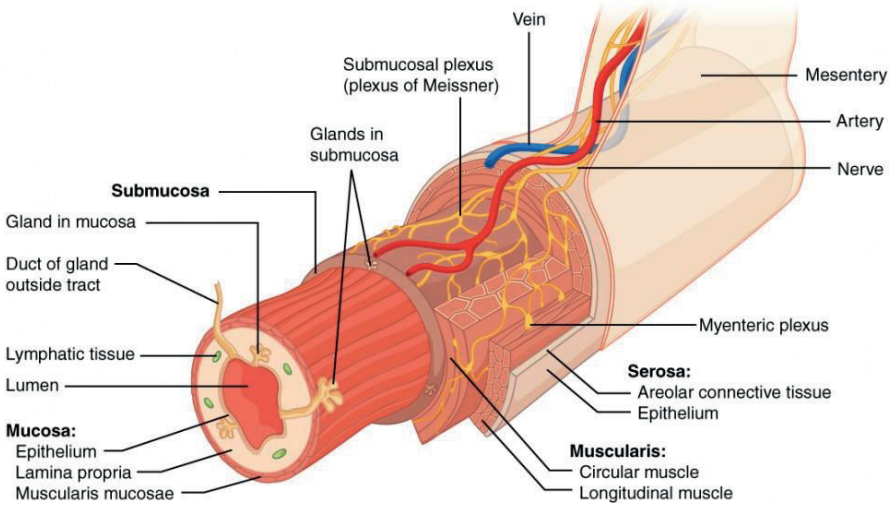

Figure 3. Diagram of the different layers of the alimentary canal. There are four basic tissue layers, and starting from the lumen they are the mucosa, submucosa, muscularis, and serosa (Chruścik et al., 2021). Copyright: *Fundamentals of Anatomy and Physiology* is licensed under a Creative Commons Attribution ShareAlike License by the University of Southern Queensland, Toowoomba, Australia.

The gastrointestinal tract first and foremost has a role in digestion and absorption of nutrients. However, it is a complex and multifunctional organ with important roles in osmoregulation, the endocrine system, and the immune system. The mucosa is an important barrier to prevent pathogens from entering the organism via the gastrointestinal tract (Grosell et al., 2011).

1.4 Immune system in fish

The aquatic environments in which fish are living are also habitat to a range of microorganisms and pathogens. The fish immune system maintains a defensive barrier between the inside and outside of the organism to protect the fish from infections, pathogens and harm. The immune system is divided into two parts – the adaptive immune

system and the innate immune system, which are also known as the specific and non-specific immune system, respectively. The two parts complement each other and work together to keep the fish healthy.

Important organs and tissues associated with the salmonid immune system are the thymus, head kidney, spleen, the recently discovered salmonid bursa, and the mucosa-associated lymphoid tissues which are found in the skin, gills, gastrointestinal tract, and olfactory organ (Bjørgen and Koppang, 2021, Press and Evensen, 1999).

1.4.1 The adaptive immune system

The adaptive immune system targets pathogens through specific receptors and antibodies produced by specialized cells. Although this immune response is slow at first, caused by the time needed to proliferate cells, express the required receptors and produce antibodies, this arm of the immune system makes the organism capable of mounting a highly targeted attack on the offending pathogen. Important components of the adaptive immune system are lymphocytes, namely T-cells and B-cells, antibodies like immunoglobulins, and cytokines like chemokines, interferons, and interleukins (Tort et al., 2003, Castro and Tafalla, 2015).

One of the greatest benefits of the adaptive immune system is its ability to remember previously encountered pathogens by producing and keeping memory B-cells with the capacity to produce specific antibodies corresponding to the antigen of the pathogens (Castro and Tafalla, 2015). The adaptive immune system enables the use of vaccines to achieve immunity against certain diseases. All salmon in Norwegian aquaculture are vaccinated against the most common bacterial diseases before they are transferred to sea cages (Lillehaug, 1997).

1.4.2 The innate immune system

The innate immune system provides general barriers and protection against all pathogens and foreign bodies inside or on the surface of the organism. Barriers created by the skin and mucosal surfaces keep the organism separated from the environment. The innate immune

system quickly removes or incapacitates discovered pathogens by secretion of antimicrobial peptides (AMPs) and enzymes. These interact with the cell wall of microorganisms and cause their lysis (Tort et al., 2003, Castro and Tafalla, 2015). The activation of immune cells such as macrophages and natural killer cells also facilitates the destruction of pathogens. For instance, receptors on the surface of macrophages bind to pathogen-associated molecular patterns (PAMPs). This results in phagocytosis of the pathogens (Castro and Tafalla, 2015).

Antimicrobial peptides (AMPs)

AMPs are short peptides with antimicrobial activity that are produced and found in all kingdoms of life, and they have broad and potent anti-bacterial, anti-viral, and anti-parasitic activities. The peptides are divided into families such as hepcidins, defensins and cathelicidins, and AMPs belonging to each of these families are found in fish. In addition, a new AMP family named piscidins has been discovered that is unique to fish (Rakers et al., 2013, Masso-Silva and Diamond, 2014). AMPs are mainly expressed and produced throughout the mucosal and immune tissues of fish such as skin, gills, intestine, head kidney and spleen, and some are expressed in fish immune cells such as granulocytes. The peptides most often disrupt the cell membrane of microorganisms, for example by pore formation as done by piscidins and defensins, though some AMPs such as the hepcidin family seem to kill microorganisms through other mechanisms. The antimicrobial activity of AMPs against specific pathogens varies between fish species (Masso-Silva and Diamond, 2014). There is also evidence that fish AMPs can have direct immunomodulatory effects, both by activating expression of immune-related compounds such as interleukins and cytokines (Masso-Silva and Diamond, 2014), which are important signalling molecules in the immune system, and by attracting immune cells such as head kidney leucocytes by chemotaxis (Cuesta et al., 2011).

Research on salmonids has demonstrated that AMP gene expression is stimulated by exposure of the fish to bacteria (Chang et al., 2006, Casadei et al., 2009). Fish AMP genes are also activated by the presence of bacterial cell components, viruses, fungi and parasites, and may be induced by cytokines (Masso-Silva and Diamond, 2014). Furthermore, Reyes-Becerril

et al. (2013) found that dietary microalgae and LAB supplementation caused an increase in head kidney AMP gene expression in gilthead sea bream (*Sparus aurata*), demonstrating that feed ingredients may affect AMPs. In addition, crowding stress has been shown to induce expression of AMPs in skin and gills of fish (Terova et al., 2011).

Both adaptive and innate components of the immune system are present in the mucosal surfaces to help fish to thrive under the demanding circumstances of the aquatic environment.

1.4.3 Mucosal surfaces – important for barrier function in fish

Mucosal surfaces covering all surfaces in contact with the environment are important barriers to keep the fish healthy. Spread throughout these mucosal surfaces are aggregates of immune cells such as B- and T-lymphocytes, and these sites are referred to as the mucosa-associated lymphoid tissue (MALT). MALT is further classified according to the mucosal sites of the fish: skin associated lymphoid tissue (SALT), gut associated lymphoid tissue (GALT), gill associated lymphoid tissue (GIALT), and nasal associated lymphoid tissue (NALT) (Castro and Tafalla, 2015).

The intestine of fish is their biggest immune organ, and the distal intestine and its mucosal surface have central roles in the fish immune system. The GALT of fish is more diffuse compared to mammals, that is, it is not organised and compartmented. Instead, the various immune cells are spread throughout the tissue of the intestine (Rombout et al., 2011, Salinas, 2015). Immune activity has generally been reported to be higher in the distal intestine compared to the proximal and mid intestine of Atlantic salmon (Harstad et al., 2008, Koppang et al., 1998a, Koppang et al., 1998b), and also in other fish (Calduch-Giner et al., 2016).

Mucus and mucins

Mucus layers cover the surfaces of the body facing the external environment and are important to protect the organism from pathogens and harm from the external environment

(Peterson, 2015). The skin and gills of fish, the lungs and airways of mammals, and the gastrointestinal tract of both are examples of such mucosal surfaces. The teleost and mammalian gut mucosa have a lot in common (Gomez et al., 2013). The mucus gel has many functions. It lubricates the mucosal surfaces to avoid injuries, it forms a barrier to prevent pathogens from reaching and entering the epithelial cells, and it forms a structure to hold components of the immune system such as AMPs and antimicrobial enzymes (McGuckin et al., 2011).

Mucins are high molecular weight glycoproteins which are both components of secreted mucus and attached to the epithelial cells of mucosal surfaces. Mucins are essential for not only maintaining the viscous and elastic properties of mucus, but also for enabling its barrier function (Thornton and Sheehan, 2004). In addition to mucins, secreted mucus contains water, ions, other proteins, and some lipids. Mucins may be classified as monomeric, which are mainly bound to the cell surface, or multimeric, which are mainly secreted (Thornton and Sheehan, 2004, Linden et al., 2008). However, this is not a strict rule, as some cell-surface mucins are multimeric and some monomeric mucins are found in secreted mucus (Carraway et al., 2003). Multimeric mucins are also known as oligomeric mucins or gel-forming mucins.

Mucins are comprised of a filamentous protein backbone with several distinct domains (Bansil and Turner, 2006). Some regions of the protein backbone often have a high number of carbohydrates found in branched oligosaccharide side chains, which are attached to the amino acids through their hydroxyl groups. This type of carbohydrate attachment is often termed as *O*-glycosylation, and gives the mucin its characteristic “bottle-brush” appearance (Bansil and Turner, 2006). Glycosylation patterns of the mucin proteins are very diverse and mucins from even neighbouring goblet cells may be differentially glycosylated (McGuckin et al., 2011). O-glycosylated mucins on the cell surface act as decoys to divert pathogens and prevent their adhesion, thereby protecting the epithelial cells (McGuckin et al., 2011, Ashida et al., 2012). The carbohydrates also take part in electrostatic interactions and bind a lot of water, contributing to the properties of the mucus gel (Yang et al., 2012).

Mucin secretion

Mucus is continuously synthesised and shed, providing an opportunity for quick modulation of responses to external signals (Linden et al., 2008). In the gastrointestinal tract, mucins are mainly secreted by goblet cells. A study on developing stages of zebrafish (*Danio rerio*) found that differentiation of goblet cells in the emerging intestine happens around 102 hours post fertilization, and that the earliest goblet cells secrete only acidic mucins. Later, the goblet cells also start synthesizing neutral mucins. The reason for the specific production of acidic mucins might be that these are less susceptible to bacterial degradation (Ng et al., 2005).

The mucus layer is relatively thick in the stomach and intestine compared to other mucosal surfaces, and new mucus is produced continuously as old mucus is shed (Moncada et al., 2003). In mammals, intestinal mucus forms two distinct layers. The outer layer is looser and more colonized by bacteria and other microorganisms, while the inner layer is denser and more impermeable to microorganisms (Johansson et al., 2008, Johansson et al., 2011). The mucins in secreted mucus and on the epithelial cell surface protect the organism from pathogens by preventing their access to the epithelial cells by steric hindrance or providing decoy binding sites (Moncada et al., 2003, Ashida et al., 2012). In addition, the gel-forming mucins create a matrix of interconnected molecules that can hold antimicrobial compounds and other immune system components to prevent the molecules from immediately diffusing away (McGuckin et al., 2011, Linden et al., 2008).

Mucus can be modulated by changing the number of goblet cells or their activity, the composition of the mucus, or the composition of the mucins. Mucin secretion is influenced by factors such as diet, stress or presence of pathogens (Salinas and Parra, 2015). Infection with an intestinal parasitic worm increased the number of goblet cells in the intestine of wild European chub (*Squalius cephalus*, Bosi et al., 2015). The same response was later observed in several other fish species infected with various intestinal parasites (Bosi et al., 2017). In gilthead sea bream, infection with an intestinal parasite caused changes to mucins isolated from intestinal mucus (Estensoro et al., 2013).

Mucin genes

Mucin proteins are encoded by MUC genes. Twenty one MUC genes are identified in humans, and they are differentially expressed throughout the mucosal sites of the human body. Homologues of most of these mucin genes have been found in other species (Lang et al., 2016). Several mucin genes have also been identified or predicted in fish genomes.

Lang et al. (2004) predicted six monomeric and multimeric mucins in the pufferfish (*Takifugu rubripes*) genome. Later, a study involving the same researchers identified several mucins and mucin-related proteins in zebrafish and pufferfish. This study also concluded that gel-forming mucins are evolutionarily related and seem to have appeared during early metazoan evolution, while cell-surface mucins originated in vertebrates (Lang et al., 2007). Later, the same group identified eleven gel-forming mucins in zebrafish and analysed the evolutionary relationship between mucins of many different species (Lang et al., 2016). Yet another study that investigated the developmental stages of zebrafish used image techniques to visualize cell-surface mucin-type *O*-linked glycoproteins in several organs and tissues (Laughlin et al., 2008). Pérez-Sánchez et al. (2013) identified six mucin genes in gilthead sea bream. van der Marel et al. (2012) characterized two mucin genes in common carp (*Cyprinus carpio*). A study on Atlantic salmon identified seven secreted gel-forming mucins across several tissues and studied their transcription (Sveen et al., 2017). It is likely that more mucin genes will be discovered in fish as more genomes are sequenced and studied. Removal of the *muc2* gene has severe negative effects, as studies on mice have shown that this may lead to development of atypical goblet cells, colon cancer, and inflammation (Velcich et al., 2002, Van der Sluis et al., 2006). Various mucin genes have been proposed as markers for issues like diseases (Sheng et al., 2012), stress (Sveen et al., 2017), and parasites (Marcos-López et al., 2018) in several animals.

Microbiota

Associated with the mucosal surfaces of all animals is the complex community of microorganisms termed as microbiota. The microbiota include a variety of viruses, yeast and bacteria, and they influence the host organism's digestion and nutrition, and its immune

system and disease resilience. The gills, skin and gut of fish are all populated with their own microbiota. The largest microbe community is found in the gut, and it has a great impact on gut development and health (Romero et al., 2014, Merrifield and Rodiles, 2015).

The composition of the gut microbiota varies both between and, to a certain degree, within fish species. Dietary factors such as feed ingredients, lipid and protein levels, and feed additives have all been reported to cause changes to the GI microbe community (Romero et al., 2014). The microbiota is also influenced by environmental factors such as seasonal changes, temperature, and water salinity. Despite all these external influences there seems to exist a core microbiota which does not change, probably caused by a set of selection pressures which are unique to each host fish (Merrifield and Rodiles, 2015).

Since the microbiota has an impact on the host organism, a promising aquaculture strategy is to modulate the composition of the microbiota to improve fish health and nutrition (Romero et al., 2014). This will be further addressed in chapter 1.5.2.

1.4.4 Antioxidant system

Oxidation of components and compounds in cells can cause harm to an organism and plays a role in many diseases. Reactive oxygen species (ROS) such as hydrogen peroxide or various oxygen radicals may react with for example cell membranes, enzymes, DNA, or lipids and damage their structure and impede their function. To prevent damages, animals have evolved an antioxidant system, the task of which is to remove ROS from the organism before any harm is done (Jacob, 1995). These ROS may originate as by-products from enzymes or from the basal cell metabolism, or they can be caused by external forces such as radiation (Forrester et al., 2018).

The antioxidants that act in animals include (1) enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), (2) endogenous antioxidants such as NADPH/NADH and glutathione, (3) dietary antioxidants such as vitamin C, vitamin E, and carotenoids, and (4) metal binding proteins like ferritin and myoglobin. All of these components facilitate or react in different ways to convert harmful ROS into various

harmless compounds (Jacob, 1995). The antioxidant system is highly active in central organelles such as the mitochondria, peroxisomes and endoplasmic reticulum (Forrester et al., 2018). While the antioxidant system is present in all tissues, the liver is the key organ involved in metabolism and detoxification of blood, and is an important site of antioxidant enzyme activity (Lemaire et al., 1994).

1.5 Impact of feed ingredients on health of the intestinal mucosa

Intestinal health is dependent on the structure, morphology and function of the intestinal mucosal surface, and is important for the intestine to be able to fulfil its normal roles. The intestine has barrier functions, immune functions, and digestive and nutrient uptake roles which are essential for the fishes' health and growth. Feed ingredients and feed additives in the diet may have major impacts on the intestinal health of farmed fish. Plant protein ingredients may contribute with antinutritional factors, and use of plant oils disturbs the n-6/n-3 fatty acid ratio and may also contain phytosterols that may interfere with lipid metabolism (Sissener et al., 2018).

Plant protein ingredients and antinutritional factors

Antinutritional factors (ANFs) present in both terrestrial and marine derived plant ingredients may have negative effects on the health of the fish, or interfere with growth and nutrient utilization, thus limiting the potential use of these ingredients in aquaculture diets (Krogdahl et al., 2010, Silva et al., 2015, Oliveira et al., 2009). The compounds have multiple functions in plants (Table 2). The ANFs can be divided into heat labile and heat stabile ANFs (Refstie, 2007, Storebakken et al., 2000). The negative effects can be avoided by (1) removing the ANFs from the ingredient, for example by selective breeding, dehulling or extraction, (2) inactivating or altering the ANFs, for example heat inactivation of enzymes, or (3) supplementing the diet to avoid specific nutrient deficiencies caused by ANFs, for example cholesterol supplementation to counter phytosterols (Krogdahl et al., 2010). Plant protein concentrates are commonly used in commercial fish diets to avoid negative effects of antinutritional factors (Ytrestøyl et al., 2015, Aas et al., 2019).

Table 2. Some common antinutritional factors in plants and their potential effect on fish.

Antinutritional factor	Found in	Potential effect on fish	HL/HS*
Enzyme inhibitors	Legumes, especially peas and beans	Inhibit digestion	HL
Lectins	Plant seeds	Bind to cell receptors in the gut and can alter metabolism, increase mucus secretion and damage intestinal villi	HL
Glucosinolates	Rapeseed	Alter thyroid structure and activity, potential liver and kidney damage	HS
Fibres, non-starch polysaccharides (NSPs)	All plants	Reduce digestibility, can bind to and interfere with digestion of other nutrients	HS
Oligosaccharides	Legumes, grains, cereals	Bind to bile acids, affect digestive enzymes, and alter the microbiota	HS
Phytic acid/phytate	Soybean, rapeseed, sesame seed	Binds minerals and contains non-bioavailable phosphorous	HS
Phytosterols	Legumes	Interfere with cholesterol uptake and metabolism	HS
Saponins	Legumes	Hinder lipid and protein digestion, possibly increase the permeability of the gut mucosa	HS
Tannins	Rapeseed, beans	Bind to enzymes, proteins or minerals, reduce uptake of vitamin B_{12}	HS

*Heat labile - HL, Heat stabile - HS. Information from (Krogdahl et al., 2010, Francis et al., 2001).

Plant lipid ingredients and fatty acid composition

Use of plant oils in fish diets may have an impact on fish morphology and health, depending on the source and inclusion levels. It is widely known that the fatty acid composition in fish is dependent on the fatty acid composition of the diet. A change in dietary fatty acid composition will also be reflected in the fish intestinal tissue (Bou et al., 2017a, Kousoulaki et al., 2020), and may be a trigger for inflammation. Plant oils have different fatty acid compositions than fish oil, as they (1) lack the long PUFAs like EPA and DHA and (2) have an imbalanced n-6/n-3 fatty acid ratio (National Research Council, 2011, Moldal et al., 2014). In mammals, n-6 fatty acids are pro-inflammatory, while n-3 fatty acids are anti-inflammatory (Moldal et al., 2014).

1.5.1 Microbial feed ingredients

Feed ingredients and additives can be used purposefully to improve intestinal health. Microbial ingredients such as microalgae, bacteria and yeast (or compounds derived from them) in fish diets have been found to influence the intestinal health of fish in several ways.

Studies are already published reporting effects of microalgal ingredients on growth of fish and digestibility of nutrients, but information about the effects on intestinal health is sparse (Batista et al., 2020a, Skalli et al., 2020, Kiron et al., 2016, Sørensen et al., 2017, Kousoulaki et al., 2015). It is therefore important to generate more information on the effect of using novel feed ingredients on gut health.

Bacterial meal incorporated in diets for Atlantic salmon (in fresh water and salt water) and rainbow trout did not cause any adverse effects on the fishes' intestinal morphology (Aas et al., 2006b, Berge et al., 2005, Storebakken et al., 2004), but rather induced positive effects (Romarheim et al., 2011). On the other hand, the use of probiotic bacteria is gaining ground in aquaculture and will be described in section 1.5.2.

In a feeding trial with Atlantic salmon, during smoltification, the fish fed 25% dietary yeast had increased feed intake and growth rate compared to the control group. Yeast-fed fish also showed decreased production of pro-inflammatory immune components (IFNγ, TNFα, IL-1β, IL-8) (Sahlmann et al., 2019). Supplementation of fish diets with β-glucans derived from yeast have been shown to improve protection and survival during a pathogen infection (Guselle et al., 2007). β-glucans may enhance fish immune responses through recognition by specific receptors and immune cell activation as in the case of phagocytosis and cytokine production. β-glucans can also bind to pathogenic bacteria, which may hinder the bacteria from attaching to or infiltrating fish enterocytes (Agboola et al., 2021). This mode of action has also been reported for mannan oligosaccharides (MOS), which are components of yeast cell walls. MOS have also been shown to boost fish growth, influence gut morphology, and improve the barrier function of mucosal surfaces (Agboola et al., 2021).

1.5.2 Feed additives and probiotics

Feed additives

Feed additives are compounds added to diets with the purpose of improving fish performance, product quality, or the physical or chemical properties of the diet. Some examples are antimicrobial agents, antioxidants, binders, pigments, feeding stimulants, enzymes, organic acids, immunostimulants, probiotics and prebiotics (National Research Council, 2011). Immunostimulants such as synthetic chemicals, animal or plant extracts, bacterial or yeast derivatives, algal derivatives, and cytokines are feed additives that stimulate the immune activity of fish (Caipang and Lazado, 2015).

Prebiotics are used as feed additives to selectively stimulate the growth or activity of beneficial bacteria, for example by creating a favourable environment for the desired bacteria or catering to their specific metabolic needs. Some examples of prebiotic compounds commonly used in aquaculture are fructo-, galacto- and mannan oligosaccharides, and inulin, which are sugars fermented by probiotics (Caipang and Lazado, 2015, Lauzon et al., 2014).

Studies have also explored the use of microalgae as feed additives, rather than as main ingredients. Some studies reported increased antioxidant enzyme activity, improved immune responses, positive effect on fillet colouration, and improved growth performance (Subramaniam et al., 2019). One study using *N. gaditana* supplemented to diets for gilthead sea bream reported that the richness of the microbiota was enhanced by the microalgae, while the micromorphology of the intestine remained unaffected (Jorge et al., 2019).

Probiotics

Probiotics are live cultures of microorganisms with the purpose of modulating the host microbiota in a way that is beneficial to the host (Caipang and Lazado, 2015, Lauzon et al., 2014). The gut microbiota influences the host in several ways. The presence of the microbiota is crucial during the development of the digestive tract (Romero et al., 2014),

and the development and continued immune activity of the GALT (Pérez et al., 2010). In addition, the commensal or symbiotic bacteria, which are part of the microbiota, compete with invasive pathogens for nutrients and adhesion sites, and some produce various antibacterial compounds to create a disadvantageous environment for other bacteria (Merrifield and Rodiles, 2015). Genes related to cell proliferation, nutrient metabolism and innate immune response have been shown to be influenced by the gut microbiota (Romero et al., 2014).

Probiotics may influence the host microbiota by excreting inhibitory compounds, hindering pathogen adhesion, producing enzymes or other bioactive compounds, or modulating the immune activity in the gut (Caipang and Lazado, 2015). Common reported beneficial outcomes of probiotics in various aquaculture species are improved growth performance, disease resistance, feed utilization, digestive enzyme activity, and increased immune-related parameters (Ringø et al., 2020). When used in aquaculture, probiotics are usually provided to the fish through their diet, though it is also possible to administer them through the water (Nayak, 2010, Klakegg et al., 2020). The probiotics used must be safe for the host as well as the environment. The label of "Generally recognized as safe" (GRAS) was launched by the United States Food and Drug Administration (FDA) and is designated to probiotics (and other food and feed ingredients) that are deemed to have no safety concerns when included in food. Lactic acid bacteria (LAB) is a group of bacteria often investigated in probiotic studies for aquaculture, and they include *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Enterococcus* (Ringø et al., 2020). LAB are known to have beneficial effects when applied to humans (Lauzon et al., 2014), and many strains of LAB have been assigned the GRAS label by the FDA (Plavec and Berlec, 2020). Other bacteria often researched for aquaculture probiotics are *Aeromonas*, *Alteromonas*, *Arthrobacter*, *Bacillus*, *Bifidobacterium*, *Clostridium*, *Paenibacillus*, *Phaeobacter*, *Pseudoalteromonas*, *Pseudomonas*, *Rhodosporidium*, *Roseobacter*, *Streptomyces* and *Vibrio* (Ringø et al., 2020).

2. Objectives

The general objective of my PhD thesis was to investigate the effect of diets containing different feed ingredients or feed additives on fish growth, nutrient digestibility and mucosal health and barrier function, with main emphasis on the intestinal health of farmed Atlantic salmon.

This thesis is based upon four feeding experiments with Atlantic salmon. The studies were designed to investigate the health of fish fed plant-derived ingredients, several species of microalgae, feed additives and probiotics in diets with high or low content of fish meal and fish oil.

The main hypothesis was that the growth, feed utilization, nutrient digestibility and the mucosal health of the intestine, skin and gills of Atlantic salmon are affected by feed ingredients.

The feed ingredients used in experimental diets in this thesis can be categorized as follows:

(1) plant-derived protein and lipid sources (Papers I, III & IV)

(2) novel ingredients, such as microalgae (Papers II & III)

(3) feed additives and probiotics (Papers III & IV)

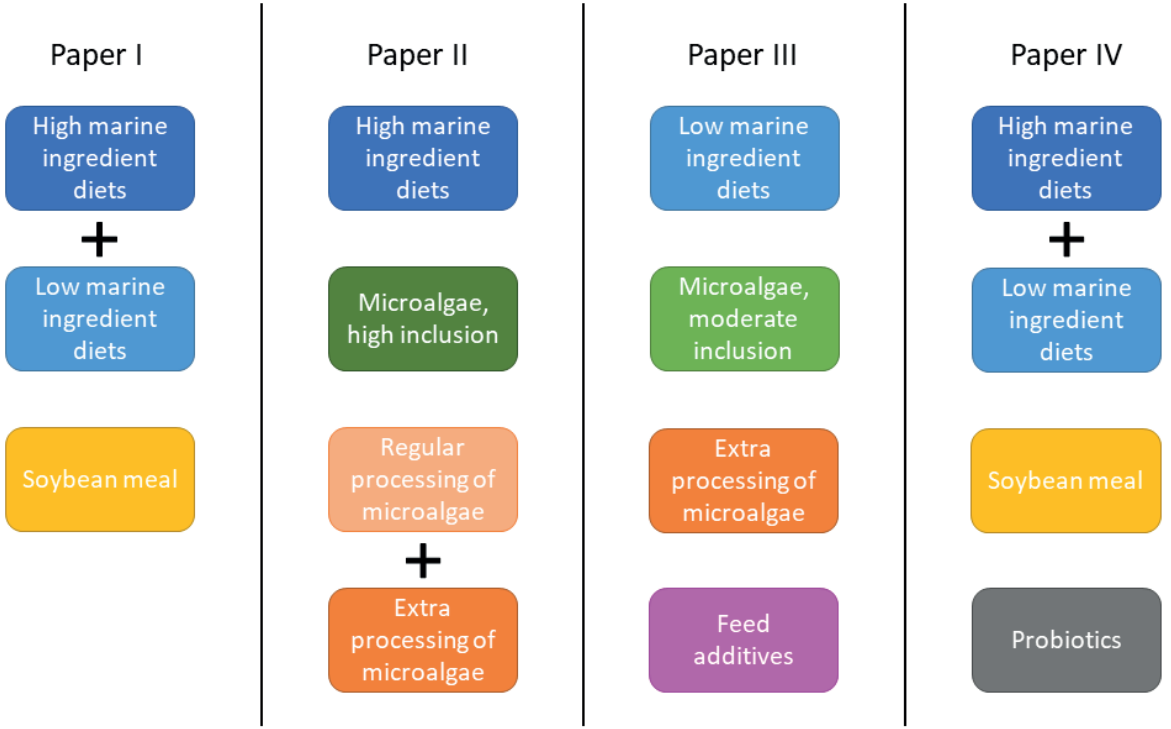

Figure 4. Overview of the experiments performed to generate data for Papers I-IV presented in this thesis.

3. Summary of papers: Main results

This thesis is based on four papers summarising findings from four feeding experiments with farmed Atlantic salmon smolt (Figure 4). All experiments were performed at the Research Station of Nord University.

Paper I: Nutrient Digestibility, Growth, Mucosal Barrier Status, and Activity of Leucocytes from Head Kidney of Atlantic Salmon Fed Marine- or Plant-Derived Protein and Lipid Sources

In this paper, Atlantic salmon smolt were fed four diets containing marine or plant-derived protein and lipid sources, plus one diet with soybean meal, which is known to cause enteritis (positive control). The mucosal barrier status of the skin, gills, and distal intestine of the fish were evaluated by analysing the gene expression of mucins (*muc2, muc5ac1, muc5ac2, muc5b*) and AMPs (*def1*, *def2*, *def3*, *def4* and *cathl1*), in addition to goblet cells and intestinal micromorphology. The activity of immune cells isolated from the head kidney, the growth of the fish, and the digestibility of macronutrients were also examined.

Our results showed that salmon fed the soybean meal diet developed enteritis and had lower expression of *muc2* in the distal intestine, indicating a reduced barrier function. Additionally, fish fed the soybean meal diet showed reduced concentration of blood cholesterol and lower weight gain compared to the other diets. Fish fed diets based on plant protein concentrates with or without rapeseed oil had a compromised intestinal barrier and increased volumetric density of goblet cells. The mucosal barrier status of the fish was thus altered either by reducing goblet cell size and volumetric density of goblet cells (soybean meal diet), or by increasing volumetric density of goblet cells (plant protein diets).

Paper II: Growth, Chemical Composition, Histology and Antioxidant Genes of Atlantic Salmon (*Salmo salar*) Fed Whole or Pre-Processed *Nannochloropsis oceanica* and *Tetraselmis* sp.

Atlantic salmon smolts were fed diets with a high inclusion (30%) of one of the two species of microalgae, either *Nannochloropsis oceanica* or *Tetraselmis* sp., or a fish meal based control diet. One set of microalgal diets was subjected to extra processing (pre-extrusion) to examine potential effects of this procedure.

The results showed that high inclusion of microalgae had minor effects on Atlantic salmon intestinal morphology, though we observed increased liver vacuolization and antioxidant response. The fish fed the unprocessed microalgae had reduced enterocyte vacuolization in the distal intestine compared to the control diet. Processing of microalgae also had an impact on the liver structure and tended to improve weight gain. Inclusion of microalgae in the diets changed the fillet fatty acid composition. A significant effect of microalgae processing was also found on the fillet fatty acid composition, but the results were not consistent for the two algae. Our conclusion was that processing of microalgae tended to increase the bioavailability of nutrients, releasing more of the bioactive compounds that impacted gene expression and fatty acid composition. The increased vacuolisation of hepatocytes and enterocytes must be studied further to ascertain their cause.

Paper III: Approaches to improve utilization of *Nannochloropsis oceanica* in plant-based feeds for Atlantic salmon

In this study, Atlantic salmon were fed four different diets; a low fish meal control diet or three low fish meal diets with 10% inclusion of the microalgae *Nannochloropsis oceanica*, without or with the commercial feed additives Digestarom or ZEOFeed.

The results showed that including 10% *N. oceanica* did not affect the growth or whole-body proximate composition of Atlantic salmon, and we did not notice any benefits of the feed additives on growth or feed utilization. Small changes in fatty acid composition were observed among the diets, but notably the contents of EPA + DHA were not altered in the

fish fed the microalgae diets, even though the microalgae diets contained 50% less fish meal and 10% less fish oil compared to the control diet. Cell proliferation in the distal intestine was increased in all *Nannochloropsis* diets, but only significantly increased for two of them, most profoundly by the ZEOFeed diet. No significant changes in intestinal morphology or villi height and width were noted among the diets.

Paper IV: Mucosal barrier status in Atlantic salmon fed marine or plant-based diets supplemented with probiotics

This experiment was performed to investigate the use of lactic acid bacteria as a probiotic in Atlantic salmon. Atlantic salmon were fed either a marine-based diet, a plant-based diet, or a diet with 20% soybean meal, all of which were produced with or without LAB probiotics (a mixture of *Lactobacillus fermentum* and *Lactobacillus plantarum*). The soybean meal diet was used to induce inflammation in the salmon, to act as a positive control group.

Our results showed that the soybean meal fed group developed intestinal inflammation, which affected the number of goblet cells, villi height and width, intraepithelial lymphocytes, lamina propria width, and supranuclear vacuoles. Supplementation of probiotics reduced some of these inflammation characteristics. Villi height and width were lower in fish fed the plant and soybean meal diets compared to fish fed the marine diet. The number of goblet cells and intraepithelial lymphocytes in the intestine were increased by addition of probiotics to the marine- and plant-based diet groups. On the other hand, the opposite effect was observed in fish fed the soybean meal diet. In the skin and gills, the number of goblet cells were higher in fish fed the plant and soybean meal diets compared to fish fed the marine diet. Addition of probiotics further increased the number of goblet cells in both the skin and gills. We concluded that the probiotics had an immune modulation potential on the observed mucosal tissues, but not under the inflammatory conditions triggered by the soybean meal diet.

4. General discussion

4.1 Feeding trials

This thesis is based on four feeding trials with Atlantic salmon. Feeding trials designed for evaluation of feed (or ingredient) quality must be performed on healthy fish, with good appetite, and they must be fed in excess. Feed intake should be carefully assessed, preferably by collecting uneaten pellets employing feed waste collection systems. The fish rearing facility, automatic feeders, and solid waste collectors used in our studies are described in detail by Sørensen et al. (2017). Feeding trials are essential to evaluate the potential of new feed ingredients, feed concepts, and their ability to support the growth and health of fish. The growth of the fish during the experiments is a key parameter to determine the utilization of the diets and feed ingredients, and this parameter also reveals the quality of the feeding trials. Feed utilization, i.e. the highest possible growth that could be achieved using a minimal amount of feed, is vital to maximise the economic output in fish farming. New ingredients and feed concepts should always be evaluated through feeding studies before their adoption by the commercial industry.

4.1.1 Growth performance

Growth in terms of weight gain and specific growth rate across four experiments (Papers I-IV) are shown in Table 3. Growth parameters are a measure of how well the diet is converted to a product.

Table 3. Overview of mean initial weight, final weight, weight gain, and specific growth rate (SGR) for all diets across Papers I-IV.

	Growth parameters				Comments
Paper I	**Initial w. (g)**	**Final w. (g)**	**W. gain (%)**	**SGR (% day^{-1})**	High/low marine ingredient diets, 65 days
BG1	72.4 ± 1.2	152.3 ± 4.5^{a}	110.2 ± 7.9^{ab}	1.1 ± 0.1	Marine protein, marine lipid
BG2	71.3 ± 1.0	138.3 ± 5.3^{b}	93.8 ± 7.0^{b}	1.0 ± 0.1	Marine protein, soybean meal, marine lipid
BG3	72.9 ± 1.7	158.4 ± 5.9^{a}	117.2 ± 3.3^{a}	1.2 ± 0.1	Marine protein, plant lipid
BG4	73.5 ± 1.4	150.7 ± 9.4^{ab}	105.1 ± 16.3^{ab}	1.1 ± 0.1	Plant protein, marine lipid
BG5	73.5 ± 0.9	150.3 ± 4.9^{ab}	104.7 ± 8.2^{ab}	1.0 ± 0.1	Plant protein, plant lipid
Paper II	**Initial w. (g)**	**Final w. (g)**	**W. gain (%)**	**SGR (% day^{-1})**	High fish meal diets, 60 days
CO	154.4 ± 0.3	307.8 ± 3.3^{a}	99.4 ± 2.0^{a}	1.15 ± 0.02^{a}	Control diet
NU	154.0 ± 0.2	288.9 ± 10.0^{b}	87.6 ± 6.3^{b}	1.05 ± 0.06^{b}	30% *N. oceanica*
NE	153.9 ± 0.7	292.9 ± 3.5^{b}	90.3 ± 3.0^{a}	1.07 ± 0.03^{a}	30% *N. oceanica*, pre-extruded
TU	154.4 ± 0.3	282.9 ± 6.8^{b}	83.3 ± 4.3^{b}	1.01 ± 0.04^{b}	30% *Tetraselmis* sp.
TE	154.3 ± 0.4	285.1 ± 7.2^{b}	84.8 ± 4.8^{b}	1.03 ± 0.05^{b}	30% *Tetraselmis* sp., pre-extruded
Paper III	**Initial w. (g)**	**Final w. (g)**	**W. gain (%)**	**SGR (% day^{-1})**	Low fish meal diets, 68 days
CO	227.94 ± 5.93	422.77 ± 22.16	85.44 ± 7.80	0.91 ± 0.63	Control diet
NC	228.51 ± 1.82	415.05 ± 25.01	81.61 ± 10.41	0.87 ± 0.08	10% *N. oceanica*, pre-extruded
ND	225.27 ± 1.48	417.28 ± 21.08	86.23 ± 4.74	0.90 ± 0.66	10% *N. oceanica*, pre-extruded, Digestarom feed additive
NZ	227.31 ± 4.24	423.26 ± 11.20	85.21 ± 8.28	0.91 ± 0.38	10% *N. oceanica*, pre-extruded, ZEOFeed feed additive
Paper IV	**Initial w. (g)**	**Final w. (g)**	**W. gain (%)**	**SGR (% day^{-1})**	High/low marine ingredient diets, 38 days
BG1÷	126.99 ± 2.03	194.30 ± 11.47	52.90 ± 6.58	1.12 ± 0.11	Marine protein, marine lipid
BG1+	126.82 ± 3.23	199.54 ± 5.74	57.33 ± 0.51	1.19 ± 0.01	Marine protein, marine lipid, with probiotics
BG2÷	123.43 ± 4.85	205.56 ± 7.12	66.58 ± 0.78	1.34 ± 0.01	Marine protein, soybean meal, marine lipid
BG2+	125.72 ± 1.86	194.24 ± 14.96	54.40 ± 9.61	1.14 ± 0.16	Marine protein, soybean meal, marine lipid, with probiotics
BG5÷	115.08 ± 4.36	187.48 ± 5.30	62.97 ± 1.57	1.29 ± 0.03	Plant protein, plant lipid
BG5+	117.61 ± 4.26	185.25 ± 6.15	57.53 ± 0.48	1.20 ± 0.01	Plant protein, plant lipid, with probiotics

Different superscripts within column indicating significant differences are applicable for each paper and the respective growth parameter.

Weight gain

The duration of the study should aim for obtaining at least a doubling of the initial fish weight. This goal was achieved in the feeding trials for Paper I and II, while the weight gain observed in Paper III was slightly lower. The duration of the feeding trial performed in Paper IV (38 days) stands out, as it only lasted for roughly 55-60% of the time compared to the other three experiments (60-68 days). The relatively short feeding period also explains the low weight gain observed in Paper IV (53-67%). However, Paper IV was designed to test effects of probiotics in functional feeds, which are commonly pulse fed (in shorter time spans) to the fish (Leclercq et al., 2020, Reyes-Cerpa et al., 2018). Thus, the duration of the trial reflected common practice.

Specific growth rate

The specific growth rate (SGR) is measured in percent weight gain per day and values from different studies can only be compared when the seawater temperature and size of fish in the experiments are in the same range (Austreng et al., 1987). Comparison is challenging when the water temperature and size of fish differ among experiments. It is known that the SGR is highest for young fish and decreases as they grow bigger. Thus, experiments using fish with a lower initial weight should have a higher SGR. In our experiments the initial weight of the fish varied from roughly 72 g (Paper I) to roughly 227 g (Paper III). Fish in Paper IV had higher initial weight compared to the other studies because three of the diet groups from Paper I were employed for the trials explained in Paper IV, and the diets were without or with the addition of probiotic LAB coated on to feed pellets.

Overall, Atlantic salmon in all experiments had good growth considering the conditions of each feeding trial (fish initial weight, feeding time, water temperature, ingredient composition). The diets were well accepted by the fish, and their appetite was good. Throughout all the experiments we observed very low to no mortality among the fish.

The SGR of Paper I and II were similar at around 1, while Paper III had slightly lower SGR at around 0.9, and the SGR of Paper IV was in the higher range between 1.12-1.34. These

results are comparable to other studies on Atlantic salmon of similar size (Albrektsen et al., 2018, Hatlen et al., 2012), and they are also comparable to the growth rates estimated by Austreng et al. (1987). The low SGR observed in Paper III is partly explained by the high initial weight of the fish and the low water temperature (7.5°C). The water temperature during the course of the experiment reported in Paper I (7.6°C) was similar to the temperature reported in Paper III, but the start weight of the fish was much lower compared to Paper III. The thermal growth coefficient (TGC) includes water temperature into the calculation as it is based on sum degree-days and not days as in the case of SGR. However, growth prediction based on TGC is not valid for a wide temperature range; such prediction is accurate only for the range where SGR increases with temperature (Jobling, 2003). Average TGCs in Papers I-IV were 2.30, 2.46, 2.71 and 2.86, respectively.

Growth performance was influenced by diet

The growth performance of the fish was influenced by the diet, especially in Papers I and II. In Paper I, the soybean meal fed fish had the lowest final weight and weight gain. In Paper II, the growth of fish fed the microalgae incorporated diets had significantly lower final weight compared to the control fish, regardless of pre-treatment of the algae. However, the experiment performed for Paper II was designed for determination of ingredient digestibility, not for growth assessment. Analysis of the diets (Paper II) upon completion of the experiment revealed that the diets did not contain the marker to determine digestibility. An alternative approach could have been to estimate digestibility of macronutrients using crude fibre in the diet as an inert marker (Krontveit et al., 2014). However, this method was not feasible because the diet and the faeces were no longer available for additional analyses. Nevertheless, it should be noted that the estimated digestibility did vary depending on the fibre content (although with no apparent trend) and fibre source (Krontveit et al., 2014). Hence, this method might have been challenging to use for the microalgae-included diets.

4.1.2 Digestibility

Digestibility of main nutrients is commonly used as a first screening parameter for the assessment of new feed ingredients. Apparent digestibility coefficients (ADCs) were calculated for macronutrients in Papers I and III, and a comparison of the ADC values across the experiments is presented in Table 4.

Table 4. Overview of digestibility of macronutrients in Papers I and III.

	Digestibility of macronutrients (ADC, %)					Comments
Paper I	**Dry matter**	**Protein**	**Lipid**	**Ash**	**Energy**	High/low marine ingredient diets, 65 days
BG1	62.1 ± 3.1^{ab}	81.3 ± 1.7^{b}	90.6 ± 1.3^{b}	-14.1 ± 10.7^{ab}	77.6 ± 1.7^{b}	Marine protein, marine lipid
BG2	66.1 ± 0.6^{a}	86.1 ± 0.3^{a}	87.4 ± 0.2^{c}	0.9 ± 4.6^{a}	77.3 ± 0.6^{b}	Marine protein, soybean meal, marine lipid
BG3	68.4 ± 1.2^{a}	85.5 ± 0.6^{a}	96.4 ± 0.2^{a}	-8.6 ± 1.8^{a}	83.8 ± 0.8^{a}	Marine protein, plant lipid
BG4	59.0 ± 3.4^{b}	86.6 ± 1.4^{a}	92.0 ± 0.9^{b}	-33.2 ± 10.6^{b}	73.1 ± 2.4^{b}	Plant protein, marine lipid
BG5	63.6 ± 5.4^{ab}	88.1 ± 2.2^{a}	95.4 ± 2.4^{a}	-21.0 ± 17.5^{ab}	77.0 ± 3.6^{b}	Plant protein, plant lipid
Paper III	**Dry matter**	**Protein**	**Lipid**	**Ash**	**Energy**	Low fish meal diets, 68 days
CO	63.3 ± 0.52^{b}	87.8 ± 0.11^{ab}	94.3 ± 0.28^{a}	-24.0 ± 2.05^{b}	NA	Control diet
NC	67.5 ± 0.41^{a}	88.5 ± 0.07^{a}	91.3 ± 0.04^{b}	12.9 ± 2.66^{a}	NA	10% *N. oceanica*, pre-extruded
ND	65.3 ± 0.34^{ab}	86.5 ± 0.54^{b}	91.1 ± 0.32^{b}	13.9 ± 1.06^{a}	NA	10% *N. oceanica*, pre-extruded, Digestarom feed additive
NZ	66.1 ± 0.89^{a}	87.9 ± 0.60^{ab}	91.9 ± 0.52^{b}	7.7 ± 0.18^{a}	NA	10% *N. oceanica*, pre-extruded, ZEOFeed feed additive

Different superscripts within column indicate significant differences for each paper.

The ADC of protein in the diets mostly ranged between 85.5% and 88.5%, which is within the expected range of protein digestibility (Albrektsen et al., 2018, Kousoulaki et al., 2015). The only exception was the BG1 diet in Paper I which had a protein ADC of 81.3%. This was unexpected because this diet mainly consisted of high-quality fish meal and fish oil, which is usually highly digestible for Atlantic salmon. In addition, the BG3 diet (Paper I) contained the same amount and the same type of fish meal, yet had a significantly higher protein ADC (85.5%). A possible explanation could be that there was some deviation during the production of the BG1 diet which negatively affected the protein digestibility in the fish meal.

The lipid ADC values ranged between 91% and 96%, which agrees with similar studies (Albrektsen et al., 2018, Hatlen et al., 2012, Kousoulaki et al., 2015). The exception was the BG2 diet (Paper I) which had a lipid ADC of 87.4%. This could be related to the soybean meal induced enteritis that was observed in salmon fed the BG2 diet. The diets containing rapeseed oil (BG3, BG5, CO), wholly or partly, yielded higher lipid digestibility compared to the diets containing only fish oil (BG1, BG4). This may have been caused by the differing content of SFAs, MUFAs and PUFAs in rapeseed oil and fish oil, as lipid digestibility in salmonids has been shown to vary depending on the SFA to total FA ratio in the diets (Hua and Bureau, 2009). Our result is in contrast to Kousoulaki et al. (2015) who observed that the lipid digestibility decreased when a mix of fish oil and rapeseed oil was replaced with only rapeseed oil. However, this result may have been caused by the concurrent increase of algae levels in the diet.

In terms of ingredient composition, the CO diet (Paper III) was almost identical to the BG5 diet (Paper I). Both diets contained marine and plant ingredients at a ratio of approximately 30:70, respectively. These similarities were reflected in their nearly identical ADCs for dry matter, protein, lipid, and ash.

4.1.3 Means to disrupt cell walls

Extrusion exposes the microalgae to a combination of high temperature, pressure, and shear, subjecting the ingredients to both physical and mechanical treatments. In Paper II, we employed extrusion in an attempt to disrupt the cells of the microalgae *N. oceanica* and *Tetraselmis* sp.. The growth data showed a trend towards improved weight gain in fish fed processed microalgae. This is in line with Gong et al. (2018), who reported that extrusion increased digestibility of dry matter and ash of *Nannochloropsis* sp., and increased digestibility of protein and ash of *Desmodesmus* sp. in a feeding trial with Atlantic salmon. No digestibility data was collected in Paper II, however, the weight gain and SGR indicated that the energy digestibility was compromised due to the connection between digestibility of feed ingredients and growth. The changes in fillet fatty acid composition in Paper II in fish fed pre-extruded or whole-cell microalgae indicated that the salmon fillet fatty acid

composition was affected by processing of microalgae. Some of the interesting observations was that the TE group showed higher levels and the NE group showed lower levels of C18:1n-9, sum MUFA, C22:6n-3 compared to their unextruded counterparts. Sum EPA + DHA was lower in TE than TU. We expected increased utilization of the nutrients in the microalgae upon disruption of cell walls through extrusion. Disruption of cell walls of *N. oceanica* cells using an extruder yielded roughly double the amount of lipid and almost 75% increase in PUFA, compared to the control without treatment Wang et al. (2018). The authors also confirmed the presence of broken cells (after extrusion) with scanning electron microscopy. Similarly, Li et al. (2020a) shortened the lipid extraction time and improved PUFA yield by over 30% by extruding *N. oceanica*. Extrusion processing seems to be an efficient method to make lipids and other nutrients more accessible, both for extraction during industrial production and for digestion by enzymes in fish. In Paper II, we also found an effect of microalgae extrusion on gene expression in fish liver, and this is further discussed in chapter 4.2.5.

4.2 Effect of feed ingredients on mucosal health

An effective barrier function in the distal intestine is important for nutrient uptake, disease prevention, and fish health in general. The most important components of the intestinal barrier are (1) mucus, including secreted mucins, AMPs, enzymes and immunoglobins, (2) the epithelial cells, which along with the tight junctions, adherens junctions, desmosomes and gap junctions prevent the entry of unwanted compounds or organisms, and (3) the gut-associated lymphoid tissue (GALT), including dendritic cells, macrophages, granulocytes, and lymphocytes (Rombout et al., 2011, Gomez et al., 2013). Histology and gene expression of an array of genes associated with mucosal health in the intestine, gills and skin of Atlantic salmon were studied in Papers I-IV. Intestine is the key tissue where the diet is in direct contact with the fishes' mucosal surfaces, whereas effects on the gills and skin will be secondary (systemic) responses. Furthermore, intestinal histomorphology was assessed in all experiments, and mucosal health related genes were analysed in all experiments except Paper III.

4.2.1 Effects of plant ingredients assessed by histomorphology

4.2.1.1 Plant protein ingredients

Plant protein ingredients such as soybean meal are known to cause soybean meal induced enteritis (SBMIE) (Baeverfjord and Krogdahl, 1996). Antinutritional factors, saponins in particular, cause SBMIE which is characterized by shortening of mucosal folds, loss of supranuclear vacuoles in the enterocytes, widening of the lamina propria, and increased infiltration of lymphocytes into the lamina propria (Baeverfjord and Krogdahl, 1996, Knudsen et al., 2007, Urán et al., 2009, Krogdahl et al., 2015). Soybean meal was fed to fish to serve as a positive control for intestinal inflammation in the studies described in Papers I and IV. The SBMIE compromises the intestine's barrier functions, leaving the fish more vulnerable to infections (Trushenski, 2015, Ringø et al., 2007). SBMIE was reported in Papers I and IV based on the assessment of the fishes' gut mucosal health status; evaluated by assessing the number and size of goblet cells in the distal intestine of the fish. The soybean meal fed fish in Paper I had reduced goblet cell size and density, while fish in Paper IV had more goblet cells, though they appeared smaller compared to other diets. Our results are in line with those of previous research which has shown that goblet cells and mucus secretion may be affected by diet. van den Ingh et al. (1991) found that Atlantic salmon fed diets containing soybean meal had more goblet cells in the intestine. Significantly more goblet cells were also reported in zebrafish larvae with SBMIE (Solis et al., 2020). The SBMIE changes in number of goblet cells were associated with lowering of *muc2* gene expression (Papers I and IV). We suggest that the fish fed soybean meal diets compensate for the decreased mucin production caused by SBMIE by creating more small goblet cells.

Fish fed diets dominated by plant protein concentrates (BG4 and BG5, Paper I) had smaller supranuclear vacuoles and appeared to have increased goblet cell size and volumetric density, indicating micromorphological changes compared to fish fed fish meal based diets (BG1, BG3). This trend was also observed in Paper IV, where the histomorphometrical parameters of fish fed BG5 without (÷) or with (+) lactic acid bacteria numerically (though not significantly) ranged in between those fed marine based (BG1÷/+) and soybean meal

(BG2÷/+) diets. The results observed in Papers I and IV are partly supported by observations in a feeding experiment with Atlantic salmon fed diets high in plant ingredients (Li et al., 2020b). The latter authors reported histomorphological changes such as shortening and thickening of mucosal folds, less enterocyte vacuolization, and infiltration of leukocytes in the proximal and distal intestine. Taken together, use of plant protein concentrate as the main protein source in fish diets led to the development of mild enteritis symptoms in the fish.

4.2.1.2 Plant lipid ingredients

Replacement of fish oil by vegetable oil can affect fish health by altering the fatty acid composition of cells and tissues. Paper II showed that fatty acid composition of the salmon fillet was altered by inclusion of microalgae in the diet, which is in line with other studies on Atlantic salmon fed microalgae (Mizambwa, 2017, Gong et al., 2019). Such changes in lipid composition also take place in mucosal surfaces, and this may affect the mucosal health of the intestine (National Research Council, 2011). Rapid turnover is a characteristic of enterocytes in the distal intestine, and these cells are therefore more prone to shift their cellular fatty acid composition in response to changes in the dietary fatty acid composition (National Research Council, 2011). A change in the fatty acid composition of the cells may impact important aspects of the cell membrane such as permeability, morphology, and transport (National Research Council, 2011, Trushenski, 2015). Bou et al. (2017b) found that different levels of EPA and DHA in Atlantic salmon diets led to significant changes in phospholipid composition of cell membranes in the skin and intestine. Fatty acid composition also affects immune cell proliferation, cytokine production, and phagocytosis (National Research Council, 2011). Lower phagocytotic activity and capability of head kidney leukocytes were observed in the BG5 group (Paper I) fed plant derived diets. It is not possible to conclude whether this was because of the overall lipid composition or low content of EPA and DHA.

The histomorphology in the gastrointestinal tract is affected by lipid source. Moldal et al. (2014) reported that replacing fish oil with plant oils (olive oil, rapeseed oil, or soybean oil)

caused a shortening of intestinal folds in the mid and distal intestine of Atlantic salmon. Fish fed the soybean oil diets had the largest changes in the distal intestine, most likely due to the unfavourable ratio of n-6/n-3 fatty acids in soybean oil. The n-3 fatty acids are known as anti-inflammatory while the n-6 are proinflammatory (Moldal et al., 2014). No such proinflammatory changes were observed when fish were fed rapeseed oil (BG3) compared to fish oil in the diet (BG1, Paper I). The level of the marine fatty acids EPA and DHA in the diets are also important for gut health; diets low in EPA and DHA may impair the gut health of fish (Bou et al., 2017a). The latter author reported that some fish fed such diets developed swollen intestines and more enterocyte vacuolisation of varying severity. None of the experimental diets in this PhD thesis were formulated with extremely low levels of EPA and DHA. Diets with the lowest content of EPA and DHA were used in Paper I (BG3 and BG5), but these diets were designed to contain 1.7% EPA and DHA, which is higher than what was used by Bou et al. (2017a). Salmon fed diets low in marine ingredients may develop inflammation in the distal intestine, and steatosis in the enterocytes of proximal intestine and pyloric caeca (Krogdahl, 2019). Steatosis refers to a lipid transport disorder resulting in accumulation of lipid droplets in the afflicted tissue, which is often the liver. Shortage of lipoprotein building component in plant based diets for salmonids as a key factor in lipid transportation disorder, and it has been found that supplementation of phosphatidylcholine or choline in plant-based diets prevents steatosis (Hansen et al., 2020b, Hansen et al., 2020a). Steatosis of the intestine or liver was not observed in any of the studies presented in Papers I-IV.

4.2.2 Microalgae

Research on microalgae in fish diets has been largely focusing on measuring parameters like growth and digestibility, and some studies have also examined various mucosal health parameters in the intestine.

In Paper II, histology slides of the pyloric caeca, the mid intestine, and the distal intestine were prepared, and we found that 30% inclusion of the microalgae *N. oceanica* or *Tetraselmis sp.*, that were not processed to disrupt the cells, had a slight impact on salmon

intestinal morphology. The pyloric caeca were overall unaffected by the addition of microalgae, while the mid intestine tended to have an increased width of villi in fish fed processed algae, and fish fed unprocessed algae tended to have more goblet cells. The distal intestine of fish fed the unprocessed microalgae had significantly lower enterocyte vacuolization compared to the control diet. However, the aberrations were only mild, and it was concluded that no severe inflammation was noticed in Atlantic salmon fed unprocessed microalgae. The study in Paper III did not report any diet induced alterations in the distal intestine morphology. However, a general lack of supranuclear vacuoles in the enterocytes was observed for all diets.

Feed challenge models have reported positive effect of microalgae on intestinal health. Grammes et al. (2013) reported that Atlantic salmon that were fed 20% *C. vulgaris* did not develop SBMIE when 20% soybean meal was also added to the diet. Thus, the inclusion of microalgae in the diet prevented the fish from developing intestinal inflammation. We have not tested any of the microalgae in Paper II and III in a SBMIE model, and more research is therefore warranted to know whether they have similar potential as *C. vulgaris*.

A recent study with European seabass fed either a seaweed or *N. oceanica* or a blend of the two reported that the feed ingredients did not negatively affect the intestinal morphology of the fish, though fish fed the blended diet or the microalgae diet had an increased number of neutral goblet cells in the anterior intestine and in the anterior and posterior intestine, respectively (Batista et al., 2020a). Skalli et al. (2020) performed a study with 5% *Scenedesmus* sp. (replacing fish meal) in diets for juvenile rainbow trout and found alterations in the intestinal goblet cell density and microbiota diversity, but otherwise the diet did not change the intestine and liver histology. In a study with Atlantic salmon fed different levels (0, 1, 6, and 15%) of *Schizochytrium* sp. there was a significant increase of intestinal mucus production, goblet cell numbers, and oxidative stress-related enzyme activity in fish fed algae diets, and these effects were enhanced with increasing algae inclusion. They observed no intestinal inflammation, and no negative effects on salmon health based on microarray analysis of liver (Kousoulaki et al., 2015). In another study with Atlantic salmon the fish were fed 0, 10 or 20% *Desmodesmus* sp.. There were no negative

effects on micromorphology, serum antioxidant capacity, or selected inflammation-related marker genes, but a protein expression analysis revealed underexpression of three intestinal proteins (Kiron et al., 2016). Another feeding study on Atlantic salmon fed 0, 10 or 20 % *N. oceanica* also reported alterations in the expression of intestinal proteins, although mainly at the highest inclusion level, and the alga diet group did not develop distal intestinal inflammation (Sørensen et al., 2017). The results from the latter studies are well aligned with the findings in Papers II and III; incorporation of microalgae up to 30% in the feed had no severe adverse effects on the intestinal health of fish.

However, extrapolation of our observations to draw a general conclusion about the effect of microalgae on fish intestinal histomorphology is not possible. Diversity in structure and chemical composition of microalgae may lead to differential nutrient utilization by fish. Hence, when included in fish feed, their effects on fish intestinal health might be different.

4.2.3 Probiotics

Research presented in Paper IV was performed to investigate if there were any positive effects of using lactic acid bacteria (a blend of *L. fermentum* and *L. plantarum*) as a probiotic in Atlantic salmon. In line with the observations from Paper IV, Atlantic salmon fed diets coated with a commercial feed additive based on the probiotic LAB *Pediococcus acidilactici* recovered faster after an artificially induced inflammation of the distal intestine (Vasanth et al., 2015).

Other studies have reported changes in the intestinal microbiota of Atlantic salmon fed lactic acid bacteria (Jaramillo-Torres et al., 2019, Gupta et al., 2019). Jaramillo-Torres et al. (2019) found in their study that supplementation of *P. acidilactici* caused changes in the intestinal microbiota of Atlantic salmon. When Gupta et al. (2019) fed Atlantic salmon with diets coated with two fish-derived LAB strains (*L. fermentum* and *L. plantarum*), the microbiota diversity and composition were altered significantly, and *Lactobacillus* dominated the distal intestine microbiota. These studies demonstrate that dietary LAB can

influence the microbiota of salmon, and that the mode of action observed in Paper IV may have been mediated by microbiota.

The experiment reported in Paper IV was performed with live LAB bacteria. Other studies with inactivated bacterial cells have also shown positive effects on fish health. A bacterial meal incorporated into a soybean meal diet prevented the development of SBMIE in Atlantic salmon. (Romarheim et al., 2011). The authors hypothesised that the bacterial meal – which was comprised mainly of *M. capsulatus*, and contained smaller amounts of *Ralstonia* sp., *Brevibacillus agri*, and *Aneurinibacillus* sp. – strengthened the intestinal barrier and helped the fish to maintain intestinal homeostasis. The response was dose dependent (Romarheim et al., 2013a) and the effective bacterial compound(s) were either large molecules or water insoluble components (Romarheim et al., 2013b).

4.2.4 Expression of mucin and AMP genes in the mucosal tissues

4.2.4.1 Mucin genes

Mucin gene expression in the distal intestine (Papers I, II and IV), skin and gills (Papers I and IV) of Atlantic salmon was determined and the values are provided in Table 5.

Table 5. Overview of relative mucin gene expression in the distal intestine, skin and gills of Atlantic salmon (Papers I, II and IV).

	Mucin gene expression relative to reference genes (mean ± SEM)					
Tissue	**Intestine**	**Skin**			**Gills**	
Paper I	*muc2*	*muc5ac1*	*muc5ac2*	*muc5b*	*muc5ac2*	*muc5b*
BG1	0.98 ± 0.06^{a}	0.69 ± 0.11^{b}	0.43 ± 0.09	0.66 ± 0.11	0.87 ± 0.10^{ab}	0.49 ± 0.10^{ab}
BG2	0.34 ± 0.02^{c}	0.57 ± 0.07^{b}	0.23 ± 0.03	0.42 ± 0.07	1.20 ± 0.21^{a}	0.73 ± 0.11^{a}
BG3	0.72 ± 0.04^{b}	0.44 ± 0.05^{b}	0.47 ± 0.09	0.44 ± 0.04	0.83 ± 0.10^{ab}	0.56 ± 0.08^{ab}
BG4	0.72 ± 0.06^{b}	0.94 ± 0.14^{ab}	0.27 ± 0.06	0.49 ± 0.08	0.67 ± 0.05^{b}	0.34 ± 0.03^{b}
BG5	0.81 ± 0.05^{ab}	1.42 ± 0.27^{a}	0.55 ± 0.10	0.94 ± 0.25	0.82 ± 0.08^{ab}	0.46 ± 0.10^{ab}
Tissue	**Intestine**	**Skin**			**Gills**	
Paper II	*muc2*	*muc5ac1*	*muc5ac2*	*muc5b*	*muc5ac2*	*muc5b*
CO	0.19 ± 0.02	-	-	-	-	-
NU	0.16 ± 0.02	-	-	-	-	-
NE	0.22 ± 0.02	-	-	-	-	-
TU	0.19 ± 0.02	-	-	-	-	-
TE	0.19 ± 0.02	-	-	-	-	-
Tissue	**Intestine**	**Skin**			**Gills**	
Paper IV	*muc2*	*muc5ac1*	*muc5ac2*	*muc5b*	*muc5ac2*	*muc5b*
BG1÷	2.79 ± 0.25^{a}	0.54 ± 0.06^{ab}	0.52 ± 0.07	0.34 ± 0.04^{ab}	1.07 ± 0.09	0.12 ± 0.01^{ab}
BG1+	2.71 ± 0.15^{a}	0.33 ± 0.04^{b}	0.53 ± 0.07	0.25 ± 0.03^{b}	1.09 ± 0.10	0.12 ± 0.02^{ab}
BG2÷	0.69 ± 0.04^{b}	0.36 ± 0.07^{b}	0.31 ± 0.04	0.26 ± 0.03^{b}	0.85 ± 0.07	0.11 ± 0.02^{ab}
BG2+	0.73 ± 0.07^{b}	0.81 ± 0.13^{a}	0.39 ± 0.05	0.37 ± 0.06^{ab}	0.98 ± 0.10	0.08 ± 0.01^{b}
BG5÷	2.49 ± 0.15^{a}	0.36 ± 0.05^{b}	0.29 ± 0.04	0.26 ± 0.03^{b}	1.12 ± 0.11	0.16 ± 0.03^{ab}
BG5+	2.56 ± 0.33^{a}	0.61 ± 0.07^{ab}	0.41 ± 0.07	0.49 ± 0.04^{a}	0.92 ± 0.06	0.20 ± 0.04^{a}

The mucin genes *muc2*, *muc5ac1*, *muc5ac2*, and *muc5b* were examined in all three tissues (except Paper II), but only genes with detectable expression are shown. Superscripts indicating significant differences are applicable within each paper and column.

The overall expression patterns of the mucin genes were the same in Papers I, II, and IV: The distal intestine expressed mainly *muc2*, the skin expressed *muc5ac1*, *muc5ac2*, and *muc5b*, and the gills expressed *muc5ac2* and *muc5b*. Similar tissue-specific expression of mucin genes has previously been confirmed in Atlantic salmon (Sveen et al., 2017), channel catfish (*Ictalurus punctatus*) (Liu et al., 2020), gilthead sea bream (Pérez-Sánchez et al., 2013), and common carp (van der Marel et al., 2012, Adamek et al., 2017). Different mucins may for example facilitate binding and removal of harmful bacteria specific to that environment, or facilitate the presence of useful bacteria by offering specific glycan side branches (Schroeder, 2019). A different mucin composition may also contribute to a change in the viscosity and/or elastic properties of the mucus gel (Bansil and Turner, 2006). The mucus gel

thickness and composition was not analysed in the studies described in this thesis, but research on Atlantic salmon has clearly demonstrated that factors such as mucin structure, mucin glycosylation, and mucus gel properties are important for the barrier function of mucosal tissues, for example during infections with pathogens (Padra et al., 2019, Benktander et al., 2020).

Intestine

Expression of *muc2* in the distal intestine was significantly lowered by the soybean meal diets (Papers I and IV). This is in line with other literature which has shown that diet can influence intestinal mucin gene expression (Salinas and Parra, 2015). Pérez-Sánchez et al. (2013) found that the expression of some intestinal mucins of gilthead sea bream would change depending on diet (replacement of fish oil by plant oil), while others do not. When fish are subjected to prolonged inflammation of the intestine, as in Papers I and IV, it has been suggested that the lowered mucin gene expression is caused by an exhaustion of the goblet cells after a longer period of increased mucus production (Dharmani et al., 2009, Kim and Ho, 2010). Studies on humans have shown that decreased *muc2* expression in the intestine is associated with inflammatory bowel disease, which is an umbrella term including several conditions characterized by chronic intestinal inflammation (Grondin et al., 2020). The mucins in the intestinal mucosa may be indirectly affected by diet, which in turn influences the abundance of certain gut bacteria (e.g. *Bacteroides thetaiotaomicron*, *Akkermansia muciniphila* and *Barnesiella intestinihominis*) that can degrade mucin glycans (Schroeder, 2019). In some instances this may allow opportunistic bacteria to pass through the mucus barrier. Upon reaching the epithelial cells, microbe-associated molecular patterns on the bacterial cell surface are recognized by pattern recognition receptors of immune cells and this may activate an immune response (Dharmani et al., 2009). The expression of *muc2* was not affected by the addition of probiotics (Paper IV). This could be due to the ability of the probiotic to reduce the degradation of the mucin, as observed for *Bifidobacterium longum* (Schroeder, 2019). Similarly, a report by van der Marel et al. (2012) found that expression of *muc2* in the intestine of carp was unaffected by dietary β-glucan, which is a common prebiotic. The microalgae included in the diets (Paper II) did not affect mucin gene

expression in the distal intestine. The mucin gene expression in the skin and gills were not analysed in the experiment performed for Paper II and thus further comparison with Paper I and IV is not possible.

Skin

The skin mucin gene expression was largely unaffected by the diets (Paper I), except for *muc5ac1* that tended to have a higher expression in the plant-protein based diets (BG4 and BG5). This result was not reproduced in Paper IV. Instead, *muc5ac2* in the skin of salmon was shown to be significantly affected by feed ingredients and had higher expression in the marine-based diet (BG1). Research by Cerezuela et al. (2016) has also shown that fish skin mucins can be affected by diet, although their study did not report mucin gene expression; instead they analysed the glycosylation patterns of mucins. Cerezuela et al. (2016) studied the effects of date fruit palm extracts and two probiotics in diets for gilthead sea bream. The higher *muc5ac2* expression in the BG1-fed groups (Paper IV) is likely a result of fewer goblet cells in the BG1 diet groups compared to the BG2 and BG5 groups. The fish may compensate for the lower number of goblet cells by increasing the mucus production, but this hypothesis contradicts with the observation on the distal intestine. The mucosal surface of salmon skin consists of 15-18% goblet cells by area (Paper IV). The probiotic diets (Paper IV) significantly affected the expression of *muc5ac1* and *muc5b*. The results showed no consistent pattern, as some of the mucin genes were upregulated in the BG2 and BG5 diet groups, but tended to be downregulated in the BG1 diet group, or were not affected by the treatment. Similar results were found by van der Marel et al. (2012), who fed carp β-glucan diets, and reported significant upregulation of one skin mucin gene, while another was unaffected. In the previously described report by Cerezuela et al. (2016), the glycosylation patterns of gilthead sea bream skin mucins were significantly affected by dietary addition of probiotics *Shewanella putrefaciens* and *Bacillus* sp.

Gills

The significant difference in gill *muc5b* expression that was observed in Paper IV, where BG1 and BG5-fed fish had higher expression compared to BG2-fed fish, was not observed in Paper I, though the same experimental diets were used. The BG2 group had the lowest expression of *muc5b* in Paper IV, while the BG2-fed fish had the highest expression of *muc5b* out of all the diet groups in Paper I. Additionally, *muc5ac2* expression was not affected by the diets in Paper IV, but was significantly affected by the diets in Paper I (BG2>BG4). The number of gill goblet cells (Paper IV) was significantly higher in BG2-fed fish compared to the BG1 and BG5 diet groups, and inversely related to the *muc5b* expression. This is similar to the results found in the intestine, and contrary to the results from salmon skin. The expression of mucin genes in the gills were unaffected by the addition of probiotics (Paper IV), but the number of goblet cells was increased in all LAB coated diets. Few studies have examined the effect of fish diet on gill mucin gene expression, as most of this research is focused on gill diseases and infections. Our results are in agreement with the study of van der Marel et al. (2012), wherein the two measured mucin genes in the gills of carp were not significantly affected by the addition of β-glucan to the diet. However, Firmino et al. (2020) found histochemical alterations consistent with changes to the mucin composition in the gills of gilthead sea bream fed diets containing an essential oil feed additive. The latter authors did not measure the mucin gene expression, but their histochemistry results indicate that fish diet may affect gill mucin composition.

4.2.4.2 AMP genes

AMP gene expression was measured in the distal intestine and dorsal skin of Atlantic salmon (Papers I and IV, Table 6).

Table 6. Overview of relative AMP gene expression in the distal intestine and skin of Atlantic salmon (Papers I and IV).

	AMP gene expression relative to reference genes (mean ± SEM)				
Tissue	**Intestine**			**Skin**	
Paper I	*def3*	*def4*	*cathl1*	*def1*	*cathl1*
BG1	1.07 ± 0.2^{a}	-	0.028 ± 0.009^{b}	0.68 ± 0.05	0.47 ± 0.07^{b}
BG2	0.16 ± 0.05^{b}	-	0.24 ± 0.06^{ab}	0.68 ± 0.09	1.14 ± 0.2^{a}
BG3	0.81 ± 0.2^{ab}	-	0.34 ± 0.1^{a}	0.84 ± 0.07	0.54 ± 0.05^{b}
BG4	0.38 ± 0.1^{b}	-	0.10 ± 0.03^{b}	0.81 ± 0.08	0.57 ± 0.08^{b}
BG5	0.67 ± 0.2^{ab}	-	0.21 ± 0.04^{ab}	0.75 ± 0.08	1.01 ± 0.1^{a}
Tissue	**Intestine**			**Skin**	
Paper IV	*def3*	*def4*	*cathl1*	*def1*	*cathl1*
BG1÷	0.32 ± 0.03^{b}	1.27 ± 0.17^{a}	0.023 ± 0.01^{b}	0.64 ± 0.08	0.47 ± 0.06^{bc}
BG1+	1.75 ± 0.47^{a}	0.93 ± 0.06^{a}	0.14 ± 0.05^{ab}	0.61 ± 0.05	0.43 ± 0.05^{bc}
BG2÷	0.46 ± 0.10^{b}	0.70 ± 0.08^{ab}	0.064 ± 0.01^{ab}	0.74 ± 0.06	0.31 ± 0.03^{c}
BG2+	0.33 ± 0.11^{b}	0.84 ± 0.08^{ab}	0.17 ± 0.05^{a}	0.76 ± 0.06	0.57 ± 0.05^{ab}
BG5÷	0.98 ± 0.24^{ab}	0.59 ± 0.07^{b}	0.028 ± 0.01^{b}	0.81 ± 0.08	0.71 ± 0.07^{a}
BG5+	1.78 ± 0.45^{a}	1.16 ± 0.21^{ab}	0.17 ± 0.06^{a}	0.76 ± 0.09	0.59 ± 0.06^{ab}

The AMP genes *def1*, *def2*, *def3*, *def4* and *cathl1* were examined in both tissues, but only genes with detectable expression are shown. Superscripts indicating significant differences, where noted, are applicable within each paper and gene.

Harte et al. (2020) reported seven β-defensin genes across several salmonids, including Atlantic salmon. The defensin genes (Harte et al., 2020) and several cathelicidin genes (Chang et al., 2006) showed a tissue-specific expression across various mucosal tissues in salmonids. These papers agree with our results, as we also found specific defensins and cathelicidins expressed in the skin (*def1*, *cathl1*) and intestine (*def3*, *cathl1*) of fish (Papers I and IV). The AMP gene *def4* was expressed in the intestine of fish reported in Paper IV, and although the gene was also detected in the intestine of fish described in Paper I, the levels were too low to quantify.

Various AMPs are produced and secreted in the fishes' mucosal surfaces, and their gene expression may relay information about the mucosal health status of the fish. Thus, induction of AMP production may be one approach to stimulate the fish immune system.

Intestine

The expression of all detected AMP genes (*def3, def4, cathl1*) in the distal intestine were affected by the feed ingredient composition (Paper IV). The intestinal AMP gene expression in Paper IV was also affected by the addition of probiotics, and this was especially evident for *cathl1*; the expression of *cathl1* was significantly increased in all probiotic feeding groups compared to the corresponding non-probiotic groups. This may be interpreted as an enhanced immune response. Our results are in line with Yang et al. (2019), who reported significantly increased expression of several AMP genes in the intestine of grouper fed diets supplemented with potential prebiotics. Casadei et al. (2013) fed rainbow trout peptidoglycan-enriched diets and their results after 14 days showed upregulation of several AMP genes, especially cathelicidins, while some defensins were downregulated. Several papers also reported increased expression of AMP genes in the gut of shrimp after probiotic treatment with LAB (Sha et al., 2016, Miandare et al., 2016). The expression of both intestinal defensins (Paper IV) was higher in the BG1 and BG5 diet groups compared to the BG2 diet group. This aligns with the scoring of two intestinal-health related parameters in the distal intestine; intraepithelial lymphocytes (IEL) and supranuclear vacuoles (SNV). The average scores were higher (better; less IEL, more SNV) in the BG1 and BG5 diet groups compared to the BG2 diet group. Thus, the increased expression of defensin genes in the intestine seems to be associated with improved intestinal health.

Skin

The expression of the skin AMP genes *cathl1* was shown to be significantly affected by LAB supplemented diets as well as feed ingredients (Paper IV). The expression of the gene *cathl1* was higher in the plant protein-based diet (BG5) compared to BG1 and BG2. Regarding the effect of probiotics, *cathl1* expression was significantly improved in the BG2+ diet group. Meanwhile, *def1* was unaffected by either variable (Paper IV). The latter result was also observed in Paper I, where the expression of *def1* in the skin was unaffected by the various diets. Our results are in agreement with previously mentioned research done by Casadei et al. (2013), who fed rainbow trout diets supplemented with different amounts of

peptidoglycan. The latter author found that several skin AMP genes were significantly upregulated in almost all peptidoglycan-fed groups after 1, 7 and 14 days of administration, and the changes were dependent on the treatment time, though some AMPs remained unaffected. van der Marel et al. (2012) found that β-glucan fed carp had significant upregulation of two AMP genes in the skin.

To conclude, we observed an inverse relationship between mucin gene expression and goblet cell number in the distal intestine, skin and gills of Atlantic salmon. A higher number of goblet cells corresponded with decreased mucin expression in the gills and intestine, while a lower number of goblet cells was associated with increased mucin production in the skin. This could be caused by the fish regulating the mucin gene expression based on the number of goblet cells present in the mucosal tissue, or possibly the opposite (regulating the number of goblet cells depending on the mucin gene expression). In general, we also conclude that the expression of fish mucosal mucin and AMP genes may be affected by both feed ingredients and addition of probiotics, and that regular expression of mucin and AMP genes throughout the fish mucosal tissues is important for maintenance of good health and barrier status.

4.2.5 Antioxidant genes

Reactive oxygen species (ROS) are naturally produced in organisms as part of various metabolic processes or may be formed by external sources. The antioxidant system continuously removes ROS, thus preventing the build-up of harmful oxidative stress. The antioxidant defence system of fish includes enzymes, peptides and proteins, and dietary vitamins, carotenoids, phenolic compounds, and minerals (Biller and Takahashi, 2018). Several species belonging to the microalgae families *Nannochloropsis* and *Tetraselmis* have been shown to exhibit antioxidant potential and to contain antioxidant phenols, sterols, vitamins, and carotenoids (Goiris et al., 2012, Sansone and Brunet, 2019).

Our results in Paper II showed that high inclusion (30%) of *N. oceanica* and *Tetraselmis* sp. initiated an antioxidant response in the salmon liver measured by gene expression analysis

of selected genes (superoxide dismutase, catalase, glutathione peroxidase, and nuclear factor erythroid 2—related factor 2). Processing of the microalgae seemed to strengthen this effect for some of the genes, which was more pronounced with the *Tetraselmis* diets. Statistical analysis revealed that there was a significant effect of processing on the expression of all the genes analysed in liver, which we interpret as successful algae cell disruption that enabled the release of antioxidant bioactive compounds.

Antioxidant capacity of *N. oceanica* and *C. vulgaris* has been reported in vitro (Ferreira et al., 2021). Batista et al. (2020a) reported that European seabass fed diets with 8% *N. oceanica* had increased liver total antioxidant capacity compared to fish fed diets with 8% seaweed, but none of them differed from the control diet. *C. vulgaris* was found to enhance plasma antioxidant enzyme activity, total antioxidant capacity, and muscle radical scavenging activity when fed to olive flounder at 10-15% dietary inclusion (Rahimnejad et al., 2017). A study on rainbow trout fed diets with different levels (2.5-10%) of *Spirulina platensis* found a dose-dependent increase in total antioxidant capacity, lower lipid peroxidation in serum and liver, and higher expression of antioxidant genes in liver (Teimouri et al., 2019). Sørensen et al. (2017) reported a significantly increased serum superoxide dismutase activity in Atlantic salmon fed 10% *N. oceanica*, but not for salmon fed 20% of the algae. Serum catalase activity tended to rise with increasing microalgae inclusion (though not significantly), and total antioxidant activity did not differ among the groups. The results from the mentioned studies emphasize the antioxidant potential of microalgae and show that this may be transferred to fish through the diet.

The elevated antioxidant genes in fish may also be explained by other constituents of the diet, such as pollutants or oxidized lipids. Li et al. (2019) reported an increase in the expression of several genes, some of them antioxidant-related, after feeding Atlantic salmon with insect meal, and they conclude that this may be caused by an “elevated detoxification response” triggered by the presence of heavy metals in the diet. Another potential explanation for their results is that the antioxidant gene expression was triggered by products from lipid oxidation in the diet, as the degree of lipid oxidation in the insect meal diet was quite high. In Paper II, we analysed volatile organic compounds in the diets to assess

the extent of lipid and protein oxidation, and our conclusion was that the expression pattern of the antioxidant genes did not correlate with the degree of oxidation of the diet. We therefore concluded that fish fed the microalgae diets had an improved capacity to handle oxidative stress.

In Paper II we also analysed antioxidant-related genes in the distal intestine, and while a majority of them did have detectable expression, statistical analyses did not show any significant differences. Thus, it seems that the distal intestine is not the best organ to focus on for detection of antioxidant system related genes. In conclusion, the main targets for various antioxidant analyses are liver and serum of fish, and the liver is the main target organ for antioxidant gene expression analysis.

4.3 Methodological aspects

Atlantic salmon is an economically important species with a big global market, and the future growth of the industry demands new feed ingredients. Novel ingredients should not compromise fish health, and studies are warranted to examine effects of feed ingredients on gut health. Evaluation of dietary effects are often done by feeding trials. To evaluate the immune status or strength of fish it is also possible to perform infection trials with pathogens. However, such trials are more taxing from a fish welfare point of view. In this thesis we chose to use feeding trials to study the effect of feed ingredients on fish barrier status by evaluating growth, digestibility, gene expression, and histology.

4.3.1 Feeding trials

Feeding trials are commonly used to evaluate new feed ingredients, feed additives or effects of processing methods. These effects are usually measured or evaluated by various parameters connected to fish growth and health.

Diet composition

In this context, diet composition refers to the different feed ingredients, and the amount of each ingredient, included in experimental diets. The composition of the diets used in the four experiments (Papers I-IV) are quite different, complicating evaluation of results across experiments. For example, the diets in Paper II were based on high fish meal with no plant oil or protein, while Paper III was based on low fish meal diets with a mixture of plant proteins and a combination of fish oil and plant oil.

In the feeding trial performed for Paper II, we chose a very high inclusion of microalgae in the diets (30%). Inclusion levels of 5-15% are more commonly seen in reports on microalgae in diets for salmonids, as higher levels tend to result in decreased nutrient digestibility (Tibbetts, 2018, Teuling et al., 2017a, Sørensen et al., 2017, Kiron et al., 2016, Kiron et al., 2012, Sørensen et al., 2016). Indeed, this is why an inclusion level of 10% *N. oceanica* was chosen for Paper III. The 30% microalgae inclusion was chosen because the experiment was designed to evaluate nutrient digestibility of the algae. Unfortunately, no markers were added to the diets. It is, however, still relevant to study high incorporation levels to explore microalgae as a main ingredient, and not only as feed additives. High inclusion levels are also warranted to study the safety of novel ingredients, or to understand adverse health effects in fish.

The diets produced for Paper II were not isoproteic or isoenergetic, as they were only balanced based on ingredient composition. The differences in growth were most likely explained by the varying content of protein (44.8-52.4% of DM), lipid (16.0-20.9% of DM) and energy (20.7-23.4 MJ/1000 g) in the diets, rather than the species of microalgae or pre-processing. For evaluation of feed ingredients in growth studies, careful balancing of nutrients and energy are warranted.

Digestibility

In both experiments reporting digestibility (Papers I and III) fish were fed ad libitum using automatic feeders. The feeding frequency, however, differed, as the fish in Paper I were fed

eight times a day, while the fish in Paper III were only fed twice a day. Fewer feedings correlate to larger portion sizes, which causes increased passage rate of feed and shorter retention time throughout the gastrointestinal tract of the fish, which has been shown to decrease nutrient digestibility (Oehme et al., 2014). The inverse relationship between the two parameters is also recognized, as slower GI passage rate has been linked to longer retention time and increased digestibility (Aas et al., 2011). It is unknown whether this has influenced our results. To compare results from different studies, there should be as few differing variables as possible between the experiments.

4.3.2 Histomorphology

Histomorphology is a well-established method for studying the morphology of organs and tissues. After fixation and embedding of the sampled tissue in paraffin, thin sections are cut, stained, and mounted on glass slides for visualization through light microscopy. The various cell types and structures that make up the tissue can be identified and characterized as normal or abnormal, and digital tools can be used for the analysis of suitable parameters.

Various quantitative or semi-quantitative analyses were employed to study histomorphology (Papers I-IV, Table 7). This makes direct comparison of results from different papers difficult, as the results generated employing different methods cannot necessarily be compared.

Table 7. Overview of quantitative and semi-quantitative methods applied for analysing the histological slides of tissues sampled from Atlantic salmon (Papers I-IV).

Paper	Method	Tissues
Paper I	Goblet cell size, volumetric density, and barrier status.	Distal intestine
Paper II	Stratum compactum and granulosum height, villi width. Number of goblet cells and area of goblet cells.	Mid intestine, distal intestine
	Scoring of enterocyte/hepatocyte vacuolization.	Liver, distal intestine
Paper III	Analysis of cell proliferation, and villi height and width.	Distal intestine
Paper IV	Total area of epithelium, total area of goblet cells and number of goblet cells.	Skin, gills, distal intestine
	Height and width of villi, height of enterocytes, and width of lamina propria. Scoring of number of intestinal goblet cells, number of intraepithelial lymphocytes, and presence of supra nuclear vacuoles in enterocytes of intestinal villi.	Distal intestine

Evaluation of intestinal health in fish across different experiments should be performed using standardized methods and analyses. The various methods and parameters used to draw conclusions about intestinal and mucosal health makes comparison challenging. For example, histomorphology was used to study the effects of microalgae on intestinal health in Papers II and III, but the methods used were different. As seen in Table 7, more measurements and scorings were performed in Paper II compared to Paper III. Paper III relied mainly on visual observation. Slight changes in structure or morphology could be difficult to observe by mere visual examination. Thus, some trends or differences in histomorphology between diet groups may have been overlooked in Paper III.

Some parameters were measured in several of the experiments (height and width of intestinal villi, goblet cell number and/or goblet cell area), however, these methods were not necessarily standardized.

Histological analysis relies on many processing steps before the final images are obtained; sampling, processing, fixation, embedding, sectioning, staining, and mounting. Mistakes during any of these steps may cause artifacts in the sample, or imprecision during the steps may cause a cumulative inaccuracy. Both cases might cause human error to be misinterpreted as experiment-related effects in the final results and measurements.

Semi-quantitative methods, such as scoring, are used to quantify features of the histology images that are not easily measured, or in other words, transforming a researcher's subjective observation into an objective number. Subjective evaluation may be biased, but quantitative methods may also be inflicted with errors or bias. For example, the results from measurements of intestinal villi width depend on exactly where the line was drawn across the villi. Thus, even quantitative measurements do have an element of subjectivity to them.

4.3.3 Gene expression analysis (by qPCR)

The assessment of gene expression by qPCR is a widely adopted method that is sensitive and can be used to quantify the relative expression of target genes, compared to other more work intensive methods such as RNA sequencing. Microarrays could have been an

alternative method and might have facilitated a more extensive gene expression analysis in the tissues of interest.

The success of qPCR depends on, among other factors, high quality and uncontaminated RNA extracted from the target tissues. Therefore, one must take precautions to prevent degradation and contamination of the sample and extracted RNA. Tissue samples were collected immediately after the fish was euthanized, and the sample was preserved instantly in either liquid nitrogen or RNA later. Before starting the labwork, all surfaces and equipment were cleaned with RNase Away. After extraction, the resulting RNA was checked for both sufficient quality (gel electrophoresis) and quantity (Qubit).

Another important factor in qPCR is the design of primers to bind the target genes. For the mucin gene primers we relied on the work of Sveen et al. (2017), who identified seven mucin-like genes expressed in Atlantic salmon mucosal tissues and designed four pairs of primers (*muc2, muc5ac1, muc5ac2, muc5b*) for six of these genes (*muc2.1, muc2.2, muc5ac1, muc5ac2, muc5ac4, muc5b*). Two sets of genes had too high sequence similarity to be able to bind selectively to different primers, so two of the primer pairs (*muc2* and *muc5ac2*) bind two genes each (*muc2.1* and *muc2.2*, and *muc5ac2* and *muc5ac4*, respectively).

As seen in Tables 5 and 6, the relative mucin and AMP gene expressions vary between the three experiments (Papers I, II and IV). While the same set of four reference genes were used for all these experiments, our protocol dictates that the two most stable reference genes out of these four are selected for normalization of the target gene expression. The two reference genes calculated as being most stable, which was done using geNorm (Vandesompele et al., 2002), varied between the three experiments.

5. Conclusions

The following conclusions are made about the effects of feed ingredients on farmed Atlantic salmon growth, nutrient digestibility, and feed utilization:

- The fish accepted all the experimental diets and had good growth throughout the feeding trials.
- The digestibility of protein was in the range 86.1-88.1%, lipid in the range 87.4-95.4%, dry matter in the range 59.0-68.4% and energy in the range 73.1-83.8% in diets containing plant derived protein or lipid. The ADCs of protein, lipid and dry matter in diets containing 10% of the microalgae *N. oceanica* were 86.5-88.5%, 91.1-91.9% and 65.3-67.5%, respectively.
- Extrusion can be used as a method to disrupt microalgae cells in order to improve salmon growth and feed utilization, but the efficiency of the process varies among microalgae species and may be more relevant in diets with high inclusion of microalgae. Extrusion of microalgae increased availability of some lipids, and may release bioactive compounds that could be utilized by the fish to improve their antioxidant capacity.

The following conclusions are drawn relating to the effects of feed ingredients on farmed Atlantic salmon mucosal health and barrier function:

- Soybean meal fed fish developed SBMIE and had a significant decrease of *muc2* gene expression in the distal intestine of Atlantic salmon, resulting in reduced mucin production. The SBMIE fish had more goblet cells in the distal intestine, most likely as a compensatory mechanism. The *muc2* gene can be a marker for intestinal health.

- Use of plant protein concentrates as the main protein source in fish diets has an adverse effect on mucosal health of the distal intestine of salmon, and the intestinal histomorphology indicated a mild enteritis-like condition.

- High inclusion level (30%) of the microalgae *N*. *oceanica* and *Tetraselmis* sp. in fish diets caused minor to no changes to intestinal morphology and did not have major impacts on intestinal health-related parameters.

- Dietary inclusion of the microalgae *N*. *oceanica* and *Tetraselmis* sp. increased the fishes' ability to cope with oxidative stress, as shown by increased expression of antioxidant-related genes in the liver. The liver should be the target organ when assessing antioxidant gene expression analysis.

- Use of LAB probiotics (*Lactobacillus fermentum* and *Lactobacillus plantarum*) had a modest but positive effect on intestinal health as some of the typical signs of SBMIE were reduced in the probiotic treatment group, though regression of SBMIE was not observed. Changes in the microbiota of fish may have contributed to this positive effect. It was also concluded that the addition of probiotics improved the mucosal barrier of skin and gills by increasing the relative area or number of goblet cells.

- Mucin gene expression in the distal intestine, skin and gills of Atlantic salmon is inversely related to the number of goblet cells, suggesting a regulatory mechanism.

- Expression of the various mucins and AMPs in mucosal surfaces of Atlantic salmon are tissue-specific and may be modulated by feed ingredients and probiotics.

- The gene expression of mucins and AMPs may be used to evaluate mucosal health in combination with other parameters. Mucosal tissues are important for maintenance of good health and barrier status.

This thesis has shown that the mucosal health of Atlantic salmon is affected by feed ingredients and highlights the necessity of addressing fish health in addition to fish growth

and feed digestibility when feeding experiments are performed to study quality of feed ingredients. The assessment of fish health must include use of multiple tools.

6. Future perspectives

The growing aquaculture industry needs more feed ingredients from sustainable sources. New feed ingredients can only be adopted by the industry when quality is thoroughly documented. The quality assessment should include studies of potential health effects. This thesis has shown that feed ingredients affect the mucosal health of Atlantic salmon. Diets with a high inclusion of plant ingredients and low inclusion of marine ingredients may compromise fish health. The microalgae *N. oceanica* and *Tetraselmis* sp. do not have negative effects on the mucosal health of salmon, even at high inclusion levels. The examined microalgae are promising salmon feed ingredient candidates, especially when processed, for example by extrusion, to increase the availability of cell contents. Future studies should expand upon this knowledge. The research in this thesis has demonstrated the importance of standardizing the parameters used for assessing mucosal health of fish. Also, it is important to examine several parameters when evaluating mucosal health.

Throughout this thesis it has been seen that expression of AMP genes did not change much when feed ingredient composition was changed, but showed clearer responses in the experiment that evaluated the effect of probiotics in feed. AMP genes may thus be good markers for studying effects of feed additives such as probiotics or immunostimulants. Future studies should also include timeseries to increase the understanding of "mode of action" of the two probiotics on mucosal health. Studying the probiotic effects on AMP expression in the mucosal tissues over a time series can give better clues about optimal feeding regime – pulsing – of functional feeds.

The presented research has shown that mucin genes can be used as markers for diet-induced inflammation in the intestine, however, future studies should further reveal the role of MUC genes in enteritis. The *muc2* expression in the intestine during the onset of inflammation should be tested – to study the correlation between the development of enteritis and the mucin depletion. Research on a human cell line under a simulated intestinal inflammation has shown increased apoptosis in goblet cells with high expression of *muc2* (Tawiah et al., 2018), and if this occurs during the early onset of inflammation it may explain

the reduced mucin production in chronic inflammation. This may also be one of the mechanisms connecting mucin expression and number of goblet cells, which we found to be inversely correlated in the intestine, skin and gills. More research is needed to confirm this correlation. We do not know whether a lower number of goblet cells will be correlated with an increased mucin expression in the gills and intestine, or a higher number of goblet cells will correspond with increased mucin production in the skin. Additionally, we do not know whether the number of goblet cells is regulated based on the mucin expression, or the opposite. More studies are needed to get a better understanding of the effect of feed ingredients on mucosal health. This information is warranted for the protection of fish welfare in the ongoing advancement of salmon aquaculture.

7. References

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Paper I

frontiers in Immunology

ORIGINAL RESEARCH
published: 19 February 2021
doi: 10.3389/fimmu.2020.623726

Nutrient Digestibility, Growth, Mucosal Barrier Status, and Activity of Leucocytes From Head Kidney of Atlantic Salmon Fed Marine- or Plant-Derived Protein and Lipid Sources

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Edited by:
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Specialty section:
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

Received: *30 October 2020*
Accepted: *31 December 2020*
Published: *19 February 2021*

Citation:
Sørensen SL, Park Y, Gong Y, Vasanth GK, Dahle D, Korsnes K, Phuong TH, Kiron V, Øyen S, Pittman K and Sørensen M (2021) Nutrient Digestibility, Growth, Mucosal Barrier Status, and Activity of Leucocytes From Head Kidney of Atlantic Salmon Fed Marine- or Plant-Derived Protein and Lipid Sources. Front. Immunol. 11:623726. doi: 10.3389/fimmu.2020.623726

Nutrient digestibility, growth, and mucosal barrier status of fish skin, gills, and distal intestine were studied in Atlantic salmon fed feeds based on marine or plant-derived ingredients. The barrier status was assessed by considering the expression of four mucin genes, five genes that encode antimicrobial proteins, distal intestine micromorphology, and design-based stereology of the midgut epithelium. In addition, the head kidney leukocytes were examined using flow cytometry; to understand the differences in their counts and function. Five experimental feeds containing the main components i) fishmeal and fish oil (BG1), ii) soybean meal (BG2; to induce enteritis), iii) fishmeal as the main protein source and rapeseed oil as the main lipid source (BG3), iv) a mix of plant protein concentrates as the protein sources and fish oil as the lipid source (BG4), and v) plant and marine ingredients in the ratio 70:30 (BG5) were produced for the study. Atlantic salmon with initial weight 72.7 ± 1.2 g was offered the experimental feeds for 65 days. The results revealed that the weights of all fish groups doubled, except for fish fed BG2. Fish fed the BG2 diet had lower blood cholesterol concentration, developed enteritis, had lower expression of *muc2* in the distal intestine, and had a compromised barrier status in the intestine. Expression of both the mucin genes and genes that encode antimicrobial peptides were tissue-specific and some were significantly affected by diet. The fish fed BG1 and BG3 had more head kidney lymphocyte-like cells compared to BG5-fed fish, and the phagocytic activity of macrophage-like cells from the head kidney was the highest in fish fed BG1. The intestinal micromorphology and the mucosal mapping suggest two different ways by which plant-based diets can alter the gut barrier status; by either reducing the mucous cell sizes, volumetric densities and barrier status (as noted for BG2)

or increasing volumetric density of mucous cells (as observed for BG4 and BG5). The results of the compromised intestinal barrier in fish fed plant ingredients should be further confirmed through transcriptomic and immunohistochemical studies to refine ingredient composition for sustainable and acceptable healthy diets.

Keywords: Atlantic salmon, enteritis, mucosal barrier status, plant ingredients, mucin gene, antimicrobial genes, stereology, distal intestine

INTRODUCTION

Mucosal surfaces of fishes, the skin, gills, and gastrointestinal tract, are important barriers that protect the host from pathogens and infections. The barriers include a mucosal epithelium which is covered by mucus and a wide range of components such as antimicrobial peptides that inhibit the entry of pathogens (1, 2). Mucus contains O-glycosylated proteins called mucins, and the expression of mucin genes in fish is altered by parasite infection (3) and fish density- and handling-related stress (4). The mucin glycosylation itself plays a key role in disease resistance in fish (5) and is affected both by the origin and size of Atlantic salmon (6). Antimicrobial peptides (AMPs) are also important components of the innate immune system in fish (2). The AMPs are classified into different families which show broad-spectrum antimicrobial activity to overcome the different resistance mechanisms activated by microbial organisms (2, 7, 8). The innate immune system plays a key role in keeping fish healthy in intensive aquaculture systems, especially the components at the semipermeable mucosal epithelia in the gut (9–11). Dietary interventions are known to strengthen the intestinal barrier in mice and humans, thereby allowing the organ to carry out its intended functions (12). However, little information is available as to how the intensive production systems and use of modern diets affect the gut barrier function of fishes.

Modern diets are formulated on the presumption that fish do not have a need for specific ingredients, but combinations of different ingredients can help meet the nutrient requirements of the farmed species. Fishmeal (FM) and fish oil (FO) are still considered to be the gold standard feed ingredients. However, their use in commercial fish feeds is reduced to a minimum because of static supply, increasing demand resulting in increasing prices and debates about sustainability when fish is used to feed fish. Commercial feeds used in Norwegian salmon farming are based on plant-derived products, which constitute 71% of the feed ingredients, while the marine feed ingredients is reduced to approximately ~25% (13). Soy protein concentrate has become the key protein source and rapeseed oil the primary oil source in present-day salmon feeds (13, 14). However, these ingredients have certain drawbacks. Feeding rapeseed oil is known to affect the n-3/n-6 ratio in the fillets of farmed salmon. Use of plant products with unfavourable n-6/n-3 ratio or diets without eicosapentaenoic acid (EPA) may bring about histomorphological changes in the intestine and can reduce fish growth (15, 16). Many studies have shown that the intestinal structure, microbiota and ion and water transport of Atlantic salmon are affected by the feed ingredients (17–19). However, further research is needed to understand the effect of feed ingredients on the immune defense of the fish, especially at the intestinal level.

Most studies have employed fishmeal-based diets to evaluate the impact of plant ingredients on salmon; the researchers have replaced either fishmeal with plant protein or fish oil with plant oil. Few studies have investigated the effect of different combinations of protein and oil derived from marine and plant origin on the growth and health of the fish. The aim of this study was to investigate the combined effect of replacing marine proteins and lipids with a mixture of plant-derived protein concentrates and oil on growth, nutrient digestibility, mucosal barrier status and systemic immune responses. The barrier status was assessed based on the expression of mucin genes in the skin, gills, and distal intestine, the expression of genes that encode antimicrobial proteins in the skin and distal intestine, histological changes in the distal intestine and information from design-based stereology of the midgut epithelium. Stereology was used to evaluate the mucosal barrier function because this type of mucosal mapping is more sensitive and independent of section orientation (11, 20). Furthermore, to understand the systemic effect, head kidney leukocytes were examined using flow cytometry.

MATERIAL AND METHODS

Experimental Design and Feeds

The study used five experimental diets: a control diet (BG1) based on fishmeal and fish oil; a diet containing 20% soybean meal and 30% fishmeal and fish oil (BG2); a diet with fishmeal and rapeseed oil (BG3); a diet based on a mix of plant protein concentrates as the main protein source (soy protein concentrate, pea protein concentrate and corn gluten meal) and fish oil (BG4); and one diet resembling a commercial diet with the same protein ingredients as in BG4 and a mix of rapeseed oil and fish oil (BG5; **Table 1**). All diets were supplemented with crystalline amino acids (lysine, histidine, methionine and threonine) and inorganic phosphate (**Table 1** and **Supplementary Table 1**). Diets also contained 0.01% yttrium oxide as an inert marker for digestibility measurements.

The five feed mixes were prepared and homogenized (30 min) using a horizontal ribbon mixer. The feed mixes were conditioned with steam and water in an atmospheric double differential preconditioner (DDC) prior to extrusion in a TX-52 co-rotating, fully intermeshing twin-screw extruder (Wenger Manufacturing Inc., Sabetha, KS, USA). The temperature of

TABLE 1 | Ingredient composition (%) of the experimental feeds.

	BG1	BG2	BG3	BG4	BG5
Fishmeal	50	30	50	10	10
Wheat meal	13.85	6.55	13.85	6.05	6.05
Wheat gluten	5	10	5	10	10
Soy protein concentrate	0	0	0	20	20
Soybean meal	0	20	0	0	0
Corn Gluten	0	0	0	9	9
Pea protein concentrate	0	0	0	9	9
Fish oil	25	26.4	3.8	27.5	7.7
Rapeseed oil	0	0	21.2	0	19.8
Mineral premix	0.59	0.59	0.59	0.59	0.59
Vitamin premix	2	2	2	2	2
Monosodium Phosphate	2.5	2.5	2.5	2.5	2.5
Carop. Pink (10% Astax)	0.05	0.05	0.05	0.05	0.05
Yttrium oxide	0.01	0.01	0.01	0.01	0.01
Choline	0.5	0.5	0.5	0.5	0.5
Methionine	0.3	0.6	0.3	0.9	0.9
Lysine	0	0.5	0	1.2	1.2
Threonine	0	0.1	0	0.4	0.4
Histidine	0.2	0.2	0.2	0.3	0.3

the feed mash entering the extruder was 86–88°C. Temperature at the extruder outlet were 120°C for BG1 and BG3, 128°C for BG2 and 137°C for BG4 and BG5. Three of the diets, BG2, BG4, and BG5 had lower wheat content in the recipe, and hence more moisture was added as heat into the DDC to ensure expansion. The extruder outlet had 24 circular 2.5 mm die holes. The wet extrudates were cut at the die surface with a rotating knife. To ensure the pellet quality, pellet samples were visually inspected after achievement of steady state conditions in the preconditioner and extruder. The extrudate was dried in a hot air dual layer carousel dryer (Paul Klockner, Nistertal, Germany) at a constant air temperature (77°C) to obtain final products of approximately 7–8% moisture. Then each of the diets were coated with oil in an experimental vacuum coater (Pegasus PG-10VC LAB, Dinnissen B.V., Netherlands). Immediately after coating, diets were packed in sealed plastic buckets and shipped to the research site.

Fish and Feeding

Atlantic salmon (*Salmo salar*) post-smolts were obtained from Cermaq, Hopen, Bodø, Norway (Aquagen strain, Aquagen AS, Trondheim, Norway) and maintained at the Research Station, Nord University, Bodø, Norway. At the start of the experiment, a total of 1100 fish (initial weight 72.7 ± 1.4 g) (mean ± SD) were randomly allocated to 20 experimental units (n = 4 tanks per treatment group).

The feeding experiment was carried out in a flow-through system. In total, 20 circular fiberglass tanks (1100 L) were used for the study. Each tank was supplied with water pumped from a depth of 250 m from Saltenfjorden. During the experiment, water flow rate was maintained at 1000 L per hour, and the average temperature and salinity of the rearing water were 7.6°C and 35‰, respectively. Oxygen saturation was always above 85% measured at the water outlet. A 24-h photoperiod was maintained throughout the experimental period. The fish were fed *ad libitum* using automatic feeders (Arvo Tech, Finland) for 12 h per day from 08:00–20:00 (divided into eight feedings: 08:00–10:00, 10:00–12:00, 12:00–14:00, 14:00–16:00, 16:00–18:00, 18:00–19:00, and 19:00–20:00) during the 65-day feeding trial.

Fish Sampling and Data Collection

At the beginning and end of the experiment, all fish (1100) were individually weighed, and their total lengths recorded. Before handling, fish were anesthetized using tricainemethanesulfonate (MS 222, 140 mg/L). Feces for digestibility determination was obtained by stripping individual fish. Feces from all individuals from a tank were pooled into one sample to obtain a value from a particular tank. The fecal samples that were immediately transferred to -20°C were used for further analyses.

For the histology and design-based stereology studies, distal intestine and mid intestine samples, respectively were collected as described in our previous publications (20–24). In addition, skin, gill and distal intestine samples were obtained for the gene expression analysis, and our standard protocols (21–23) were used in the present study also. For the cell study, the head kidney (HK) was collected at the end of experiment. These tissues were immediately transferred to 15 ml tubes to make a total volume of 4 ml in ice-cold Leibovitz's L-15 Medium (L-15; Sigma-Aldrich, Oslo, Norway), supplemented with 100 µg/ml gentamicin sulphate (Sigma), 2 mM L-glutamine (Sigma), and 15mM HEPES (Sigma).

Biochemical and Cholesterol Analyses

Frozen fecal samples were freeze dried (VirTis benchtop, U.S.A.) for 72 h at -76°C and at a pressure of 20 bar. The moisture, protein, lipid, ash, energy and yttrium contents of the feed and freeze-dried feces were determined as described in Sørensen et al. (22). Blood was drawn from the caudal vein of 12 fish/feed, into lithium heparin vacutainers and immediately spun at 703.2 x *g* for 10 min at 4°C. Cholesterol level in the plasma was measured by application of 115 µl plasma to a T4/Cholesterol rotor cassette (Profile #500-0037, Abaxis, CA, US), and analyzed by a VETSCAN Chemistry Analyzer (VETSCAN VS2, Abaxis, CA, US). Cholesterol was only analyzed in fish from BG1-BG4 due to lack of cassettes to analyze fish from BG5.

Mucosal Mapping

Samples for mucosal mapping with design-based stereology were collected at the end of the feeding experiment (day 65). Approximately 2 cm of the anterior part of the mid intestine from four fish (three tanks per diet group) were collected for this study—in total 12 samples per diet group. Luminal contents were first rinsed out with 10% neutral buffered formalin, and then the tissues were fixed in 10% formalin for 48 h. The fixed samples were dehydrated in an alcohol gradient, equilibrated in xylene and embedded in paraffin blocks. Approximately, 5 µm thick longitudinal sections were cut using a microtome and mounted onto a glass slide. The sections were stained with Alcian Blue pH 2.5—Periodic Acid Schiff's reagent (25) and mounted with Pertex medium.

All slides were scanned in batches using a Hamamatsu NanoZoomer S60 with a source lens; at 40x magnification and saved as high-resolution digital images in NDPI-format. The digital files were examined using NDP.view 2.6.8 (Free edition, Hamamatsu Photonics K.K. 2016). Mucosal mapping of the digitized slides was performed using the MucoMaster2 (Quantidoc AS, 2019) software according to Pittman et al. (26, 27). Blinded stereological analysis was done, maintaining the anonymity of the diet groups until the completion of the analysis. Regions of interest were manually drawn over the mucosal folds and lamina propria of each fish midgut. An unbiased selection of about 100 mucosal cells was performed to carry out the measurements for each slide as described in Pittman et al. (26) Epithelial area and mucous cell area were measured using stereological probes, followed by counting of mucous cells. Mean area of the mucous cells and percentage of epithelial with mucous cells were exported to Microsoft Excel for Office 20 365 MSO version 1908 (Microsoft Corporation, 2019). The barrier status as described in Dang et al. (20) was calculated using the mean mucous area, mucous number and epithelial area.

Distal Intestinal Micromorphology

Sections of the distal intestine were prepared as described under *Mucosal Mapping*. Slides were examined using microscope Olympus BX51 at 100x total magnification and photomicrographs were captured employing Camera SC180 (Olympus Europa GmbH, Hamburg, Germany) and processed using the imaging software CellEntry (Soft Imaging System GmbH, Munster, Germany).

Gene Expression Analysis

Tissues for gene expression analysis were sampled from the second gill arch (left side of the fish), skin (below dorsal fin), and distal intestine of 16 fish per diet group (four fish per tank). These tissues were immediately placed in tubes filled with RNA later® (Ambion Inc., Austin, Texas, United States), and stored at -20°C until further analysis.

The relative mRNA levels of mucin genes (*muc2*, *muc5ac1*, *muc5ac2*, and *muc5b*) in the distal intestine, skin and gills and antimicrobial protein genes (*defensin 1 - def1; defensin 2 - def2, defensin 3 - def3, defensin 4 - def4;cathelicidin 1 - cathl1*) in the distal intestine and skin were examined in this study. The primer sequences for all target and reference genes are presented in **Supplementary Table 2**. Primers were purchased from Eurofins Genomics (Luxembourg, Luxembourg).

RNA was extracted from the samples using E-Z 96 Total RNA Kit (Omega Bio-Tek, USA). Roughly 100 mg of the tissue sample was removed from RNA later® and homogenized using Zirconium oxide beads (1.4 mm; Percellys, Tarnos, France) and TRK lysis buffer in a capped free standing tube (VWR International, Oslo, Norway) at 6000 rpm. The resulting mixture was centrifuged (18,000 × g, 20°C) to obtain a clear supernatant. Briefly, 300 µl supernatant was added to 300 µl of 70% ethanol and mixed, before this mixture was added to the E-Z 96 RNA plate which contains an RNA HiBind® matrix in each well. Centrifugation (3000 rpm, 15 min) was used to draw the sample through the well, followed by several steps of buffer washes according to the kit instructions. Finally, the purified RNA was obtained by adding 65–75 µl of RNase-free water (5 Prime GmbH, Hilden, Germany) to each well and a final centrifugation.

Extracted RNA was quantified by Qubit™ RNA broad-range assay kit (Life Technologies, Carlsbad, USA) on a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, USA) and diluted with RNase-free water if necessary. cDNA synthesis was done with QuantiTect™ Reverse Transcription Kit (Quiagen GmbH, Hilden, Germany) employing 1000 ng of RNA and a reaction volume of 20 µl per sample, according to the manufacturer's instructions. The cDNA samples were diluted with nuclease free water by a factor of 10 before continuing with qPCR.

The qPCR was performed on a LightCycler® 96 (Roche Life Science) using Fast SYBR® Green Real-Time PCR Master Mix (Applied Biosystems, Carlsbad, USA). Each reaction contained 5 µl of Fast SYBR® Green PCR Master Mix, 1 µl primer mix (200 nM), and 4 µl cDNA (0.5 ng/µl). Reactions (n = 16 per diet) were performed in duplicate. Thermal cycling conditions were: initial holding at 95°C for 20 s, 40 cycles of denaturation at 95°C (3 s), and annealing/extension at 60°C (30 s).

A standard curve with known concentrations was prepared for each primer in order to calculate the gene expression. This was done by pooling RNA from every sample, reverse transcribing the pooled RNA as described above, and using the resulting cDNA to create a 6-point threefold dilution series. The equation E = (10^(−1/m)− 1) *100 was used to calculate the efficiency of the primers; E, efficiency and m, slope of the standard curve (**Supplementary Table 2**). Using geNorm (28) a normalization factor was computed for each sample based on the relative quantities of the two most stable genes from among the set of four reference genes, namely elongation factor 1AB (*ef1ab*), ribosomal protein L13 (*rpl13*), ribosomal protein S29 (*rps29*), and ubiquitin (*ubi*). The expression levels of all the target genes were calculated relative to the normalization factor.

Head Kidney Leucocytes

Head kidney (HK) cells (six fish/group) were harvested employing the protocols described for Atlantic salmon HK cells (29). The leucocyte fraction was employed for analysis of the lymphocyte counts. The monocyte/macrophage fraction was allowed to adhere on a petri dish for 3 days at 12°C. The adherent cells were detached by washing three times with 1.5 ml ice-cold phosphate-buffered saline (PBS) supplemented with 5mM EDTA (Sigma), and centrifuged at 500 × g for 5 min at 4°C. The cells were counted using a portable cell counter (Scepter™ 2.0 cell counter, EMD Millipore, Darmstadt, Germany). The flow cytometric analyses were performed as described by Park et al. (29), employing ImageStream®X Mk II Imaging Flow Cytometer (Luminex Corporation, Austin, TX, USA). Cell analyses were performed on 20,000 cells; lymphocyte-like cell population was determined based on the positivity of cells to salmon IgM while other cell populations (monocyte/macrophages) were identified based on morphological characteristics (29). Phagocytosis was studied using fluorescent bio-particles designed for flow cytometry, as detailed in our previous publication (29). Phagocytic ability and phagocytic capacity are presented to indicate phagocytosis; the former parameter is the percent of phagocytic cells, and the latter one is calculated as the mean number of particles per phagocytic cell.

Calculations and Statistical Analysis

Fish growth performance was analyzed using the following equations.

$$\text{Weight gain}(\%) = \left(\frac{W_f - W_i}{W_i}\right) \times 100$$

Where Wf = final body weight of fish (g/fish) and Wi = initial body weight of fish (g/fish)

$$\text{Specific Growth Rate } (\%\ day^{-1}) = \left(\frac{\text{Ln } (W_f) - \text{Ln } (W_i)}{\text{No.of feeding days}}\right) \times 100$$

$$\text{Thermal growth coefficient (TGC)} = \frac{(W_f)^{1/3} - (W_i)^{1/3}}{(T \times d)} \times 1000$$

where T is the temperature in °C and d is time in days.

Apparent Digestibility Coefficient (ADC) of nutrients and dry matter were calculated according to following equations:

$$ADC_{nutrient} = \left[1 - \left(\frac{Marker_{feed} \times Nutrient_{feces}}{Marker_{feces} \times Nutriten t_{feed}}\right)\right] \times 100$$

$$ADC_{\ dry\ matter} = \left[1 - \left(\frac{Marker_{feed}}{Marker_{feces}}\right)\right] \times 100$$

where $Marker_{feed}$ and $Marker_{feces}$ represent the marker content (% dry matter) of the feed and feces, respectively, and $Nutrient_{feed}$ and $Nutrient_{feces}$ represent the nutrient contents (% dry matter) in the feed and feces, respectively. Tank was used as the experimental unit.

The mucous cell-based barrier status was calculated using the following formula:

$$\left[\frac{1}{Mucous\ cell\ area \Big/ \left[Mucous\ cell\ area\ x\ mucous\ number \Big/ Epithelial\ area \times 100\right]}\right] \times 1000$$

Statistical analyses were performed using SPSS 22.0 software and R packages for Windows. The data were tested for normality (Shapiro–Wilk normality test) and equality of variance (Levene's test). For parametric data, one way analysis of variance (ANOVA) was performed after checking for equal variance. Tukey's multiple comparison test was used to identify the significant differences among the means of the dietary groups. For non-parametric data, Kruskal-Wallis test, followed by Dunn's multiple comparison test, was performed to decipher the significant differences between the groups. A significance level of $p < 0.05$ was chosen to indicate the differences.

RESULTS

Apparent Digestibility Coefficients

The dry matter content in feces was significantly higher in BG1- and BG3-fed fish (14%–15%) compared with BG2-, BG4-, and BG5-fed fish (10%–11%). We observed significant differences for the digestibility values of dry matter (DM), protein, lipid, ash and energy of the five feeds (**Table 2**). The DM digestibility was significantly lower in BG4-fed (59%) fish compared to BG2 (66%) and BG3 (68%), while no differences were noted among fish fed BG1, BG2, BG3, and BG5. Protein digestibility was lowest (significantly) in fish fed the BG1 (81%) compared to the other groups (85%–88%). Lipid digestibility was the highest in fish fed BG3 (96%) and BG5 (95%), and the lowest in fish fed BG2 (87%). Digestibility value of ash in BG2-fed fish was positive (1%), while those of fish fed other diets were negative (9%–33%). Energy digestibility was significantly higher in fish fed the BG3 (84%) compared to the other groups (73%–78%).

Growth Performance

The weight gain and growth rate are given in **Table 3**. The fish grew from an initial average weight of 70 g to a final average body weight of 150 g during the experimental period of 65 days. Significantly lower final body weight (138 g), weight gain (94%), thermal growth coefficient (2.1) was noted in fish fed the BG2 compared to the fish fed BG3 (158 g, 117%, 2.5, respectively). No differences in final body weight, weight gain, specific growth rate and thermal growth coefficient were noted for fish belonging to the different dietary treatments. Five fish died during the experiment, but mortality was not related to feed groups.

Cholesterol

Cholesterol concentration in blood ranged from 7 to 10 Mmol/L, and certain values were significantly differences (**Figure 1**). Cholesterol level was the highest in fish fed fishmeal-based

TABLE 2 | Dry matter content in feces and apparent digestibility coefficients (ADC %) of dry matter (DM), lipid, protein, ash, and energy in Atlantic salmon fed the experimental diets.

	BG1	BG2	BG3	BG4	BG5	p value
DM	14.5 ± 0.5^{a}	10.4 ± 0.4^{b}	13.8 ± 0.8^{a}	11.2 ± 0.4^{b}	11.4 ± 0.4^{b}	<0.001
ADC %						
DM	62.1 ± 3.1^{ab}	66.1 ± 0.6^{a}	68.4 ± 1.2^{a}	59.0 ± 3.4^{b}	63.6 ± 5.4^{ab}	0.007
Protein	81.3 ± 1.7^{b}	86.1 ± 0.3^{a}	85.5 ± 0.6^{a}	86.6 ± 1.4^{a}	88.1 ± 2.2^{a}	<0.001
Lipid	90.6 ± 1.3^{b}	87.4 ± 0.2^{c}	96.4 ± 0.2^{a}	92.0 ± 0.9^{b}	95.4 ± 2.4^{a}	<0.001
Ash	-14.1 ± 10.7^{ab}	0.9 ± 4.6^{a}	-8.6 ± 1.8^{a}	-33.2 ± 10.6^{b}	-21.0 ± 17.5^{ab}	0.005
Energy	77.6 ± 1.7^{b}	77.3 ± 0.6^{b}	83.8 ± 0.8^{a}	73.1 ± 2.4^{b}	77.0 ± 3.6^{b}	<0.001

BG1: Fishmeal + Fish oil diet; BG2: Soybean meal diet; BG3: Fishmeal + Plant oil diet; BG4: Plant ingredients + Fish oil diet; BG5: Plant ingredients + Plant oil diet. Values are expressed as mean ± SD (n=4 replicates). Values in the same row with different superscript letters indicate significant differences (p <.05).

TABLE 3 | Growth performance of Atlantic salmon for the experimental period.

	BG1	BG2	BG3	BG4	BG5	p value
IBW	72.4 ± 1.2	71.3 ± 1.0	72.9 ± 1.7	73.5 ± 1.4	73.5 ± 0.9	0.15
FBW	152.3 ± 4.5^{a}	138.3 ± 5.3^{b}	158.4 ± 5.9^{a}	150.7 ± 9.4^{ab}	150.3 ± 4.9^{ab}	0.01
WG	110.2 ± 7.9^{ab}	93.8 ± 7.0^{b}	117.2 ± 3.3^{a}	105.1 ± 16.3^{ab}	104.7 ± 8.2^{ab}	0.04
SGR	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	0.11
TGC	2.4 ± 0.1^{ab}	2.1 ± 0.1^{b}	2.5 ± 0.1^{a}	2.3 ± 0.3^{ab}	2.3 ± 0.1^{ab}	0.05

BG1: Fishmeal + Fish oil diet; BG2: Soybean meal diet; BG3: Fishmeal + Plant oil diet; BG4: Plant ingredients + Fish oil diet; BG5: Plant ingredients + Plant oil diet.
IBW, Initial body weight (g); FBW, Final body weight (g); WG, Weight gain (%); SGR, Specific growth rate (% day^{-1}); TGC, Thermal growth coefficient.
Values are expressed as mean ± SD (n=4 replicates). Values in the same row with different superscript letters show significant differences (p <.05).

diets, BG1 and BG3, and the lowest in those fed BG2. Fish fed the BG4 had lower cholesterol than those fed BG1, but not significantly different from BG3-fed fish.

Histology of the Distal Intestine

Micromorphology of the distal intestine samples is shown in **Figure 2**. Inflammatory response in BG2-fed group was evident from the aberrant lamina propria, widened villi, villi fusion and infiltration of inflammatory cells into lamina propria from base of intestinal mucosa. In addition, nuclei of intestinal absorptive cells were displaced and supranuclear vacuoles were also absent in the distal intestine of BG2-fed fish.

Fish fed the BG1 and BG3 had distal intestine with normal features. Enterocytes had a columnar shape, with nuclei situated near the lamina propria. Supranuclear vacuoles were present and the tissue had a normal distribution of goblet cells. Lamina propria had a slender and delicate core, and normal intraepithelial leucocyte infiltration was observed in BG1- and BG3-fed fish. Fish fed BG4 and BG5 also had normally positioned cell nuclei, and the typical distribution of goblet cells. However, the supranuclear vacuoles were smaller in size compared to BG1.

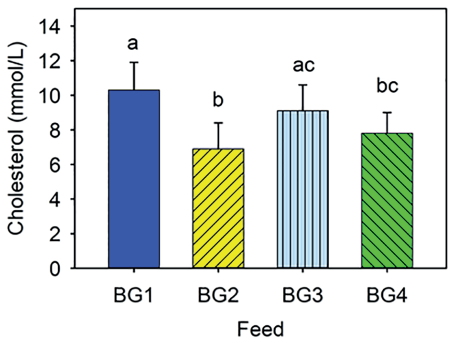

FIGURE 1 | Cholesterol level in the blood of Atlantic salmon fed different experimental diets. BG1: Fishmeal + Fish oil diet; BG2: Soybean meal diet; BG3: Fishmeal + Plant oil diet; BG4: Plant ingredients + Fish oil diet. Values are expressed as mean ± SD (n=12 fish per diet group). Different letters above the bars indicate significant differences (p < .05).

Mucosal Mapping

The mean area of intestinal mucous cells for the 60 fish sampled was around 155.3 ± 3,6 μm^2, for the five diet groups. The mucous cells' mean area per diet group was not significantly different (**Figure 3A**).

Average intestinal mucous cell density ranged from about 6% to about 11% and density of the mucous cells differed among diet groups ($p < 0.05$; **Figure 3B**). Fish fed BG2 and BG3 had mucous cell volumetric densities that was significantly lower than fish fed diets BG4 and BG5 ($p < 0.001$). Interestingly the marine diet BG1 also had a volumetric density of mucous cells in the epithelium that was significantly lower than BG4 ($p < 0.05$), and the values indicated a strong tendency towards a lower volumetric density than fish fed BG5 ($p = 0.057$).

The mucous cell-based barrier status values of the different fish groups also indicated a strong tendency to differ ($p = 0.062$). Fish fed BG2 had the lowest average barrier status (0.440 ± 0.055) and those fed BG1, BG2 and BG3 had a significantly lower barrier status than fish fed diets BG4 and BG5 (**Figure 3C**, $p < 0.01$).

Expression of Mucin Genes and Antimicrobial Protein-Encoding Genes

The relative expression of mucin genes in Atlantic salmon skin, gills, and distal intestine is shown in **Figure 4**, respectively. Expression of all four mucin genes were analyzed for all three tissues, and the expressional pattern was found to be tissue-specific.

The skin expressed *muc5ac1, muc5ac2*, and *muc5b* (**Figure 4A**). The expression of *muc5ac1* was relatively higher than those of the other two genes, and significant differences were observed only for the *muc5ac1* gene. The fish fed BG5 diet had the highest relative expression of the *muc5ac1* gene; approximately 3-fold higher compared to other groups. On the other hand, fish fed BG4 tended to have higher expression (2-fold) than those fed BG1-BG3 but lower (-1.5-fold) than BG5-fed fish.

The gills expressed the two genes *muc5ac2* and *muc5b*, and these genes showed an overall higher relative expression (**Figure 4B**). A similar relative expression pattern was noted for both the genes; the highest value (2.1-fold) for fish fed BG2 and lowest in fish fed BG4.

The distal intestine expressed *muc2* (**Figure 4C**). Fish fed BG2 had a significantly reduced (-3.2-fold compared to BG1) expression compared to all the other fish groups. The BG1 group had the highest relative expression (1.3-fold) and was significantly different compared to BG2, BG3 and BG4.

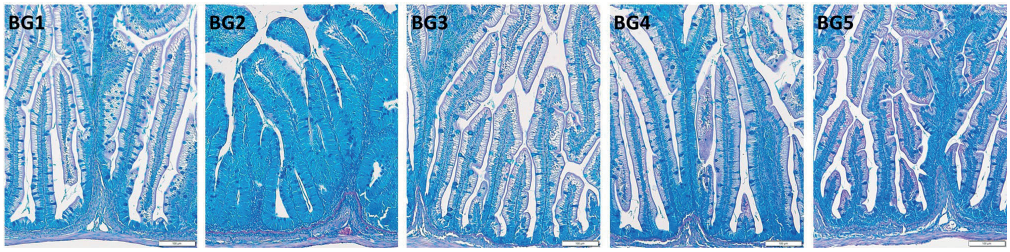

FIGURE 2 | Histomorphology of the distal intestine of Atlantic salmon fed diets BG1-5. BG1: Fishmeal + Fish oil diet; BG2: Soybean meal diet; BG3: Fishmeal + Plant oil diet; BG4: Plant ingredients + Fish oil diet; BG5: Plant ingredients + Plant oil diet.

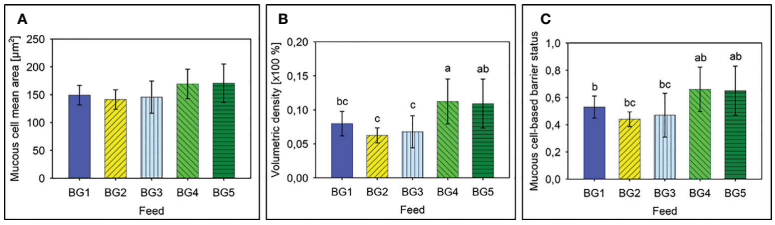

FIGURE 3 | Mucus cell-based analysis to assess the barrier status in the mid intestine of Atlantic salmon. **(A)** Mean area of mucus cells present in the mid intestinal epithelium of Atlantic salmon. **(B)** Mean volumetric density of mucous cells present in the mid intestine of Atlantic salmon. **(C)** Barrier status of the mid intestine of Atlantic salmon. BG1: Fishmeal + Fish oil diet BG1: Fishmeal + Fish oil diet; BG2: Soybean meal diet; BG3: Fishmeal + Plant oil diet; BG4: Plant ingredients + Fish oil diet; BG5: Plant ingredients + Plant oil diet. Values are expressed as mean ± SD (n=12 fish per diet group). Different letters above the bars indicate significant differences among diet groups.

As for the relative expression of AMPs in Atlantic salmon skin (**Figure 5A**) and distal intestine (**Figure 5B**), the relative expression of *cathl1* and *def1* in the skin of Atlantic salmon was relatively high and the expression of *cathl1* was significantly higher in fish fed BG2 (2.5-fold compared to BG1 and BG3-4) and BG5 (2-fold, **Figure 5A**). We did not observe any differences in the expression of *def1* in the different fish groups. In the distal intestine, *def3* had higher relative expression than *cathl1* (**Figure 5B**). Expression of both genes in the diet groups differed significantly. The expression of *cathl1* was significantly higher in fish fed BG3 compared to those fed BG1 and BG4. The *def3* had the highest expression (3.7-fold) in fish fed BG1 and lowest for those fed BG2 and BG4.

Salmon Head Kidney Lymphocyte-Like Cell Population and Phagocytic Activity of Macrophage-Like Cells

The percentages of lymphocyte-like cells in the head kidney from fish fed BG1 (39%) and BG3 (41%) were significantly higher than that of fish fed BG5 (24%; **Figure 6**; $p < 0.05$). However, there was no significant difference between the counts of fish fed BG1 and BG3 (41%; $p > 0.05$) or those fed BG2 (30%) and BG4 (32%).

Phagocytic ability (**Figure 7A**) and capacity (**Figures 7B, C**) of HK macrophage-like cells from fish fed BG1 were significantly higher than those fed the other diets ($p < 0.001$). There were no significant differences among the fish fed BG2-5 ($p > 0.05$).

DISCUSSION

The experimental diets were formulated to investigate nutrient digestibility, growth, mucosal barrier status, and activity of leucocytes from head kidney of the fish fed marine- or plant-derived protein and lipid sources. Plant protein concentrates were chosen to evade the negative effect of carbohydrate and antinutritional factors in plant ingredients on fish health, as noted by other researchers (30–33). Furthermore, feeding studies with Atlantic salmon have shown good growth performance with fishmeal incorporation at 3% or even without the finite ingredient; in such cases amino acids in the feed should be well balanced (32) and the feed should contain attractants derived from marine ingredients (31, 32). Hence, we included the essential amino acids in all the feeds. Rapeseed oil was chosen as the plant oil because it is commonly used to replace fish oil in

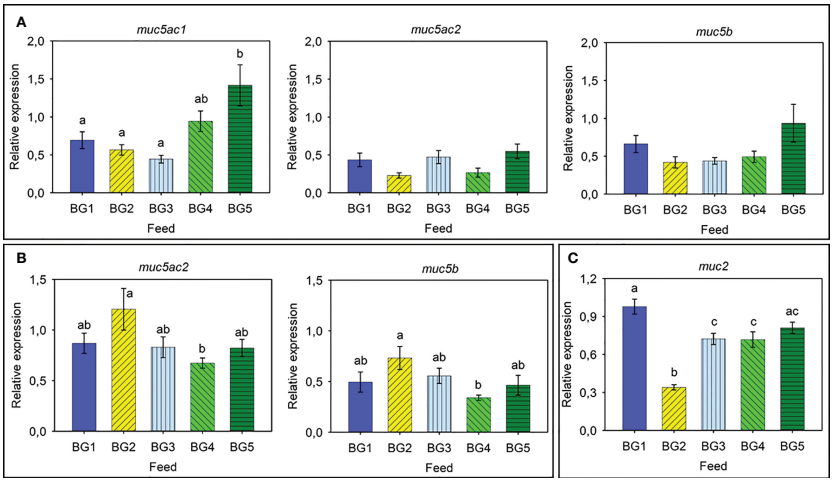

FIGURE 4 | Relative expression of mucin-related genes in Atlantic salmon. **(A)** Skin: *muc5ac1*, *muc5ac2*, and *muc5b*. **(B)** Gills: *muc5ac2* and *muc5b*. **(C)** Distal intestine: *muc2*. BG1: Fishmeal + Fish oil diet; BG2: Soybean meal diet; BG3: Fishmeal + Plant oil diet; BG4: Plant ingredients + Fish oil diet; BG5: Plant ingredients + Plant oil diet. Values are expressed as mean ± SD (n=12 fish per diet group). Different letters above the bars indicate significant differences ($p < .05$). Expression of *muc2* was too low to be quantified in the skin. Expression of *muc5ac1* and *muc2* was too low to be quantified in the gills. Expression of *muc5ac1*, *muc5ac2*, and *muc5b* was too low to be quantified in the distal intestine.

modern aqua diets (14). The soybean meal diet (SBM; BG2) was deliberately designed to study enteritis; based on earlier reports (34–37). Soybean meal-induced inflammation model is often used to study effects of the ingredient on gut health as well as bile acid levels and hypocholesterolaemia (36–38).

This experiment was not designed as a typical growth performance trial with feed intake measurements. Nevertheless, the results showed that except for the fish fed soybean meal (BG2), all diet groups doubled their weights during the 65 days feeding trial; this result indicates that the diets generally performed well. The lower weight gain of fish fed BG2 is not an unexpected finding because previous studies have already reported such a consequence of soybean meal feeding. Fish fed BG3 diet that contains 50% fishmeal and 21% rapeseed oil had the best growth; the good growth is likely to be due to the high protein and lipid digestibility of this diet.

The lowest protein digestibility was observed for the fish fed the fishmeal and fish oil diet (BG1). Protein digestibility of fishmeal-based diet can vary between 82% to almost 90% (39, 40). However, the difference in protein digestibility between BG1 and BG3 was unexpected because both diets contained the same amount and source of fishmeal. Lipid digestibility was lower in the diets containing fish oil (BG1, BG2 and BG4). The result may be explained by the higher content of saturated fatty acids in fish oil compared to rapeseed oil (41). The lowest lipid digestibility was noted for the fish fed the SBM diet, and the finding corroborates with those of earlier studies (36–38). The highest energy digestibility was observed in fish fed fishmeal and plant oil (BG3), reflecting the high protein and lipid digestibility. Reduced DM content in feces from Atlantic salmon fed SBM or SPC is in line with other studies on salmonids (17, 36, 42, 43), and could likely to be an effect of altered expression of genes encoding aquaporins, ion transporters, tight junction and adherence junction proteins (17), leading to a loss of junction barrier integrity.

The morphological changes observed in the distal intestine of the fish fed the SBM diet were consistent with soybean meal-induced enteritis and in line with several other studies that employed 20% SBM in diets for salmonids. For the other diet groups, there were no severe signs of enteritis. Saponin is the antinutritional factor responsible for inducing enteritis in soybean meal fed Atlantic salmon (44), but severity is potentiated by other bioactive components of the plant ingredients (34). Soy protein concentrate is devoid of saponins (45) and incorporation up to 45% into marine based diets do not cause severe gut inflammatory and immune responses in Atlantic salmon (46, 47). Fish fed the fishmeal-based diets (BG1 and BG3) had normal distal intestine features and the only dietary difference between these two groups was the inclusion of rapeseed oil (BG3). The reduction of supranuclear vacuoles noted for fish fed BG4 and BG5 compared to BG1 indicated subtle plant-based diet-induced aberrations as reported in other studies. Loss of absorptive vacuoles was also reported by Katerina et al. (48); they evaluated the effect of replacement of fish oil with the alga *Schizochytrium limacinum* throughout the entire life cycle of Atlantic salmon by feeding the fish with diets low in marine ingredients. The final grow-out diets in the latter experiment contained either 10% fishmeal and 4.3% fish, or 10% fishmeal and 6.25% alga. Irrespective of the diet, the authors

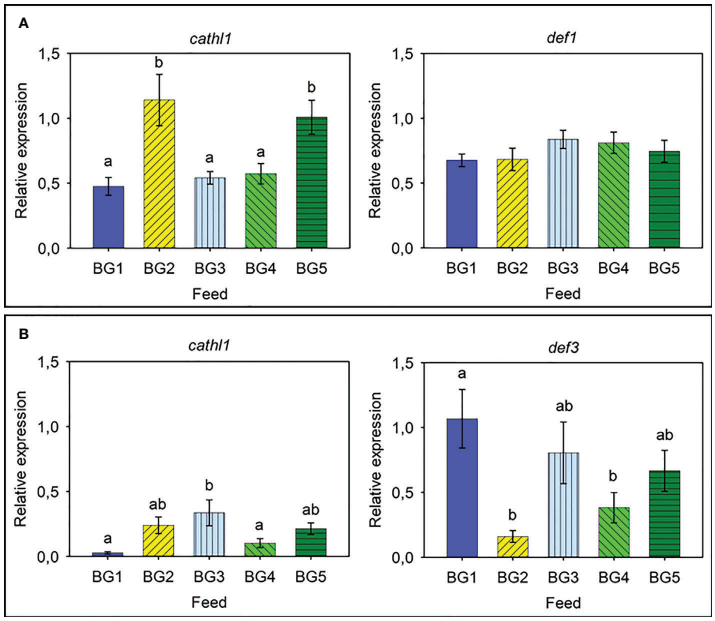

FIGURE 5 | Relative expression of antimicrobial protein genes in the skin and distal intestine of Atlantic salmon. **(A)** Skin *cathl1* and *def1*. **(B)** Distal intestine *cathl1* and *def3*. BG1: Fishmeal + Fish oil diet; BG2: Soybean meal diet; BG3: Fishmeal + Plant oil diet; BG4: Plant ingredients + Fish oil diet; BG5: Plant ingredients + Plant oil diet. Values are expressed as mean ± SD (n=12 fish per diet group). Different letters indicate significant differences ($p < .05$). Expression of *def2*, *def3*, and *def4* was too low to be quantified in the skin. Likewise, the expression of *def1*, *def2*, and *def4* was too low to be quantified in the distal intestine.

observed abnormal histomorphology in the distal intestine of the fish, characterized by enterocytes lacking vacuoles, abnormally tall folds with extensively developed branches and infiltration of inflammatory cells into the connective tissue. Taken together these two studies suggest that salmon compensate the lack of absorptive vacuoles by hypertrophy of the primary and secondary folds in the distal intestine. Based on the histology results from the present study we state that plant protein concentrates (not rapeseed oil) can also induce mild enteritis similar to the micromorphological changes that were noted in BG4 and BG5 that contained a mix of plant protein concentrates. It should be noted that all the diets in the present study were optimized to contain at least 1.7% EPA and docosahexaenoic acid (DHA) in the diets. The EPA+DHA content was also higher than the levels used by Katerina et al. (48). Other studies have pointed out the importance of fish oil to maintain a healthy barrier status and to maintain a good host disease resistance status. European seabass fed low levels of fish oil was not able to resist the invasive pathogens; an infection with *Vibrio anguillarum* resulted in increased translocation of the bacteria and increased fish mortality (49).

The lower cholesterol level in the fish fed the SBM diet is in line with other experiments that noticed hypocholesterolaemia as well as changes in the expression of genes involved in cholesterol biosynthetic pathways in fish fed soybean meal and lupin meal (36, 38, 50–52). The reduction in cholesterol level in the plasma of SBM fed fish is associated with saponins in SBM (34, 44, 51, 53). Fish fed the fishmeal and rapeseed oil diet (BG3) also had a numerically lower cholesterol level than BG1 but slightly higher than in fish fed plant protein mix and rapeseed oil (BG4). Sissener et al. (54) found a correlation between cholesterol level in the feed and its concentration in plasma, bile and whole fish. Therefore, the lower cholesterol in fish fed BG3 and BG4 can be partly explained by the lower content of cholesterol in these diets.

The mucosal mapping of the five diet groups revealed a consistent relationship with the growth data; the soybean meal diet group (BG2) had the smallest mean area, lowest volumetric density and an ensuing poor mucous cell-based barrier status compared to the other groups. Mucosal mapping results agree with more traditional analyses of gut health and with the overall growth performance. In contrast, both feeds containing a mix of plant protein concentrates (BG4 and BG5) had the largest mean mucous cell area, highest volumetric density and aberrant barrier status. These results suggest that the plant proteins cause enteritis in two ways; either by reducing (BG2) or increasing (BG4 and BG5) the mucous cell sizes and volumetric densities.

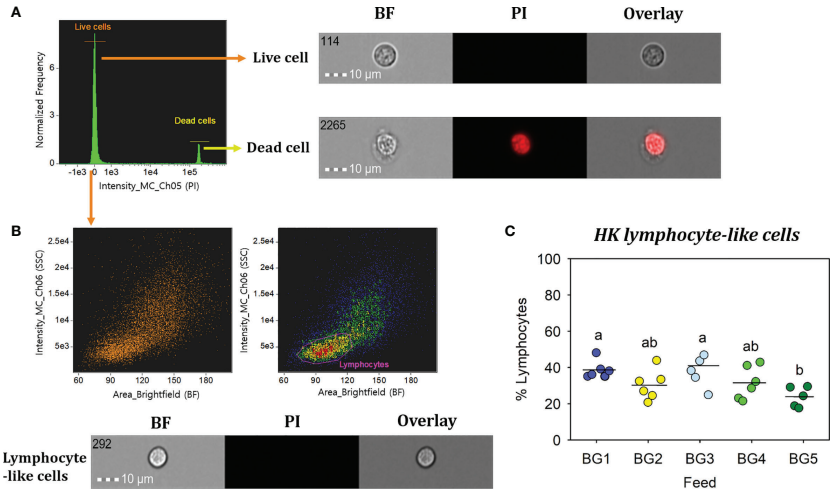

FIGURE 6 | Percentage of head kidney lymphocyte-like cells from Atlantic salmon fed different experimental diets. BG1: Fishmeal + Fish oil diet; BG2: Soybean meal diet; BG3: Fishmeal + Plant oil diet; BG4: Plant ingredients + Fish oil diet; BG5: Plant ingredients + Plant oil diet. **(A)** Live cells (orange) were separated by excluding the dead cells (yellow); by staining with propidium iodide (PI). **(B)** Brightfield (BF) area (cell size) vs. side scatter (SSC) intensity (cell internal complexity) plot showing the HK leucocyte population. **(C)** Percentage of HK lymphocyte-like cells from fish (n=6) fed different experimental diets. Statistically significant differences ($p < 0.05$) between dietary groups are indicated by different letters. All cell images were captured with 40× objective. Scale bar = 10 µm. BF, brightfield; PI, propidium iodide.

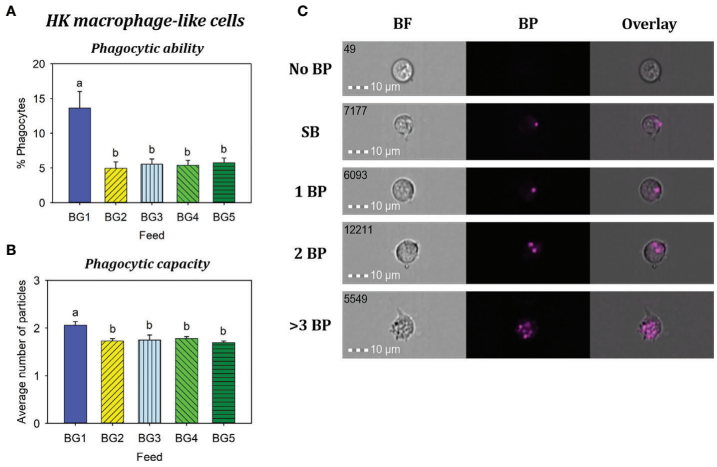

FIGURE 7 | Phagocytosis of head kidney macrophage-like cells from Atlantic salmon fed different experimental diets. BG1: Fishmeal + Fish oil diet; BG2: Soybean meal diet; BG3: Fishmeal + Plant oil diet; BG4: Plant ingredients + Fish oil diet; BG5: Plant ingredients + Plant oil diet. **(A)** Percent of phagocytic cells. **(B)** mean number of particles ingested per phagocytic cell. **(C)** Representative cell images indicate cells with no BP, SB, and 1BP, 2BP, and >3BP. Statistically significant differences ($p < 0.05$) between dietary groups are indicated by different letters. Bar plots show mean ± SD, n = 6. All cell images were captured with 40× objective. Scale bar = 10 µm. SB, surface-binding particles; 1 BP, 2 BP, and > 3 BP, one to three internalized bio-particles; BF, brightfield.

The present study focused on the secreted mucin genes that are expressed on certain mucosal tissues of salmon (4). Earlier studies have mainly investigated the expression of mucin genes in relation to stress (4), or as markers of parasite infestation (3, 55). Mucin genes are diagnostic markers of severe human diseases; e.g. airway disorders, inflammatory diseases, cancers (56–58). Tissue-specific expression of mucin genes–*muc2*-like genes in the distal intestine and *muc5*-like genes in the skin and gills–is consistent with previous research on Atlantic salmon (4). Sveen et al. (4) reported high expression of *muc5ac1* and *muc5b* in the skin and *muc5ac2* in the gills. In the present experiment only the expression of *muc5ac1* in the skin of fish fed the experimental diets differed significantly. Fish fed diets with high levels of plant protein concentrates had the highest expression of *muc5ac1*, but only the group fed diet with the highest incorporation of plant ingredients (BG5) had significantly higher *muc5ac1*. In the gills, the relative expression of *muc5ac2* was slightly higher than *muc5b* and the expression of both genes was significantly higher in fish fed soybean meal in the diet (BG2) and lowest in those fed the combination of plant protein ingredients and fish oil (BG4). Higher expression of *muc2* in the distal intestine of fish fed the marine ingredient-based diet (BG1) and down regulation in fish fed soybean meal (BG2) clearly indicate that this gene is correlated to intestinal health. The *muc2* has an anti-inflammatory and tumor suppressive role, and experiments with *muc2* knockout mice have shown abnormal goblet cells followed by development of colitis and colorectal cancer (59, 60). Mucus layer and microbiota structure are interdependent on each other, and the major and minor forms of O-glycosylated entities of *Muc2* in mice colon are known to have key roles in host-microbiota symbiosis (61). Diet is an important determinant of gut microbiota, and it is known that these microorganisms enhance the expression of e.g. *Muc2* and *Fut2* (galactoside 2-alpha-L-fucosyltransferase 2) to strengthen the mucus barrier and mucin glycan structure, thereby preventing the entry of microbes into the intestinal epithelium (62). The lower expression of *muc2* in fish fed BG3 compared to BG1 can only be explained by the different oil sources since both contained fishmeal. The difference between BG1 and BG4 could be due to the replacement of fishmeal with plant protein concentrates. The lower expression of *muc2* for fish fed the soybean meal diet (BG2) suggests that this gene may be used as a marker for severe intestinal inflammation.

Antimicrobial peptides are defense molecules that have key roles in disease prevention in fishes (63). The expression of both cathelicidins and defensins has been induced in salmonids subjected to bacterial challenge (64, 65). In the present study, *cathl1* was expressed in both the skin and distal intestine, but the expression was the highest in the skin of Atlantic salmon. The observation corroborates with that of Chang et al. (65); they also observed differential expression of the two cathelicidin genes in different tissues. After a bacterial challenge the expression of *cathl1* increased in some tissues but not in all (65). In the present experiment, the relative expression in the skin and distal intestine was affected by the feeds. As for the defensin genes, the gene *def1* was only expressed in the skin and it was unaffected by the feeds. On the other hand, *def3* was expressed in the distal intestine and was affected by the feeds. Similar to the *muc2* expression in the distal intestine, the expression of *def3* was the highest for fish fed BG1 and the lowest for fish fed BG2. Increased production of AMPs can be considered as a strategy of the fish to stimulate its immune system, and could serve as an alternate approach to reduce disease outbreaks in fish farms (2, 8).

In the present study, percentages of lymphocyte-like cells from the major hematopoietic organ (HK) of fish fed more plant ingredients (BG5) was significantly lower compared to those fed fishmeal-based diets (BG1 and BG3). The low content of fishmeal and fish oil in the BG5 diet may have influenced the counts of HK lymphocyte-like cells. In a study on European sea bass (66), the total number of circulating leucocytes in fish fed 100% fish oil diet was significantly higher than in fish fed plant oil diets. A study on mice has reported that a diet rich in fish oil promotes hematopoiesis (67); mice fed fish oil diet had significantly higher hematopoietic stem cells and hematopoietic progenitors in the spleen compared to mice fed low or high-fat diets. Increased phagocytic activity by macrophages is indicative of increased disease resistance competence (68). The significantly increased phagocytic ability and capacity of HK macrophage-like cells observed in the fish fed BG1 compared to those of other diets could be linked to increased dietary n-3 fatty acid, as reported previously (69–72).

Plant protein concentrates was used in the present experiment to reduce the effect of antinutritional factors. However, fish fed the plant protein concentrates (BG4 and BG5) in the present experiment also had altered histology and mucosal barrier status–loss of absorptive vacuoles, increased mucous cell volumetric densities and barrier status– compared to those fed the fishmeal as protein source (BG1 and BG3). There seems to be a close connection between nutritional status, modulations of the immune cell populations and their functions (73). We assume that higher *muc2* expression in the distal intestine of fish fed BG1 could contribute to the enhanced intestinal barrier protection as well as increase in immune cell counts and their function in head kidney indicating the importance of fishmeal and fish oil for the health of the fish.

In conclusion, the ADC values were within the normal ranges and the fish grew well on all diets, except the fish fed SBM. Fish fed the plant protein ingredients (BG4 and BG5) had lower DM content in the feces and had mild enteritis. Decreased mucous cell size and low barrier status were hallmarks of fish fed soybean meal, but increased cell size and abnormal barrier status were the features of fish fed plant protein mixes irrespective of lipid source. These results suggest two types of impact on gut health over the long term; either reduce mucosal protection or over-activate it. The four mucin genes in Atlantic salmon skin, gills, and distal intestine were affected by the ingredient composition. The expression of the antimicrobial peptide genes, *cathl1* and *def3* were also affected by the ingredients in the diets. Furthermore, higher numbers of lymphocyte-like cells, increased phagocytic ability and capacity of macrophage-like cells in head kidney as well as higher *muc2* expression in the distal intestine of fish fed the marine based diet (BG1) points to the compromised intestinal barrier in fish fed plant ingredients. These data can be combined with marker

gene information to further refine dietary compositions for sustainable and acceptable healthy diets.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by National Animal Research Authority (FDU: Forsøksdyrutvalget ID-5887) in Norway.

AUTHOR CONTRIBUTIONS

KK and MS designed the study and feed formulation. Feeding trials were supervised by MS, YG, and SS. GV helped SS in conducting the qPCR study. YP performed the cell study. TP, SØ, and KP were responsible for the stereology part of the manuscript. DD performed the histology study. SS wrote the first version of the manuscript. VK and MS edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was part of the project "*Blodanalyser av laks som metode for vurdering av tarmhelse*" funded by MABIT, project number AF0082.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.623726/full#supplementary-material

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Conflict of Interest: KK is employed by BioVivo Technologies AS, Bodø, Norway. KP is employed by Quantidoc AS, Bergen, Norway.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Paper II

fishes

MDPI

Article

Growth, Chemical Composition, Histology and Antioxidant Genes of Atlantic Salmon (*Salmo salar*) Fed Whole or Pre-Processed *Nannochloropsis oceanica* and *Tetraselmis* sp.

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Abstract: New sustainable feed ingredients are a necessity for the salmon aquaculture industry. In this study, we examined the effect of pre-extrusion processing of two microalgae, *Nannochloropsis oceanica* and *Tetraselmis* sp., on the growth, fatty acid content in the flesh and health of Atlantic salmon. The fish were fed one of the following five diets for nine weeks: (1) CO: a fish meal-based control (basal) diet, (2) NU: a *Nannochloropsis* diet, (3) NE: a pre-extruded *Nannochloropsis* diet, (4) TU: a *Tetraselmis* diet, and (5) TE: a pre-extruded *Tetraselmis* diet. The algae-incorporated diets contained 30% of the respective microalgae. Our results showed that the best growth performance was achieved by the CO diet, followed by the NE diets. Feeding of unprocessed *Nannochloropsis* and *Tetraselmis* resulted in a significant reduction in enterocyte vacuolization compared to the CO feeding. A significant effect of processing was noted in the fillet fatty acid content, the intestine and liver structure and the expression of selected genes in the liver. The expression of antioxidant genes in both the liver and intestine, and the accumulation of different fatty acids in the fillet and liver of the extruded algae-fed groups, warrants further investigation. In conclusion, based on the short-term study, 30% inclusion of the microalgae *Nannochloropsis oceanica* and *Tetraselmis* sp. can be considered in Atlantic salmon feeds.

Keywords: aquaculture; feed ingredients; fishmeal replacement; novel ingredients; nutrition

Citation: Sørensen, S.L.; Ghirmay, A.; Gong, Y.; Dahle, D.; Vasanth, G.; Sørensen, M.; Kiron, V. Growth, Chemical Composition, Histology and Antioxidant Genes of Atlantic Salmon (*Salmo salar*) Fed Whole or Pre-Processed *Nannochloropsis oceanica* and *Tetraselmis* sp. *Fishes* **2021**, *6*, 23. https://doi.org/10.3390/fishes6030023

Academic Editors: Vikas Kumar, Beth Cleveland, Janet Genz and Eric Hallerman

Received: 1 June 2021
Accepted: 20 July 2021
Published: 23 July 2021

1. Introduction

Marine microalgae are primary producers of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and, based on their chemical composition, certain microalgae can be good candidates for ingredients in fish feeds if they can be produced on a large scale [1]. A variety of microalgae species has been studied to assess their suitability as feed ingredients for aquaculture in general, and as replacements for fish meal or fish oil in feeds for Atlantic salmon (*Salmo salar*) [2–8]. Some of the microalgae are also good sources of vitamins, minerals and pigments, all with good antioxidant effects [9]. Antioxidant activity has been reported in extracts from different microalgae, amongst others *Tetraselmis* sp. and *Nannochloropsis* sp. [10]. Feeding fish with microalgae meal, which are sources of natural antioxidants, may increase their cells' capacity to scavenge reactive oxygen (ROS) or nitrogen species. ROS, the products of basal metabolism that regulate cellular homeostasis, are produced in cell organelles such as endoplasmic reticulum, mitochondria, peroxisomes and even in cell membranes and cytoplasm [11]. The vital ROS buffering systems in mitochondria and peroxisomes include glutathione peroxidases (GPx), glutathione (GSH), superoxide dismutase (SOD) family of proteins and catalase that help in converting radicals

into unharmful substances [12]. Thus, living organisms have developed different antioxidant defence systems to counterbalance the adverse effects of ROS. In addition to these antioxidant mechanisms, rapid and continuous renewal of secreted mucus safeguards the epithelial cells such as those of the intestine [13]. Furthermore, intestinal health and welfare could be considered as interconnected because new aquafeed ingredients can negatively affect the structure and barrier functions of the different intestinal segments, and intestinal microbiota is a key player in the immune maturation, growth, health and behaviour of farmed animals [14]. The gastrointestinal tract of fish is covered by a protective layer of mucus and each part of the intestine performs different functions [15]. Mucins are a diverse group of glycoproteins that are vital components of mucus, and these molecules are responsible for the viscoelastic properties of mucus. Mucins are produced and secreted by goblet cells in the intestine [16]. Mucous cell quantification is efficient in revealing aberrant responses in tissues [17]. Mucins can be either monomeric or multimeric, and the former group includes transmembrane mucins mainly associated with the glycocalyx, while the latter group are gel-forming mucins, like MUC2, MUC5AC, MUC5B and MUC6 [18–20]. Some MUC genes have been identified in fish or predicted in fish genomes and are in general similar to mammalian mucin genes [21–25]. Recently, mucin genes have been identified in several tissues in Atlantic salmon [26].

It is suggested that microalgae such as *Nannochloropsis* sp. and *Tetraselmis* sp. can provide the essential vitamins, pigments and polyphenols for the aquaculture industry [27–29]. In addition, *Nannochloropsis* and *Tetraselmis* are sources of high-value essential n-3 long-chain polyunsaturated fatty acids such as EPA and alpha linolenic acid (ALA) [30]. The protein and lipid content of *Nannochloropsis* vary between 18 and 48% and 2 and 68%, respectively. *Tetraselmis* has a protein content ranging between 27 and 54% and a lipid content in the range 3–45% [30]. Carnivorous species such as Atlantic salmon have a relatively short and simple digestive system with a limited capacity to digest complex carbohydrates present in intact microalgae. However, it is now known that the rigid microalgae cell walls can be broken to release the essential nutrients and antioxidant elements from microalgae [29,31–33]. Since the microalgae processing technology has not matured yet [34,35], it has been reported that the current microalgae inclusion level should be below 10% to avoid negative effects on energy digestibility and to obtain a good feed conversion ratio (FCR) [3–6,30,34]. Previous studies have indicated the suitability of microalgae for farmed fishes; 14.2% of a blend of *N. oculata* defatted biomass and whole cell *Schizochytrium* sp. was efficient enough to impart good growth in Nile tilapia (*Oreochromis niloticus*) [36], and low levels of Spirulina can help rainbow trout in recruiting lipids during times of starvation [37]. Studies have also shown that extrusion improves the digestibility of *Nannochloropsis* [2] and the process can also be used as a pre-treatment method to increase extraction of lipid [32] as well as other intracellular components. It is therefore hypothesized that extrusion as a pre-treatment of the algae biomass may enhance the bioavailability of lipids from *Nannochloropsis* and *Tetraselmis*, which, along with the released antioxidants, could improve the growth as well as the health of the fish. Hence, we have investigated the effects of feeding whole and pre-extruded microalgae meal on weight gain, whole body chemical composition, expression of selected genes in the liver and intestine and histological features in these organs of Atlantic salmon. Additionally, we have examined the feed quality based on the content of volatile organic compounds. It is reported that pre-processing of ingredients as well as the heat and shear forces from the extrusion process itself may also induce volatile organic compounds (VOCs) from oxidation of lipids [38–40] as well as oxidized proteins, indicating that VOCs may interfere with feed utilization [41].

2. Results

2.1. Volatile Organic Compounds

Dynamic headspace gas chromatography–mass spectrometry (GC-MS) analysis detected approximately 130 different volatile organic compounds (VOCs) in the experimental feeds, and the information of selected compounds is provided in Table 1. The molecules

detected in the feeds were typical protein (low molecular weight (LWM) amines, branched chain volatiles) and lipid oxidation products (aldehydes, alcohols, ketones). As observed, pre-treatment of the algae increased the concentration of LMW amines, hexanal, butanal, 2-methyl-propanal, 1-penten-3-one, (E)-2-pentenal, (E)-2-hexenal, and 1-penten-3-ol.

Table 1. Volatile oxidation products (ng/g) in feeds.

VOCs \ Diet Groups	CO	NU	NE	TU	TE
SUM LMW Amines	790	694	722	595	657
Propanal	5	6	2	7	12
Propanal, 2-methyl-	27	49	96	33	45
2-Propenal	0	3	9	2	2
Butanal	18	33	37	33	39
Butanal, 2-methyl-	53	101	4	72	102
Butanal, 3-methyl-	310	651	406	372	550
1-Penten-3-one	33	38	62	31	35
2-Butenal	7	33	77	13	16
Hexanal	51	107	116	48	77
2-Pentenal, (E)-	16	32	98	16	34
1-Penten-3-ol	307	428	678	244	580
2-Hexenal, (E)-	43	80	72	36	82
2-Penten-1-ol, (E)-	135	57	189	5	170

2.2. Growth

All the feeds were well accepted by the fish and there was no mortality during the course of the experiment. The weight gain of the fish ranged from 83 to 99 g (Table 2). The final weight of the fish fed the CO diet was significantly higher compared to the fish fed algae diets. Pre-extrusion of *Nannochloropsis* tended to increase fish growth compared to the other algae-fed fish groups, as reflected in the increased weight gain (WG; %) and specific growth rate (SGR; % day^{-1}). The WG and SGR in fish fed NU, TU and TE were also significantly lower compared to the CO group. A significant effect of the extrusion process was not evident from the 2-way ANOVA (Supplementary Table S1).

Table 2. Initial, final weight and specific growth rate of Atlantic salmon fed one of the five experimental diets for 9 weeks.

Growth Indices	CO	NU	NE	TU	TE
IBW(g)	154.4 ± 0.1	154.0 ± 0.1	153.9 ± 0.3	154.4 ± 0.2	154.3 ± 0.2
FBW (g)	307.8 ± 1.6 ^{a}	288.9 ± 5.0 ^{b}	292.9 ± 1.7 ^{ab}	282.9 ± 3.4 ^{b}	285.1 ± 3.6 ^{b}
WG (%)	99.4 ± 2.0 ^{a}	87.6 ± 6.3 ^{b}	90.3 ± 3.0 ^{ab}	83.3 ± 4.3 ^{b}	84.8 ± 4.8 ^{b}
SGR (% day^{-1})	1.15 ± 0.01 ^{a}	1.05 ± 0.03 ^{b}	1.07 ± 0.01 ^{ab}	1.01 ± 0.02 ^{b}	1.02 ± 0.02 ^{b}

Tanks were the experimental units (n = 4 replicates). Values are expressed as mean ± sem. Values in the same row with different superscript letters ($^{a, b}$) show significant differences ($p < 0.05$). CO: Control fishmeal-based diet, NU: *Nannochloropsis*, NE: *Nannochloropsis* pre-extruded, TU: *Tetraselmis*, TE: *Tetraselmis* pre-extruded. IBW, Initial body weight; FBW, Final body weight; WG, Weight gain; SGR, Specific growth rate.

2.3. Whole Body Proximate Composition

The whole body proximate composition of fish from the experimental groups at the end of the feeding study is presented in Table 3. The dry matter in the TE group was higher compared to the CO group, which had values significantly lower than the NE group. The NE group had higher dry matter values than the NU group. The highest protein content was found in salmon fed diet CO, but the values were not significantly different from the protein content in fish fed diets NU and TU. Fish fed NE and TE had significantly lower protein content than fish from the CO diet groups. In addition, the protein content of the TU and TE were significantly different. The lipid content was significantly higher in fish fed diet TE than fish fed NU, NE and TU. Ash content was significantly higher in fish fed

diets NU and NE. Ash content was significantly lower in fish fed diets TE compared to the fish fed CO, NE and NU diets. The ash content in NU was significantly higher compared to the TU group. Energy content was significantly higher in fish fed the diets TU and TE compared to fish from the other diet groups.

Table 3. Whole body proximate composition (%) of fish from the different experimental groups.

Parameter	CO	NU	NE	TU	TE
Dry matter (g/kg)	28.8 ± 0.09 ^{a}	28.8 ± 0.19 ^{ac}	29.6 ± 0.11 ^{b}	29.3 ± 0.16 ^{abc}	29.6 ± 0.28 ^{bc}
Dry matter (%)					
Protein	62.6 ± 0.42 ^{a}	61.1 ± 0.45 ^{abc}	60.4 ± 0.29 ^{bc}	61.4 ± 0.6 ^{ac}	59.6 ± 0.43 ^{b}
Lipid	32.5 ± 0.54 ^{ab}	31.7 ± 0.53 ^{a}	32.0 ± 0.3 ^{a}	32.0 ± 0.57 ^{a}	35.0 ± 0.54 ^{b}
Ash	6.8 ± 0.09 ^{ab}	7.0 ± 0.13 ^{a}	7.0 ± 0.21 ^{ab}	6.1 ± 0.2 ^{bc}	5.6 ± 0.05 ^{c}
Energy	25.6 ± 0.07 ^{a}	25.6 ± 0.02 ^{a}	25.6 ± 0.06 ^{a}	26.1 ± 0.11 ^{b}	26.3 ± 0.13 ^{b}

Values are expressed as mean ± sem (n = 4 replicates). Values in the same row with different superscript letters ($^{a, b, c}$) indicate significant differences ($p < 0.05$).

The fatty acid composition of salmon fillet after 9 weeks of feeding with the experimental diets are presented in Table 4. The highest content of SFA in the flesh lipids was found in the fillet of salmon fed diet NE. SFA content was significantly lower in fish fed diets CO, NU and TU. The MUFAs in the fillet were significantly higher in fish fed diets NU compared to all fish groups except those fed on the TE diet. Fish fed the TU diet showed higher PUFA content compared to the other groups, but we did not detect any significant differences. Fillets from fish fed the TU diets had higher content of EPA+DHA compared to fish fed the TE diet. The ratio of n-6 to n-3 fatty acids in the fillet were found to be significantly higher in fish fed the NU diet compared to the CO and TU diets.

Table 4. Content of fatty acids (% of lipid) in the fillets of fish fed the experimental diets for 9 weeks.

Fatty Acids \ Diet Groups	CO	NU	NE	TU	TE
C14:0	3.1 ± 0.04 ^{ab}	3.1 ± 0.02 ^{b}	3.3 ± 0.01 ^{a}	3.4 ± 0.4 ^{b}	3.1 ± 0.02 ^{b}
C16:0	13.0 ± 0.18 ^{a}	13.0 ± 0.13 ^{a}	14.5 ± 0.26 ^{b}	12.5 ± 0.67 ^{a}	13.8 ± 0.23 ^{ab}
C18:0	2.9 ± 0.05 ^{a}	2.8 ± 0.05 ^{ab}	2.9 ± 0.05 ^{a}	2.7 ± 0.07 ^{b}	2.9 ± 0.05 ^{ab}
∑SFA	19.0 ± 0.24 ^{a}	18.8 ± 0.15 ^{a}	20.7 ± 0.31 ^{b}	17.98 ± 0.94 ^{a}	19.70 ± 0.27 ^{ab}
C16:1	3.4 ± 0.01 ^{ab}	4.0 ± 0.04 ^{ac}	4.6 ± 0.05 ^{c}	3.2 ± 0.03 ^{b}	3.4 ± 0.07 ^{ab}
C18:1n-9	21.4 ± 0.51 ^{ab}	22.8 ± 0.2 ^{b}	20.6 ± 0.39 ^{a}	19.5 ± 1.67 ^{a}	22.7 ± 0.22 ^{b}
C18:1n-7	2.8 ± 0.01 ^{ab}	2.9 ± 0.02 ^{b}	2.7 ± 0.02 ^{a}	2.7 ± 0.04 ^{a}	2.7 ± 0.01 ^{a}
C20:1n-9	3.8 ± 0.15 ^{a}	4.3± 0.02 ^{b}	3.9 ± 0.09 ^{a}	4.0 ± 0.04 ^{a}	4.1 ± 0.06 ^{ab}
C22:1n-9	3.8 ± 0.02 ^{a}	4.3 ± 0.03 ^{b}	3.9 ± 0.04 ^{abc}	3.8 ± 0.07 ^{ac}	3.9 ± 0.01 ^{bc}
∑ MUFA	35.1 ± 0.56 ^{ab}	38.3 ± 0.23 ^{c}	35.7 ± 0.38 ^{ab}	29.8 ± 5.08 ^{b}	36.9 ± 0.20 ^{ac}
C18:2n-6	6.8 ± 0.19 ^{a}	7.7 ± 0.15 ^{b}	7.1 ± 0.15 ^{a}	6.8 ± 0.33 ^{a}	7.3 ± 0.12 ^{ab}
C18:3n-3	2.3 ± 0.20 ^{ab}	2.3 ± 0.08 ^{ab}	2.1 ± 0.06 ^{a}	2.8 ± 0.05 ^{bc}	3.2 ± 0.17 ^{c}
C18:4n-3	4.4 ± 0.28 ^{a}	1.3 ± 0.01 ^{b}	1.2 ± 0.03 ^{b}	1.4 ± 0.02 ^{ab}	4.1 ± 0.11 ^{a}
C20:4n-3	1.1 ± 0.05 ^{abc}	1.0 ± 0.00 ^{ab}	1.0 ± 0.01 ^{a}	1.3 ± 0.03 ^{bc}	1.3 ± 0.01 ^{c}
C20:5n-3	4.9 ± 0.62 ^{a}	5.4 ± 0.25 ^{ab}	6.5 ± 0.16 ^{b}	4.9 ± 0.40 ^{a}	5.2 ± 0.08 ^{a}
C22:5n-3	1.5 ± 0.09 ^{a}	1.1 ± 0.07 ^{b}	1.2 ± 0.06 ^{b}	2.2± 1.03 ^{ab}	1.2 ± 0.09 ^{ab}
C22:6n-3	21.4 ± 0.51 ^{ab}	22.8 ± 0.22 ^{b}	20.6 ± 0.39 ^{a}	19.5 ± 1.67 ^{a}	22.7 ± 0.22 ^{b}
∑ PUFA	34.2 ± 4.77	34.1 ± 0.25	29.9 ± 3.95	34.4± 0.91	33.1 ± 3.97
∑ n-6FA	6.8 ± 0.19 ^{a}	7.7 ± 0.15 ^{b}	7.1 ± 0.15 ^{a}	6.8 ± 0.33 ^{a}	7.4 ± 0.12 ^{ab}
∑ n-3FA	27.4 ± 4.59 ^{a}	26.3 ± 0.18 ^{b}	22.9 ± 4.03 ^{ab}	27.6 ± 0.65 ^{ab}	25.8 ± 3.87 ^{ab}
∑ EPA+DHA	18.6 ± 4.30 ^{a}	20.8 ± 0.11 ^{ab}	17.6 ± 3.71 ^{ab}	21.3 ± 0.50 ^{a}	16.0 ± 3.55 ^{b}
∑ n-6/n-3	0.28 ± 0.06 ^{a}	0.29 ± 0.01 ^{b}	0.36 ± 0.10 ^{ab}	0.24 ± 0.01 ^{a}	0.31 ± 0.06 ^{ab}

Values are expressed as mean ± sem (n = 4 replicates). Values in the same row with different superscript letters ($^{a, b, c}$) indicate significant differences ($p < 0.05$). SFA—Saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; n-6—omega-6 polyunsaturated fatty acids; n-3—omega-3 polyunsaturated fatty acids; EPA + DHA—Eicosapentaenoic acid and Docosahexaenoic acid; n-6/n-3—ratio of n-6 PUFA/n-3 PUFA.

We did not find an effect of pre-treatment on whole body composition (Supplementary Table S1), but the factor had a significant effect on most of the fatty acids deposited in the fillets of Atlantic salmon (Supplementary Table S2).

2.4. Histology

The morphology of the pyloric caeca, mid intestine, distal intestine and liver are shown as supplementary material (Figures S1–S4), and the associated parameters are given in Figures S5–S7. We did not observe any changes related to any diet in the pyloric caeca of the different study groups. The mid and distal intestine of the fish fed the CO diet had normal micromorphological features. On the other hand, fish fed the microalgae-incorporated-feeds (NU, NE, TU, and TE) had some alterations, although not statistically significant. The altered features included the thickening of the stratum granulosum and compactum in the mid intestine of the NU, NE and TU groups, wider villi and increased mucous cell area in the TE group, wider distal intestine villi in the TE group and increased distal intestine mucous cell area in the NE group. In addition, enterocyte vacuolization in the NU and TU groups was significantly lower compared to the CO group.

Liver morphology of the CO fed group was normal with centrally located nuclei and homogenous structure of hepatocytes. Vacuolization in the NU and TU group was not as visible as in the case of the other algae-fed groups. In the pre-extruded algae feeding groups (NE, TE), noticeable vacuoles were present in the hepatocytes, with significant differences detected for the CO vs. TE, NE vs. TU and TU vs. TE comparisons.

Pre-treatment was found to significantly influence mid intestine villi width and vacuolization in the distal intestine and liver (Supplementary Table S3).

2.5. Gene Expression Analysis

The relative expression of the mucin gene *muc2* in Atlantic salmon distal intestine is shown in Figure 1; there were no significant differences in the gene expression. Expression of *muc5ac1*, *muc5ac2* and *muc5b* were also examined, but the values were too low to be quantified.

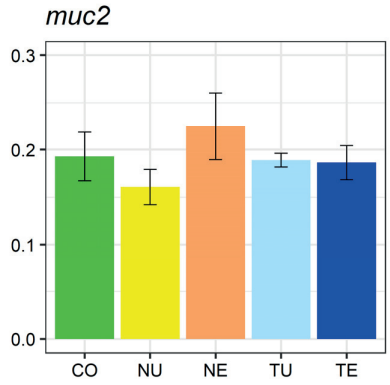

Figure 1. Relative expression of *muc2* in the distal intestine of Atlantic salmon fed on different diets (CO, NU, NE, TU, TE) (n = 4 per diet).

The relative expressions of antioxidant and immune-related genes in Atlantic salmon distal intestine and liver are shown in Figures 2 and 3, respectively. Expression of the antioxidant-related genes *sod1* and *gpx* in the distal intestine of the study groups were not significantly different (Figure 2). Nevertheless, the non-significant differences in the expression of *sod1* in the NU, NE and TU groups and the expression of *gpx* in the NU and NE groups deserve attention. The expression of *cr3* in the distal intestine of the study groups did not vary significantly.

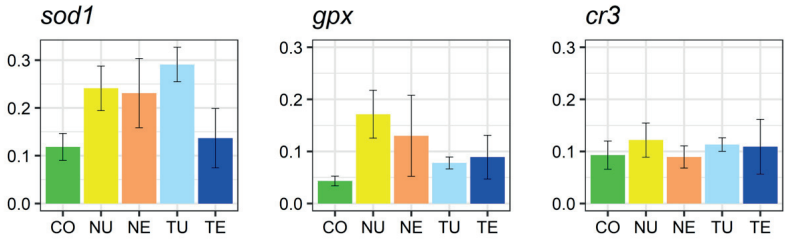

Figure 2. Relative expression of antioxidant and immune-related genes. Shown are the expression of *sod1*, *gpx* and *cr3* in the distal intestine of Atlantic salmon fed on different diets (CO, NU, NE, TU, TE) (n = 4 per diet).

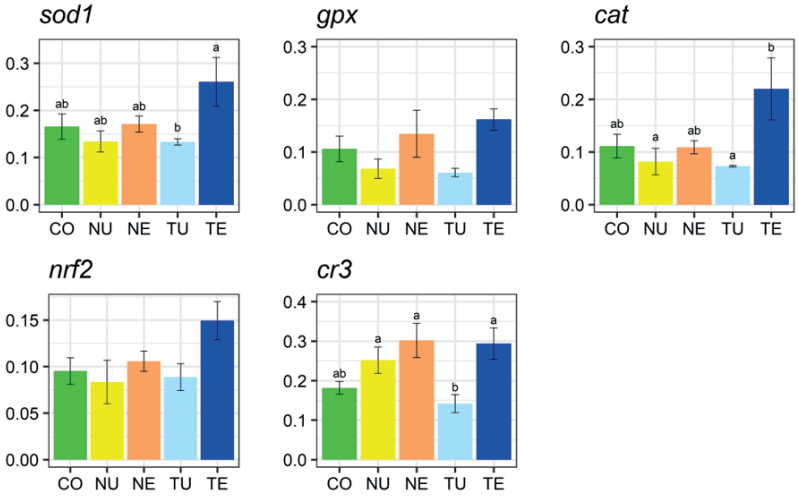

Figure 3. Relative expression of antioxidant and immune-related genes. Shown are the expression of *sod1*, *gpx*, *cat*, *nrf2* and *cr3* in the liver of Atlantic salmon fed on different diets (CO, NU, NE, TU, TE) (n = 4 per diet). Different letters (a, b) above the bars indicate significant differences ($p < 0.05$).

The extruded *Tetraselmis*-fed fish had elevated the expression of selected antioxidant and immune-related genes in the liver compared to the untreated *Tetraselmis* diet (TE vs. TU; Figure 3). The antioxidant genes, namely *sod1*, *gpx*, *cat*, *nrf2* and *cr3*, were higher in the extruded *Tetraselmis*-fed fish (TE). The gene *cr3* had significantly lower expression in the TU diet group compared to the other algae-fed groups. The gene *cat* was significantly higher in the TE group compared to the NU and TU groups. Furthermore, *sod1* was significantly higher in the TE group compared to the TU group.

The effect of pre-treatment was reflected in the expression of all the genes examined in the liver of Atlantic salmon (Supplementary Table S4). On the contrary, such an effect was not evident in the case of genes that were examined in the distal intestine of the fish (Supplementary Table S5).

3. Discussion

To obtain a well-balanced, cost-optimal feed, the aquafeed industry employs ingredients from agricultural crops, products from wild aquatic animals and land animals, insect meal and algal meal as well as oils. However, we have to reduce the dependence on finite resources from the wild and human food to make aquaculture more sustainable [42].

Among the new aquafeed ingredients, insect (black soldier fly larvae; *Hermetia illucens*, L.) meal incorporation levels of 60% in feeds is reported to lower the apparent digestibility coefficients of protein, lipids and amino acids, and increase both hepatosomatic and viscerosomatic indices in Atlantic salmon maintained in freshwater [43]. On the other hand, 20% of insect meal in feeds was found to increase the dry matter, gross energy and mineral digestibility coefficients in Atlantic salmon [44]. Another study also indicated the positive effects of 60% insect meal in the diets of Atlantic salmon in freshwater [45]. Nevertheless, it is essential to gather information regarding the suitability of algae, which lies at the bottom of the aquatic food web, for farmed fishes. In the present study, we employed meals from two microalgae, *Nannochloropsis oceanica* and *Tetraselmis* sp. to understand their ability to alter growth, accumulate fatty acids in the fillets and alter selected genes of Atlantic salmon. Furthermore, we have described the histological changes in the intestine and liver and potential impact of algae and feed VOCs on the antioxidant genes of the fish.

3.1. Growth and Chemical Composition

Earlier studies on feeding microalgae-incorporated feeds to Atlantic salmon tested inclusion levels up to 20% [2–7,46–48]. The current experiment was designed to examine the effects of 30% incorporation of two microalgae strains on growth and health parameters. The 30% inclusion level had a negative effect on growth. However, the growth data must be interpreted carefully as the feeding period was short, and the protein and lipid levels varied among the experimental feeds (Table 5). All the microalgae-incorporated diets contained less protein and lipid, and consequently less energy, compared to the CO diet, and this may explain the lower weight gain observed in algae-fed fish [49].

Table 5. Ingredient composition, analyzed chemical composition and energy of the experimental diets.

Ingredients (%) \ Diet Groups	CO	NU	NE	TU	TE
Fishmeal LT70 [1]	52.0	36.4	36.4	36.4	36.4
Nannochloropsis [2]		30.0			
Nannochloropsis (pre-extruded) [2]			30.0		
Tetraselmis [2]				30.0	
Tetraselmis (pre-extruded) [2]					30.0
Wheat gluten [3]	15.0	10.5	10.5	10.5	10.5
Wheat meal [4]	8.480	5.936	5.936	5.936	5.936
Pea starch	6.0	4.2	4.2	4.2	4.2
Fish oil [5]	17.50	12.25	12.25	12.25	12.25
Vitamin Mineral Premix PV01 [6]	1.0	0.7	0.7	0.7	0.7
Proximate composition (g/100 g)					
Dry matter (DM)	92.6	92.5	91.9	93.4	90.4
Per 100 g DM					
Protein	52.4	47.0	47.0	44.8	45.3
Lipid	20.9	18.4	18.8	16.0	17.5
Ash	8.7	17.2	16.9	16.6	16.1
Carbohydrates	18.0	17.4	17.3	22.6	21.1
Energy (MJ/1000 g)	23.4	21.5	21.7	20.7	21.2

[1] NORVIK 70, Sopropeche, France, [2] Allmicroalgae—Natural Products, Portugal, Values are expressed as mean ± SD (n = 4 replicates), [3] ROQUETTE Frères, France, [4] Casa Lanchinha, Portugal, [5] SAVINOR UTS, Portugal, [6] PREMIX Lda, Portugal.

Biochemical composition of fish varies with their life stages and is influenced by environmental and dietary factors [50]. Significant differences were observed in the proximate composition of whole fish from the different feeding groups of the present experiment; this result is in line with other studies that examined the effect of feeding microalgae on fish performance [51,52]. In the present study, the highest protein content was noted in fish fed the CO diet compared to the extruded microalgae-incorporated diets, and the unextruded *Tetraselmis*-fed fish had higher whole body protein content compared with extruded *Tetraselmis*-fed fish. These observations indicate that the pre-treatment of the

microalgae biomass can reduce the utilization of nutrients from the microalgae. Damage to proteins during heat processing is a function of temperature, time, moisture and the presence of reducing substances [53]. The amino acids that are most susceptible to heat treatment-induced degradation are arginine, cysteine, lysine, serine and threonine [54]. Amino acids with reactive side chains may link to reducing agents present in feeds [55]. Cysteine and methionine are sulfur amino acids, but the sulfur atom of cysteine that is present in the side chain is involved in the formation of reactive sulfhydryl group, and cysteine can be easily oxidized to form cystine dimer containing disulfide bridge [55]. Maillard reaction readily takes place between lysine and reducing sugars, and it is known to reduce fish growth [56]. Overheating of fish meal during drying increased the cross-linking between proteins [57] and reduced digestibility of nearly all amino acids, especially cysteine [58–61]. Heat-induced changes in cysteine and the ensuing effect on protein and amino acid digestibility in rainbow trout (*Salmo gairdneri*) has been reported previously [57]. The whole body lipid content of the experimental fish was in the same range [4], or higher than earlier studies with Atlantic salmon fed microalgae [3] and lower than lipid content reported when salmon were fed 10% pre-extruded *N. oceanica* in plant-based feeds [48]. In the present study, the highest lipid content and lowest protein content was noted for fish fed TE and this result could suggest that the protein quality was too inferior to support efficient growth. The ash content of the experimental fish was in agreement with other reported values on fish fed with microalgae [4] and higher than the whole body ash values reported by us previously [48].

As for the fatty acid composition in the fillet of Atlantic salmon, SFAs were higher in the NE fed fish and MUFAs were higher in the NU fed fish. On the other hand, the PUFAs, n-3 PUFAs and EPA + DHA were higher in fish fed the TU diet. It is known that the fatty acid composition of Atlantic salmon fillet reflects the levels present in the diet [62–64]. Feeding defatted *N. oculata* biomass and whole cell *Schizochytrium* sp. to Nile tilapia (*Oreochromis niloticus*) did not alter the total SFAs, MUFAs or PUFAs but significantly increased DHA and decreased the EPA in the fillets of the fish fed algae [36]. Another study that employed *Schizochytrium* spp. to replace fish oil in the diets of Atlantic salmon, also reported lower concentration of EPA and higher amounts of DHA in the alga fed fish [65]. Muscle EPA and DHA contents were not altered by feeding a combination of dried *Nannochloropsis* sp. and *Isochrysis* sp., but it elevated the concentration of arachidonic acid [66].

3.2. Intestinal Health

Nannochloropsis or *Tetraselmis*, at high (30%) levels, in the marine-based diets did not induce strong signs of inflammation in the intestine of the fish. The noted alteration can be characterized as minor to moderate, as the typical intestinal inflammation features such as shortening of mucosal folds and absence of absorptive vacuoles were not observed in the algae-fed groups [67,68]. Microalgae might not induce enteritis because they may not contain antinutritional factors. It has been shown that DHA deficiency in diets can affect the intestinal morphology of Atlantic salmon, causing swollen enterocytes and unusual vacuoles [69]. As discussed above, the high inclusion of microalgae in the diets used in our study may have caused an alteration of fatty acid composition in the fish, which could have contributed to the observed changes in the intestinal and liver morphology. A recent study from our research group found minor changes in the distal intestinal morphology of Atlantic salmon fed diets with a mix of plant proteins [68]. Nevertheless, we noted higher palmitic acid (C16:0) in the fillets from fish that consumed the NE diet. It is reported that dietary palmitic acid can increase the number of goblet cells, expression of Muc2 and transmucosal electrical resistance in the colon of rats [70]. Certain long-chain polyunsaturated fatty acids reduce goblet cell number [70] and, in our study, Σn-6/n-3 was lower and EPA+DHA was higher in the fillets of the TU group.

Even though the inclusion level of algae in the present experiment was higher than those employed in other studies performed with Atlantic salmon, the observed micromor-

phological features are somewhat comparable with those of other studies where *N. oceanica* was incorporated either at 10 or 20% [5]. *N. oceanica* included at 10% in a plant-based diet with or without feed additives did not change the intestinal structure of Atlantic salmon [48]. On the other hand, a blend of *Tisochrysis lutea* and *Tetraselmis suecica*, which replaced 15 and 45% fish meal protein, increased the height and thickness of the proximal intestine villi of European sea bass [71]. Studies with gilthead seabream (*Sparus aurata*) fed 10% *T. chuii* or 10% *Phaeodactylum tricornutum* reported morphological changes in the intestinal mucosa of the fish [72]; fish fed *T. chuii* showed signs of oedema and inflammation, while such changes were not evident in those fed *P. tricornutum*. In a long-term study on Atlantic salmon, covering most of the aquaculture production cycle (18 g to 3 kg), around 2.5–6% *Schizochytrium limacinum* in modern low-fishmeal diets caused a downregulation of some genes related to intestinal inflammatory response, but intestinal histology study did not indicate any diet-related changes [73]. However, all the samples, including those from the control group, had aberrant distal intestine morphology [73]. Another study reported morphological changes in the intestinal villi and swollen goblet cells in Atlantic salmon fed 15% *Schizochytrium* sp. and yeast extract, but there were no signs of toxicity, stress or inflammation [7].

The changes in hepatocyte morphology that were visible in fish fed single and double extruded microalgae in the feeds were not evident in the CO group. Vacuoles were visible in the hepatocytes of the pre-extruded groups (NE, TE). These vacuoles may indicate accumulation of glycogen or fat in the hepatocytes prompted by the algae incorporation in the feeds, as noted in previous studies [74,75]. A study revealed that the vacuoles in the liver of juvenile Senegalese sole (*Solea senegalensis*) disappeared after 90 days of feeding of the macroalga, *Ulva ohnoi* [76]. On the other hand, the microalga *Scenedesmus* sp., when included in feeds, did not cause any adverse effects in both the intestine and liver of rainbow trout [77]; here, the alga inclusion was only 5%. Our findings suggest that incorporation of microalgae at 30%, and in particular the double extrusion of microalgae, may have affected the nutrient availability or metabolism of nutrients. Feed processing temperature was also found to have an effect on the metabolism of fish, as indicated by Jasour et al.; extrusion at a higher temperature may produce undesirable protein oxidation products in the feeds [78]. Furthermore, fatty acid content can affect the liver morphology of Atlantic salmon, as observed in a feeding trial with EPA or DHA deficient diets [69]. On the contrary, large amounts of lipid droplets were noted in the liver and intestinal cells of another salmonid, rainbow trout, fed diets containing a mix of plant oils [79]. Even though we did not set out to replace fish oil in this experiment, the algae meal does contain some lipids, which may have affected the lipid metabolism and caused the observed changes in hepatocyte morphology. It is likely that the observation in the present study is lipid-based vacuolization rather than glycogen-based because glycogen should have been dyed pink by the PAS staining. However, we are unable to ascertain this, and, thus, it warrants further investigation. High levels of dietary n-6 fatty acids, especially 18:2n-6, leads to more accumulation of n-6 fatty acids in all tissues, and neutral lipids in the liver of Atlantic salmon had more linoleic acid. Linoleic acid and EPA were found to increase and C22:5n-3 and DHA were decreased in the liver of Genetically Improved Farmed Tilapia (*O. niloticus*) fed a high-fat diet (soybean oil) [80]. The TU group, which was found to have a similar liver morphology as that of the CO group, had a profile of the abovementioned fatty acids that plausibly would not have favored the accumulation of linoleic acid.

As for the mucin genes, high expression of *muc2* and low expression of *muc5ac* and *muc5b* in the distal intestine of Atlantic salmon corroborated the result of others [26] who reported the predominance of *muc2* in the intestine. Both *muc2* and *muc5b* were differentially expressed in the skin, gills and intestine of common carp [25]. The MUC2 mucin is the primary intestinal mucin across many species, including mammals and birds [81], and *muc2* is constitutively expressed in the intestine of fishes. It is reported that other intestinal mucin genes are altered by diet or intestinal parasite infection [24]. The expression of *muc2* in the intestine of carp was altered by dietary glucan [25]. Our

recent study has revealed the reduction in the expression of *muc2* in the intestine of Atlantic salmon fed a diet containing 20% soybean meal and 30% fishmeal and fish oil [68]. However, in the present study, *muc2* was not significantly altered in the distal intestine of Atlantic salmon fed algae diets.

The higher gene expression of antioxidant-related genes in the liver of *Tetraselmis*-fed fish could be pointing to an improved response to oxidative stress. Superoxide dismutase, serum catalase and glutathione-S-transferase activities and total antioxidant capacity of catfish (*Clarias gariepinus*) exposed to sodium dodecyl sulfate were restored through an additional exposure to *Spirulina platensis* [82]. The protective role of dietary *Chlorella vulgaris* against arsenic toxicity was revealed through increased antioxidant activities (catalase, glutathione and glutathione peroxidase) in the liver of Nile tilapia fed 10% of the alga [83]. We did not perform a challenge study, instead we investigated the effect of double extrusion on increased utilization of the algae biomass. Expression of *cat* and *sod1* was significantly higher in the liver of TE, which was the group with higher liver vacuolization. Oxidative stress is observed during steatosis, and cells respond to the condition by increasing the molecules of the antioxidant system. Antioxidant enzymes such as Cu/Zn-SOD, GPx and catalase activities in the liver of non-alcoholic fatty liver disease patients were significantly higher [84]. In addition, there exists a correlation between lipid accumulation and expression of C3d and *C3* in the liver of mice fed a high-fat diet [85]. CR3 on macrophages recognizes the C3d fragment of C3 [86], and we observed a higher expression of *cr3* in the liver of the TE group. In our previous study with Atlantic salmon fed diets with up to 20% defatted *Desmodesmus* sp., we did not observe any change in the antioxidant capacity or the expression of intestinal immune and inflammatory marker genes [4]. An alternate interpretation of the present results is that the double extrusion of the microalgae may have caused quality changes in the feeds, which in turn may have triggered oxidative stress in the fish. However, the patterns of change in VOCs in feeds and alteration of the liver antioxidant genes are not similar. In addition, the pattern of expression of the antioxidant genes in the distal intestine and liver were different. This may be explained by the role of the liver in the metabolism of nutrients and handling of ROS.

The experimental feeds were high in polyunsaturated fatty acids and because the feeds had high content of marine ingredients, the lipid profile was dominated by long-chain n-3 fatty acids that are prone to oxidation during processing and storage. Double extrusion of the two microalgae may have challenged the stability of the products [87,88]. The aldehyde, hexanal which is formed from linoleic, gamma-linolenic and arachidonic acids was found in higher concentrations compared to propanal, the aldehyde formed from ALA, EPA and DHA [89]. GC-MS analysis indicated that the amount and type of volatile oxidation products in the experimental feeds were similar to those associated with Norwegian fish meal. Although extrusion can eliminate enzymatic rancidity, oxidative rancidity can occur during the feed manufacturing process [90]. Hence, the higher concentration of certain volatile compounds that are linked to extrusion could be indicative of the fact that double extruded microalgae may need stabilization with antioxidants. To overcome this issue, super critical fluid extrusion can be considered because this method aided vitamin retention in addition to restraining lipid oxidation in puffed rice [91].

To embrace sustainability, the aquafeed industry could employ microalgae that have high-value lipids, high-quality proteins and antioxidants. Such a strategy will reduce the dependency on human food-based meals and oils and our finite resources. Through the current study we assessed the potential of microalgae, their ability to impart growth and affect health in fishes.

4. Materials and Methods

The study was approved by the Norwegian Animal Research Authority (FDU: Forsøksdyrutvalget ID-5887). Atlantic salmon smolt of average weight 154.2 g (AquaGen strain) were purchased from a local commercial producer (Cermaq Norway AS, Hopen, Norway), and randomly sorted into the rearing tanks (800 L, A-plast, Skodje, Norway) at the Research

Station of Nord University. Each tank contained 39 fish, and there were four replicate tanks per diet group. All tanks were supplied with filtered and aerated seawater (8.6 °C ± 0.2) in a flow-through system, and the average oxygen saturation was 86.7% measured at the outlet of each tank. Tanks were kept under 24 h photoperiod with artificial light.

The following five diets were fed for 60 days to the respective fish group: (1) CO: a fish meal-based control (basal) diet, (2) NU: a *Nannochloropsis* diet, (3) NE: a pre-extruded *Nannochloropsis* diet, (4) TU: a *Tetraselmis* diet, and (5) TE: a pre-extruded *Tetraselmis* diet. All diets, except the CO diet, contained 30% of the respective microalga (Table 5). These feeds were produced by Sparos LDA, Olhão, Portugal. For NE and TE, the dried algal meal was subjected to a pre-extrusion before they were blended with the other ingredients, as earlier described by Gong, Sørensen, Dahle, Nadanasabesan, Dias, Valente, Sørensen and Kiron [48]. In brief, this was carried out by passing the algal meal through a twin-screw extruder (model BC45, Clextral, Firminy, France). All diets were then produced by mixing the ingredients with a double-helix mixer (model 500 l, TGC Extrusion, Roullet-Saint-Estèphe, France), milled in a hammer mill (model SH1, Hosokawa-Alpine, Augsburg, Germany), extruded through a twin-screw extruder (model BC45, Clextral), and vacuum coated (model PG-10VCLAB, Dinnissen, The Netherlands) according to common fish feed production practices. The ingredient and proximate composition of the feeds are listed in Table 5. Fish in each tank were fed twice a day (08.00–9.00 and 15.00–16.00) using a programmed automatic feeding system (Arvo-Tec, Huutokoski, Finland). The feeding rate was initially 1% of biomass and the rate was gradually increased to 1.4% taking the appetite of the fish into consideration.

Chemical analysis of whole body (dry matter, protein, lipid, ash and energy; 6 fish per tank were pooled to obtain a sample for analysis) and fatty acid composition of fillet was performed on freeze-dried samples. Dry matter content was determined by differences; by oven drying (2.0 g) samples to a constant weight at 104 °C for 20 h (ISO 6496-1999). The oven dried samples were combusted in a muffle furnace to a constant weight at 540 °C for 16 h to determine the ash content (ISO 5984-2002). Crude protein in 0.5 g of samples were determined by Kjeldahl titration method (N × 6.25, KjeltecTM 2300, Foss Tecator AB, Höganäs, Sweden; ISO 5983-1987). Crude fat in 2.0 g of samples were determined gravimetrically using the diethyl ester extraction method, according to the Norwegian Standard Association [92]. Energy was analyzed by bomb calorimeter (IKA C200, Staufen, Germany: ISO 9831: 1998). Carbohydrates were calculated as follows: 100 − (water + protein + lipid + ash).

Total lipid extractions of freeze-dried whole bodies or fillets (100 mg/sample) were carried out according to the chloroform/methanol (2:1, v/v) gravimetric determination described by Bligh and Dyer [93]. Briefly, freeze-dried samples were homogenized by mixing 1.8 mL of distilled water, 2 mL of methanol and 1 mL of chloroform followed by adding 1 mL of chloroform and 1 ml of distilled water. Samples were then centrifuged (4000 rpm) to separate the phases. The lower chloroform phase containing lipids was transferred into a glass tube and dried under a gentle nitrogen flow to prevent fatty acid oxidation. Fatty acid methyl esters (FAMEs) of samples were obtained by transesterification and methylation according to the method described previously [94]. FAMEs analyses were performed in duplicate in a gas chromatograph (SCION 436-GC, Scion Instruments, Goes, The Netherlands) fitted with a flame ionization detector at 250 °C. The separation was achieved using a wax embedded column of 25 m length, 0.25 mm internal diameter and 0.2 µm film thickness (Agilent Technologies, Middelburg, The Netherlands). Individual FAME was identified and quantified by comparison to known standard mixtures of common fatty acids (FAME MIX 2/GLC-473, Nu-Chek Prep, Elysian, MN, USA) and results were expressed as relative area percentage of the total fatty acid using a software Compass CDS, Bruker Co-operation.

Oxidation stability of the experimental diets were analyzed by dynamic headspace gas chromatography–mass spectrometry (GC-MS) of volatile oxidation products. Samples (2 g) were weighed into Erlenmeyer flask and methanol spiked with ethyl heptanoate

(internal standard). The samples were then placed in a water bath at 70 °C and purged for 20 min with a stream of nitrogen, (100 mL/min). Volatile compounds were trapped on an adsorber (Tenax GR), desorbed at 280 °C for 5 min in a Markes Thermal Desorber and transferred to an Agilent 6890 GC with an Agilent 5973 Mass Selective Detector (EI, 70 eV). The volatiles were separated in a DB-WAXetr column (30 m, 0.25 mm i.d., 0.5 µm film) with a temperature program starting at 30 °C, held for 10 min, increasing 1°/min to 40 °C, 3°/min to 70 °C, and 6.5°/min to 230 °C, with a hold time of 5 min. The peaks were integrated and compounds tentatively identified with HP Chemstation software, and NIST11 Mass Spectral Library. System performance was checked with blanks and standard samples before and after analysis. The feed samples were analyzed in duplicate.

The specific growth rate (SGR) of the fish, expressed in %/day, was calculated as follows

$$\mathrm{SGR} = \frac{\ln\left(w_f\right) - \ln(w_i)}{t} \times 100$$

where w_f and w_i are the mean final and initial weight of the fish, respectively, measured in grams, and t is time measured in days.

Three fish per tank for liver gene expression analysis, and five fish per tank for intestinal gene expression analysis were collected at the end of the feeding trial. These fish were randomly selected for sampling from each tank, anaesthetized with tricaine mesylate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA), and killed with a sharp blow to the head.

For histology, 3 fish/tank or 12 fish per diet group were processed and employed for the study. A small part of the liver, pyloric caeca, mid intestine and distal intestine were dissected out and placed in neutral buffered formalin (NBF, 4%). The samples were processed using standard histology procedures and embedded in paraffin. The paraffin blocks were sectioned using a microtome (Microm HM3555, MICROM International GmbH, Walldorf, Germany), stained using a staining machine (Microm HMS 760×, MICROM International GmbH) with Alcian Blue-Periodic Acid Schiff's reagent (AB-PAS, pH 1). The stained slides were mounted with Pertex® mounting medium and later viewed with light microscope Olympus BX61/Camera Color View IIIu (Olympus Europa GmbH, Hamburg, Germany). The captured images were analyzed using the software Cell P (Soft Imaging System GmbH, Munster, Germany). ImageJ 1.53e was employed to quantify the features of interest. In all cases, we employed images from four fish per group. Measurements such as villi width (at the villi base), combined height of stratum granulosum and stratum compactum (measured by drawing a straight line parallel to lamina propria) were taken after setting the scale. Goblet cell counts and their area in each villus were recorded using freehand selection tool. The selection was first converted to an 8-bit image, after clearing and subtracting the background. Next, after converting to binary, particles were analyzed; thus, we obtained the cell counts and area. A semi-quantitative approach was also employed to score the vacuoles in both distal intestine enterocytes and liver tissue; 5 indicated high vacuolization and 1 was assigned for images with no or hardly any vacuoles.

For gene expression analysis, a small piece of the liver and distal intestine was dissected out and gently rinsed with PBS. The samples were immediately placed in cryotubes and frozen in liquid nitrogen, and later stored at -80 °C.

The relative mRNA levels of mucin genes (*muc2*, *muc5ac1*, *muc5ac2* and *muc5b*) in the distal intestine, and antioxidant genes (catalase—*cat*; glutathione peroxidase—*gpx*; nuclear factor erythroid 2—related factor 2—*nrf2*; superoxide dismutase 1—*sod1*) plus complement receptor 3—*cr3* in the distal intestine and liver were examined in this study. Detailed information for all target and reference genes are presented in Table 6. Primers were purchased from Eurofins Genomics (Luxembourg, Luxembourg).

Table 6. Primer sequences, amplicon size, PCR efficiency and accession numbers for the target genes and reference genes involved in this study.

Gene Name	Primer Sequence	Amplicon Size (bp)	PCR Efficiency (%)	Accession # (UniProt/GenBank)
		Target genes		
muc2(.1/2)	GAGTGGGCTCTCAGATCCAG-F GATGATGCGGACGGTAGTTT-R	99	96.8	XM_014184683.1/XM_014170386.1
muc5ac1	GACCTGCTCTGTGGAAGGAG-F AGCACGGTGAATTCAGTTCC-R	120	96.7	XM_014127075.1
muc5ac2 (/4)	TTTTCTCAGTTGCCGCTTTT-F AGTCGGAGCCCATAAGAGGT-R	92	98.9-99.8	XM_014182329.1
muc5b	ATTAAGAGCGATGTCTTCACAGC-F AAGCACATGAGTCTCTCACACAA-R	85	97.4-102.4	XM_014175874.1/XM_014126057.1
cat	CCGACCGTCCGTAAATGCTA-F GCTTTTCAGATAGGCTCTTCATGTAA-R	140	96.1	BT045615.1
gpx	GCAATCAGTTCGGACATCAGG-F GTCCTTCCCATTCACATCCAC-R	131	91.1	XM_014133872
nrf2	TCAACAGGACATCGACCTAAT-F GGCAGTAGTCAAACACCTCT-R	83	81.9	BT059007.1
sod1	CCACGTCCATGCCTTTGG-F TCAGCTGCTGCAGTCACGTT-R	141	94.1	AY736282.1
cr3 (itgb2)	ATGACATGGACTACCCATCTGTT-F TCTGACAATACTCCCACCTCA-R	151	110.5	BT058776.1
		Reference genes		
ef1ab	TGCCCCTCCAGGATGTCTAC-F CACGGCCCACAGGTACTG-R	59	100.8	BG933853
rpl13	CGCTCCAAGCTCATCCTCTTCCC-F CCATCTTGAGTTCCTCCTCAGTGC-R	79	94.0	BT048949.1
rps29	GGGTCATCAGCAGCTCTATTGG-F AGTCCAGCTTAACAAAGCCGATG-R	167	91.1	BT043522.1
ubi	AGCTGGCCCAGAAGTACAACTGTG-F CCACAAAAAGCACCAAGCCAAC-R	162	91.1	AB036060.1
hprt1	CCGCCTCAAGAGCTACTGTAAT-F GTCTGGAACCTCAAACCCTATG-R	255	82.5	BT043501

RNA was extracted from the samples using E-Z 96 Total RNA Kit (Omega Bio-Tek, Norcross, USA). Roughly 100 mg of the tissue sample was cut from the frozen sample and homogenized using Zirconium oxide beads (1.4 mm; Percellys, Tarnos, France) and TRK lysis buffer in a pre-cooled capped freestanding tube (VWR International, Oslo, Norway) at 6000 rpm. The resulting mixture was centrifuged (18,000× *g*, 20 °C) to obtain clear supernatant. Briefly, 300 µL supernatant was added to 300 µL 70% ethanol and mixed, before this mixture was added to the E-Z 96 RNA plate, which contained HiBind® matrix in each well. Centrifugation (3000 rpm, 15 min) was used to draw the sample through the well, followed by several steps of buffer washes according to the kit instructions. Finally, the RNA was obtained by adding 65–75 µL of RNase-free water (5 Prime GmbH, Hilden, Germany) to each well and a final centrifugation.

The purity and quality of RNA was checked in Nanodrop™ 1000 (ThermoFisher Scientific, Waltham MA, USA). Extracted RNA was quantified by Qubit™ RNA broad-range assay kit (Life Technologies, Carlsbad, USA) on a Qubit 3.0 Fluorometer (Life Technologies) and diluted with RNase-free water if necessary. cDNA synthesis was performed with QuantiTect™ Reverse Transcription Kit (Quiagen GmbH, Hilden, Germany) with 1000 ng of RNA and a reaction volume of 20 µL per sample, according to the manufacturer's instructions. The cDNA samples were diluted with nuclease free water by a factor of 10 before continuing with qPCR.

qPCR was performed on a LightCycler® 96 (Roche Life Science) using Fast SYBR® Green Real-Time PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). Each reaction contained 5 µL of Fast SYBR® Green PCR Master Mix, 1 µL primer mix (200 nM), and 4 µL cDNA (0.5 ng/µL). Reactions (n = 20 and n = 12 per diet for distal intestine and liver, respectively) were performed in duplicate. Thermal cycling conditions were initial holding at 95 °C for 20 s, 40 cycles of denaturation at 95 °C (3 s), and annealing/extension at 60 °C (30 s).

A standard curve with known concentrations was prepared for each primer in order to calculate the gene expression. This was performed by pooling RNA from every sample, reverse transcribing the pooled RNA as described above, and using the resulting cDNA to create a 6-point threefold dilution series that was used for qPCR. The equation

$$E = \left(10^{-\frac{1}{m}} - 1\right) \times 100 \quad (1)$$

was used to calculate the efficiency of the primers (E) based on the slope (m) of the standard curve. The normalization factor was computed for each sample based on the relative quantities of the two most stable genes from among the set of four reference genes using geNorm [95]. The reference genes chosen were *elongation factor 1AB (ef1ab)*, *ribosomal protein L13 (rpl13)*, *ribosomal protein S29 (rps29)* and *ubiquitin (ubi)*. The expression levels of all the target genes were then calculated relative to the normalization factor.

Statistical analysis was carried out using SigmaPlot. The data were tested for normality (Shapiro–Wilk normality test) and equality of variance (Levene's test). For parametric data, one-way analysis of variance (ANOVA) was performed, followed by Tukey's multiple comparison test to identify the significant differences among the means of the dietary groups. For non-parametric data, Kruskal–Wallis test, followed by Dunn's multiple comparison test, was performed to decipher the significant differences between the groups. The data from the algae-fed groups were subjected to 2-way ANOVA to understand the effect of the extrusion process. A significance level of $p < 0.05$ was chosen to indicate the differences.

5. Conclusions

Atlantic salmon fed *Nannochloropsis oceanica* and *Tetraselmis* sp. did not exhibit any signs of intestine inflammation other than enterocyte vacuolization. A significant effect of processing was noted on the fillets' fatty acids, liver histology and expression of genes in the liver. The correlation between vacuolization in the mid and distal intestine, and the changes in the fillets' fatty acids and hepatocytes vacuolization, should be studied further.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fishes6030023/s1. Supplementary Table S1. Results of 2-way ANOVA of fish growth and proximate composition. Supplementary Table S2. Results of 2-way ANOVA of fatty acid data from fillets of the algae-fed fish. Supplementary Table S3. Results of 2-way ANOVA of parameters measured from the mid and distal intestine and liver of Atlantic salmon. Supplementary Table S4. Results of 2-way ANOVA of gene data from the liver of the algae-fed fish. Supplementary Table S5. Results of 2-way ANOVA of gene data from the distal intestine of the algae-fed fish. Figure S1: Comparison of the photomicrographs of the pyloric caeca from Atlantic salmon fed control feed (CO) or algae-containing diets (NU, NE, TU, TE). Scale bar: 200 μm, Figure S2: Comparison of the photomicrographs of the mid intestine from Atlantic salmon fed control feed (CO) or algae-containing diets (NU, NE, TU, TE). Black arrow: Lamina propria. Asterisk: Stratum granulosum. Scale bar: 100 μm. Figure S3: Comparison of the photomicrographs of the distal intestine from Atlantic salmon fed control feed (CO) or algae-containing diets (NU, NE, TU, TE). Black arrow: Lamina propria. Asterisk: Stratum granulosum. Red arrow: Supranuclear vacuolization. Scale bar: 100 μm. Figure S4: Comparison of the photomicrographs of the liver from Atlantic salmon fed control feed (CO) or algae-containing diets (NU, NE, TU, TE). A. Original images. B. Green channel of the original images. Red arrow: Small vacuoles. Black arrow: Large vacuoles. Scale bar: 50 μm., Figure S5: Comparison of the histological parameters that were assessed in the mid intestine of Atlantic salmon fed control feed (CO) or algae-containing diets (NU, NE, TU, TE), Figure S6: Comparison of the histological parameters that were assessed in the distal intestine of Atlantic salmon fed control feed (CO) or algae-containing diets (NU, NE, TU, TE), Figure S7: Comparison of vacuolization in the liver of Atlantic salmon fed control feed (CO) or algae-containing diets (NU, NE, TU, TE).

Author Contributions: Conceptualization, V.K. and M.S.; methodology, V.K. and M.S.; validation, V.K. and M.S.; formal analysis, S.L.S., A.G., G.V., D.D. and Y.G.; investigation, Y.G., A.G. and S.L.S.; resources, M.S. and V.K.; data curation, S.L.S., M.S. and V.K.; writing—original draft preparation,

S.L.S. and A.G.; writing—review and editing, V.K. and M.S.; visualization, S.L.S.; supervision, V.K. and M.S.; project administration, V.K.; funding acquisition, V.K. and M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the Research Council of Norway (Project No. 260190, Alger4laks) and is part of the COFASP ERA-NET project MARINALGAE4aqua.

Institutional Review Board Statement: The study was conducted according to the guidelines of the National Animal Research Authority (FDU: Forsøksdyrutvalget ID-5887) in Norway.

Data Availability Statement: Data is contained within the article and supplementary material. Supplementary material contains figures necessary for interpreting the results.

Acknowledgments: This study was funded by the Research Council of Norway (Project No. 260190, Alger4laks) and is part by the COFASP ERA-NET project MARINALGAE4aqua. The authors acknowledge the support of the staff at the Research Station of Nord University. Yangyang Gong was financially supported by a fellowship from the China Scholarship Council as well as by the funding from East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Paper III

Aquaculture 522 (2020) 735122

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Approaches to improve utilization of *Nannochloropsis oceanica* in plant-based feeds for Atlantic salmon

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ARTICLE INFO

Keywords:
Microalgae
Extrusion processing
Digestarom®
ZEOFeed
Fillet PUFA
Intestine

ABSTRACT

Rigid cell walls of microalgae lower the digestibility and nutrient bioavailability in carnivorous fish. Extrusion is a thermo-mechanical process and a scalable technology that may break cell walls and improves nutrient utilization. It can be hypothesized that certain feed additives may further improve microalgae nutrient digestibility and feed utilization by fish. The aim of the current study was to investigate i) the effect of incorporation of 10% pre-extruded *Nannochloropsis oceanica* on nutrient digestibility, growth and feed utilization of Atlantic salmon post smolts, and ii) the ability of feed additives in improving the feed utilization. Four low fish meal feeds were formulated; a control without the microalga *N. oceanica* (CO), a feed containing 10% of the pre-extruded microalga (NC), and two feeds containing 10% of the pre-extruded microalga and supplemented with either 0.06% Digestarom® (ND) or 1% ZEOFeed (NZ). Fish (initial average weight of 227.3 ± 3.4 g) in 5 replicate tanks of each of the study groups were fed one of the experimental feeds for 68 days. The apparent digestibility of dry matter in the NC and NZ groups were significantly higher compared to the control group (CO). The digestibility of lipid was significantly lower, and digestibility of ash was higher in the alga-fed groups (NC, ND and NZ) compared to the control group (CO). The incorporation of 10% pre-extruded *N. oceanica* in plant-based commercial-like feeds did not affect the growth, feed utilization and whole body proximate composition of salmon. No effects of the feed additives were observed on growth, feed utilization and histomorphology of distal intestine of salmon, but cell proliferation (PCNA) was higher for fish fed the alga alone as well as the alga-ZEOfeed combination. There were no differences in polyunsaturated fatty acids in whole body of fish fed the different feeds. It is noteworthy that whole-body EPA + DHA levels of fish fed the algae feeds were maintained at the same levels as fish fed the control feed that contained 50% more fish meal and 10% more fish oil.

1. Introduction

Aquaculture production in Norway has increased from around 150, 000 t in the 1990s to more than 1.35 million tonnes in 2018, and is dominated by Atlantic salmon, accounting for around 95% of the total volume (SSB, 2018). Future growth of salmon farming depends on high-quality sustainable ingredients that promote good growth and feed utilization, maintain fish health and preserve the nutritional quality of the end product.

Chemical composition of some microalgae signifies their potential as feed ingredients for Atlantic salmon (Becker, 2007; Shields and Lupatsch, 2012; Tibbetts, 2018). These microorganisms are good sources of amino acids, n-3 polyunsaturated fatty acids (n-3 PUFAs) and astaxanthin (Shah et al., 2018). However, only a few of them are successfully commercialized and used in salmon feeds. The heterotrophic microorganism *Schizochytrium* sp. is a good source of the n-3 PUFA, docosahexaenoic acid (DHA), and hence may be a good replacer of fish oil (Kousoulaki et al., 2015; Sprague et al., 2017; Sprague et al., 2015). On the other hand, the photoautotrophic microalga *Haematococcus* sp. accumulate astaxanthin, and therefore, can be a good alternative to

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https://doi.org/10.1016/j.aquaculture.2020.735122
Received 31 December 2019; Received in revised form 13 February 2020; Accepted 13 February 2020
Available online 14 February 2020

synthetic astaxanthin in salmon feeds (Griffiths et al., 2016).

Replacement of both fish meal and currently used plant-derived ingredients in salmon feeds with microalgae remains a challenge. Thorough testing is essential to understand the effects of microalgae on feed quality, growth, feed utilization, nutrient digestibility, and health of the animal, and end product quality (Glencross et al., 2007; Ringø et al., 2009). Our previous studies have shown that microalgae such as *Nannochloropsis oceanica (N. oceanica)* can be used at modest inclusion levels; up to 10%, without negatively affecting the performance and health of salmon (Sørensen et al., 2017). However, we found that nutrient digestibility (e.g. lipid) of the microalga-incorporated salmon feeds was lower compared to the fish-meal-based reference feeds (Gong et al., 2018; Sørensen et al., 2017). By proving that cost-effective processing technologies can disrupt cell walls or special feed additives can improve nutrient availability of microalgae, the salmon feed industry can be encouraged to rely on microalgae (Teuling et al., 2017; Tibbetts et al., 2017). Recent research suggested that extrusion can effectively disrupt the cells of *Nannochloropsis* and make the intracellular biocompounds available for further use (Gong et al., 2018; Wang et al., 2018).

Feed additives such as Digestarom® (a phytogenic ingredient) and zeolites (microporous aluminosilicate) are known to improve the performance and health of farmed animals (Jeney et al., 2015; Papaioannou et al., 2005). Studies with rainbow trout (*Oncorhynchus mykiss*) reported that supplementation of feeds with 0.1% Digestarom® PEP 1000 (containing 1.2% carvacrol) or 0.1% Digestarom® PEP MGE 1000 (containing 0.6% thymol) improved feed efficiency compared to control feed, although the body weight of the fish was unaltered (Giannenas et al., 2012). Furthermore, Digestarom® P.E.P. MGE was found to lower the fillet fat and slightly increase protein content in channel catfish (*Ictalurus punctatus*) (Peterson et al., 2014).

Clinoptilolite, a natural zeolite, in feeds for farmed animals had positive effect on nutrient digestibility, growth and feed utilization (Ghasemi et al., 2016; Kanyılmaz et al., 2015). Not many studies have reported the effects of zeolite on fish, but a previous study on gilthead sea bream suggested that inclusion of clinoptilolite into the feed can promote growth rate and feed efficiency (Kanyılmaz et al., 2015). Furthermore, zeolite (bentonite and mordenite) improved the growth and feed utilization in rainbow trout (Eya et al., 2008). The improved growth and nutrient utilization in the fish fed zeolites were attributed to the detoxifying effects of the compound (Ghasemi et al., 2016).

In our previous studies, we evaluated the potential of microalgae in high fish meal and fish oil feeds of Atlantic salmon (Kiron et al., 2012; Kiron et al., 2016; Sørensen et al., 2017). In the present study, we aimed to understand the nutritional value of microalgae in commercial-like feeds; i.e. feeds high in plant and low in marine ingredients. In addition, we tried to understand the effect of two feed additives on Atlantic salmon. The aims of the present study were to investigate the potential of: i) thermo-mechanical processed (extruded) *N. oceanica* as an ingredient in high plant-low marine ingredient salmon feed and ii) two different feed additives to improve the nutrient digestibility and utilization of the *Nannochloropsis*-incorporated feeds.

2. Material and methods

2.1. Experimental design and feeds

This feeding trial was approved by the National Animal Research Authority (FDU: Forsøksdyrutvalget ID-5887) in Norway.

Four nearly isoproteic (42–44% of dry matter) and isolipidic (28–30% of dry matter) feeds were formulated. The ingredient composition is provided in Table 1, chemical and amino acid composition is given in Table 2 and the information of the fatty acids is presented in Table 3. Four low fish meal feeds were employed in the current study; the control feed containing 15% fish meal and no *N. oceanica* (CO), a basal test feed containing 7.5% fish meal and 10% of the microalgae (NC), and two other test feeds similar to the feed NC, but supplemented with either 0.06% Digestarom® PEP MGE150 (Biomin GmbH, Getzersdorf, Austria; ND), or 1% ZEOFeed (ZEOCEM AS, Bystré, Slovakia; NZ). Digestarom® PEP MGE150 contains a blend of essential oils from oregano, anise, and citrus peel and the main active compounds are carvacrol, thymol, anethol, and limonene (Peterson et al., 2014; Rodrigues et al., 2018). ZEOFeed is a clinoptilolite and a natural zeolite that comprise a microporous arrangement of silica and alumina tetrahedral

Table 1
Ingredient composition (%) of the four experimental feeds.

Ingredients	CO	NC	ND	NZ
Fishmeal 70 LT FF (NORVIK)[a]	15.00	7.50	7.50	7.50
Nannochloropsis extruded[b]	–	10.00	10.00	10.00
Soy protein concentrate[c]	16.00	16.00	16.00	16.00
Pea protein concentrate[d]	10.00	10.00	10.00	10.00
Wheat gluten[e]	11.30	13.00	13.00	13.24
Wheat meal[f]	9.44	7.04	6.98	5.80
Faba beans[g]	7.00	7.00	7.00	7.00
Fish oil (SAVINOR)[h]	10.00	9.05	9.05	9.05
Rapeseed oil[i]	15.00	15.00	15.00	15.00
Vitamin & Mineral Premix INVIVO[j]	1.00	1.00	1.00	1.00
Lutavit C35[k]	0.03	0.03	0.03	0.03
Lutavit E50[l]	0.05	0.05	0.05	0.05
Choline chloride[m]	0.20	0.20	0.20	0.20
Monocalcium phosphate[n]	2.00	2.90	2.90	2.90
Calcium carbonate[o]	2.22	0.00	0.00	0.00
L-lysine[p]	0.40	0.60	0.60	0.60
L-threonine[q]	0.20	0.30	0.30	0.30
L-tryptophan[r]	0.04	0.11	0.11	0.11
DL-methionine[s]	0.10	0.20	0.20	0.20
Yttrium oxide[t]	0.02	0.02	0.02	0.02
Digestarom®[u]			0.06	
ZEOFeed[v]				1.00

CO: Plant based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed.

[a] NORVIK 70: 70.3% crude protein (CP) 5.8% crude fat (CF), Sopropeche, France.
[b] Allmicroalgae, Portugal.
[c] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands.
[d] NUTRALYS F85F: 78% crude protein, 1% crude fat, ROQUETTE Frères, France.
[e] VITAL: 80% CP, 7.5% CF, Roquette Frères, France.
[f] Wheat meal: 11.7% CP, 1.6% CF, Casa Lanchinha, Portugal.
[g] Faba beans: 28.5% CP; 1.2% CF, Ribeiro & Sousa Cereais, Portugal.
[h] SAVINOR UTS, Portugal.
[i] Henry Lamotte Oils GmbH, Germany.
[j] PREMIX Lda, Portugal. Vitamins (IU or mg/kg feed): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg feed): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middling.
[k] ROVIMIX STAY-C35, DSM Nutritional Products, Switzerland.
[l] ROVIMIX E50, DSM Nutritional Products, Switzerland.
[m] ORFFA, The Netherlands.
[n] MCP: 21.8% phosphorus, 18.4% calcium, Fosfitalia, Italy.
[o] CaCO3: 40% Ca, Premix Lda., Portugal.
[p] Biolys: 54.6% Lysine, Evonik Nutrition & Care GmbH, Germany.
[q] ThreAMINO: 98% L-Threonine, Evonik Nutrition & Care GmbH, Germany.
[r] TrypAMINO: 98% Tryptophan, Evonik Nutrition & Care GmbH, Germany.
[s] DL-Methionine for Aquaculture: 99% Methionine, Evonik Nutrition & Care GmbH, Germany.
[t] Sigma Aldrich, USA.
[u] BIOMIN Holding GmbH, Austria.
[v] ZEOCEM, Slovak Republic.

Table 2
Chemical composition of the four experimental feeds.

	CO	NC	ND	NZ
Proximate composition				
Dry matter	94.98	94.06	94.79	95.35
% of dry matter				
Protein	44.43	43.06	42.30	42.89
Lipid	29.48	28.17	30.28	29.47
Ash	8.90	8.85	9.04	9.63
Carbohydrate[a]	17.2	19.9	18.4	18.0
Energy (KJ g^{-1})[b]	23.8	23.0	23.5	23.3
Amino acids (% of dry matter)				
Alanine	1.9	1.7	1.8	1.8
Arginine	2.8	2.6	2.6	2.5
Aspartic acid	4.1	3.6	3.9	3.8
Cysteine	0.5	0.5	0.6	0.6
Glutamic acid	9.5	9.0	9.4	9.2
Glycine	2.1	1.8	1.9	1.8
Histidine	1.0	0.9	1.0	0.9
Leucine	3.4	3.1	3.2	3.2
Lysine	3.0	2.7	2.8	2.8
Isoleucine	1.8	1.7	1.8	1.7
Methionine	0.8	0.8	0.8	0.7
Phenylalanine	2.2	2.1	2.1	2.1
Proline	3.1	3.0	2.9	2.9
Serine	2.3	2.1	2.1	2.1
Threonine	1.9	1.8	1.9	1.8
Tryptophan	0.5	0.6	0.6	0.6
Tyrosine	1.5	1.4	1.4	1.4
Valine	2.0	1.9	2.0	2.0

CO: Plant-based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed.

[a] Carbohydrate (% of dry matter) was calculated as 100 - (Protein of dry matter + Lipid of dry matter + Ash of dry matter).

[b] The gross energy content of feeds was not analyzed but calculated based on 23.7, 39.5 and 17.2 KJ g^{-1} for protein, lipids and starch, respectively.

Table 3
Fatty acid composition (% of total fatty acids) of the experimental feeds.

Fatty acids	CO	NC	ND	NZ
C14:0	2.8	2.7	2.7	2.7
C15:0	0.3	0.2	0.2	0.2
C16:0	10.2	9.9	10	9.9
C16:1n-7	3.2	3.4	3.4	3.4
C17:0	0.3	0.2	0.2	0.2
C18:0	2.3	2.2	2.2	2.2
C18:1n-9	39.1	39.9	40.0	40.1
C18:2n-6	14.3	14.5	14.4	14.4
C18:3n-3	6.0	6.1	6.1	6.1
C18:3n-6	0.1	0.1	0.1	0.1
C18:4n-3	0.9	0.8	0.9	0.9
C20:0	0.5	0.5	0.5	0.5
C20:1n-9	1.6	1.5	1.5	1.5
C20:2n-6	0.1	0.2	0.2	0.2
C20:4n-6	0.4	0.4	0.4	0.4
C20:4n-3	0.3	0.2	0.2	0.2
C20:5n-3	5.5	5.7	5.6	5.6
C22:0	0.3	0.3	0.3	0.3
C22:1	1.6	1.4	1.4	1.4
C22:5n-6	0.1	0.1	0.1	0.1
C22:5n-3	0.7	0.7	0.7	0.7
C22:6n-3	4.5	4.0	4.0	4.0
C24:0	0.1	0.1	0.1	0.1
C24:1n-9	0.3	0.3	0.3	0.3
ΣSFAs	16.8	16.3	16.4	16.3
ΣMUFAs	45.9	46.6	46.7	46.8
ΣPUFAs	33.2	33.0	32.8	32.8
Σn-6 PUFAs	15.2	15.5	15.3	15.4
Σn-3 FUFAs	18.0	17.6	17.5	17.5
n-3/n-6	1.19	1.14	1.14	1.14
EPA + DHA	10.0	9.7	9.6	9.6

CO: Plant-based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed.

(EFSA, 2013).

The test microalga *N. oceanica* (contained 2.8% moisture, 36.6% protein, 14.3% lipid, 9.4% fiber, 22.8% ash, 17.5 KJ g^{-1} of energy, 2.1% lysine and 0.9% methionine) used in the feeds was cultured in closed photobioreactors at Allma®, Lisbon, Portugal. After harvesting and dewatering by centrifugation, the biomass was spray dried at Algafarm (Pataias, Portugal) and marketed by Allmicroalgae – Natural Products® (Lisbon, Portugal).

SPAROS LDA (Olhão, Portugal) performed the extrusion treatment of the microalgae and manufactured the experimental feeds. The microalgae were pre-processed, by passing them through an extruder, prior to mixing them with other ingredients to prepare the experimental feeds. The pre-extrusion of algae was carried out as follows: *N. oceanica* (98.5%) powder was blended with wheat meal (1.5%) in a double-helix mixer (model 500 l, TGC Extrusion, France). The mixture was then passed through a pilot-scale twin-screw extruder (model BC45, CLEXTRAL, France) with a screw diameter of 55.5 mm to produce pellets (2.0 mm diameter size). The extrusion conditions were as follows: feeder rate 65 kg/h; screw speed 243 rpm; steam addition at conditioner 3%; water addition at extrusion barrel 1295 mL/min; temperature in the barrel was 112–113 °C recorded in section 3; moisture level of the dough at die exiting was 26%. The extruded alga pellets were dried in vibrating fluid bed dryer (model DR100, TGC Extrusion, France). The chemical composition of pre-extruded *N. oceanica* + wheat meal was 3.3% moisture, 36.4% protein, 14.2% lipid, 9.3% fiber, 22.6% ash, 17.4 KJ g^{-1} of energy, 2.0% lysine and 0.9% methionine.

The experimental feeds were produced by mixing all the powder ingredients and pre-extruded alga pellets in a double-helix mixer (model 500 l, TGC Extrusion, France) and ground (below 400 μm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Feeds (pellet size: 3.0 mm) were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extrusion conditions for the experimental feeds were: feeder rate (80–89 kg/h), screw speed (235–244 rpm), water addition (approximately 230 mL/min), temperature recorded in barrel section 1 was 34–36 °C and highest temperature was observed in barrel 3, varying between 124–127 °C. Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, oils were added by vacuum coating (700 mbar, for approximately 50 s) (model PG-10VCLAB, Dinnissen, The Netherlands). Immediately after coating, feeds were packed in sealed plastic buckets and shipped to Nord University Research Station, Bodø, Norway for the feeding trial.

2.2. Fish and feeding

Atlantic salmon (*Salmo salar*) post-smolts were obtained from Cermaq, Hopen, Bodø, Norway (Aquagen strain, Aquagen AS, Trondheim, Norway) and maintained at the Research Station, Nord University for approximately 5 months. The fish were fed Spirit Supreme 75 and Spirit Supreme 150 (Skretting, Stavanger, Norway) during the holding period. At the start of the experiment, a total number of 600 fish with initial weight 227.3 ± 4.0 g were randomly allocated to the experimental units (n = 30 fish per tank). The fish were starved for 2 days after the distribution to the experimental tanks and then switched directly to the experimental feeds.

The feeding experiment was carried out in a flow-through system. In total, 20 circular fiberglass tanks (800 l) were used for the study. Each tank was supplied with sea water pumped from Saltenfjorden, from a depth of 250 m. During the experiment, water flow rate was maintained at 1000 l per hour, and the average temperature and salinity of the rearing water were 7.5 °C and 35‰, respectively. Oxygen saturation was always above 85% recorded for water at the outlet. A 24-h

photoperiod was maintained throughout the feeding period. The fish were fed ad libitum using automatic feeders (Arvo Tech, Finland); administered at two time points every day, from 08:00–09:00 and 14:00–15:00 during the 68-day trial. After each feeding, the uneaten feeds that settled in the steel wire mesh of each experimental tank were collected.

2.3. Fish sampling and data collection

At the beginning and end of the experiment, all the fish (600) were individually weighed and their lengths were recorded. Before handling, fish were anesthetized using tricainemethanesulfonate (MS 222, 140 mg/l). Fish that were sampled for histology, whole body composition and organosomatic indexes were humanely euthanized by a sharp blow to the head. At termination of the experiment, six fish per tank were pooled to assess the final chemical composition. These fish were packed in plastic bags, immediately frozen and kept at −40 °C until analyses. Three fish from each tank were weighed, dissected and the visceral organs (without heart and kidney) and liver from each fish were removed and weighed for calculation of organosomatic indexes. The distal intestine of these fish was sampled for histomorphology evaluation. Faeces were collected from the remaining fish in the tanks. Fecal matter was obtained from individual fish by stripping and pooled to obtain enough material for chemical analysis.

2.4. Chemical analyses

The fish samples from each tank were homogenized using an industrial food processor (Foss Tecator, 2096 homogenizer, Hilleroed, Denmark) before analyzing the whole body proximate composition of fish fed the experimental feeds. Both fecal samples and whole body samples were freeze dried (VirTis benchtop, Warminster, PA, USA) for 72 h prior to the chemical analysis.

The fish, experimental feeds and freeze-dried faeces were finely ground by mortar and pestle and homogenized prior to analyses of dry matter (105 °C for 20 h; ISO 6496:1999), crude protein (Kjeldahl Auto System, Tecator Systems, Höganäs, Sweden; ISO 5983:1987), crude lipid (Soxtec HT6, Tecator, Höganäs, Sweden; ISO 6492:1999), ash (incineration in a muffle furnace at 540 °C for 16 h; ISO 5984:2002) and energy (IKA C200 bomb calorimeter, Staufen, Germany; ISO 9831:1998). The amino acid analyses were performed according to ISO 13903:2005. Yttrium in both faeces and feeds was analyzed by employing inductive coupled plasma mass spectroscopy (ICP-MS) by Eurofins (Moss, Norway; NS-EN ISO 11885). All the samples were analyzed in duplicate.

Total lipid content of the fish was determined by ethyl-acetate extraction method. Total lipid content of the faeces was analyzed employing the Soxhlet method with acid hydrolysis (Soxtec HT 6209, Tecator, Höganäs, Sweden; modified AOAC method 954.020), by Eurofins® (Moss, Norway). Fatty acid composition of fish and feed was measured by gas chromatography (GC) of methyl-ester derivatives of the fatty acids of the lipids extracted from the samples. For this, the homogenized samples were lyophilized for 72 h before the lipids were extracted and analyzed in duplicate. Total lipid from the samples was extracted according to the method of Bligh and Dyer (1959). The fatty acid methyl esters (FAMEs) were prepared according to the AOCS Official Method Ce 1b-89. FAMEs were separated and quantitated using a Scion 436 GC equipped with a flame ionization detector, a splitless injector and a DB-23 column (Agilent Technologies, Santa Clara, USA). Standard mixtures of FAMEs were used for identification and quantitation of common fatty acids in samples (GLC-473, Nu-Chek Prep, Elysian, MN, USA).

2.5. Histological analysis

Approximately 1 cm of the anterior part of the distal intestine was sampled and luminal contents were rinsed off with 10% neutral buffered formalin (NBF), and the tissue was fixed in 10% NBF for 24 h. Formalin-fixed samples were dehydrated in an alcohol gradient, equilibrated in xylene and embedded into paraffin blocks. For each fish, approximately 5 μm thick longitudinal sections were cut using microtome, after which they were mounted onto a glass slide.

2.5.1. Immunohistochemistry

Samples of the distal intestine from six fish per feed group were used for the immunohistochemistry analysis of the proliferating cell nuclear antigen (PCNA). The sections were dewaxed and rehydrated. Antigen retrieval was done by autoclaving the sections for 10 min at 120 °C in citrate buffer (10 mM/l citric acid monohydrate, pH 6). For quenching of endogenous peroxidase, sections were incubated with 3% hydrogen peroxide in water for 30 min. To prevent nonspecific binding, the sections were blocked with normal horse serum containing 5% BSA in PBS for 20 min at room temperature. Sections were then incubated with the primary antibody anti-PCNA mouse monoclonal antibody to PCNA (M0879, Dako Cytomation, Bath, United Kingdom) at a dilution of 1:500 in 1% BSA/TBS overnight at 4 °C. The sections were then incubated with secondary antibody horse anti-mouse biotinylated against IgG at dilution 1:1000 for 30 min at room temperature. Subsequently the slides were incubated with ABC reagent (Vectastatin PK6102, Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Brown staining was obtained by dropping 3,3-Diaminobenzidine (DAB) substrate (D7679 Sigma-Aldrich Corp. St.Louis, MO, USA) on top of the slides to form a dark brown insoluble precipitate. Hematoxylin was used for counterstaining. The sections were washed with PBS (3 × 5 min) between each step in the protocol.

For the analysis of cell proliferation, 20 well-oriented and intact villi per fish were selected. This generated 120 microphotographs per feed group that were captured at × 40 magnification by a camera (Leica MC170HD, Heersbrugg, Switzerland) mounted on light microscope (Leica DM1000, Wetzlar, Germany) using a software, Leica Microsystems Framework (LAS V4.12.INK, Heerbrugg, Switzerland). All the images were analyzed with ImageJ 1.52a (Schneider et al., 2012).

The total area of a villus (TVA) was demarcated by 'Freehand selections' tool, and measured by 'Analyze' menu in ImageJ. The PCNA stained area of a villus (PSA) was estimated using 'Colour Threshold' in ImageJ. For that, 'Brightness' in the 'Colour Threshold' was decreased until only the PSA was covered, while 'Thresholding method' was set to 'Default', 'Threshold colour' to red and 'Colour space' to HSB (hue, saturation and brightness). The PSA could then be selected and measured (Fig. 1). This value was used to calculate the cell proliferation index (CPI), ratio between PSA and TVA. Mean ± SEM values of CPI are presented.

2.6. Calculations and statistical analysis

Fish growth performance was assessed based on different indices, derived employing the following equations:

$$\text{Weight gain (\%)(WG)} = \left(\frac{W_f - W_i}{W_i}\right) \times 100$$

$$\text{Feed intake (\%BW day}^{-1}\text{) (FI)} = \left(\frac{\text{Daily feed intake in dry basis (g)}}{\sqrt{W_f \times W_i}}\right) \times 100$$

$$\text{Specific growth rate (\%day}^{-1}\text{) (SGR)} = \left(\frac{\text{Ln}\,(W_f) - \text{Ln}\,(W_i)}{d}\right) \times 100$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Total feed intake in dry basis (g)}}{\text{Weight gain (g)}}$$

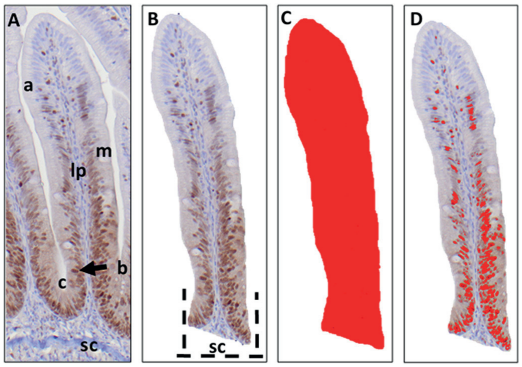

Fig. 1. Morphometric technique used to calculate the cell proliferation index (CPI) of the distal intestine of Atlantic salmon (*Salmo salar*). A. Simple intact villus at x10 magnification. a- absorptive vacuoles, b- PCNA-negative enterocyte, c- crypt, lp- lamina propria, m- mucous cell, sc- stratum compactum, arrow- PCNA-positive enterocyte. B. The selected boundaries of the villus included the epithelial part from tip of villus to its base and the crypt boundary was perpendicular to sc (which were not included) and parallel to lp. C. Total area of villus (TVA). D. The PCNA stained area of a villus (PSA).

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Weight gain (g)}}{\text{Total protein ingested (g)}}$$

$$\text{Thermal growth coefficient (TGC)} = \frac{(W_f)^{1/3} - (W_i)^{1/3}}{(T \times d)} \times 1000$$

$$\text{Hepato} - \text{somatic index (\%)(HSI)} = \frac{\text{Liver weight of fish (g)}}{W_f\,(g)} \times 100$$

$$\text{Viscero} - \text{somatic index (\%)(VSI)} = \frac{\text{Viscera weight of fish (g)}}{W_f\,(g)} \times 100$$

$$\text{Condition factor (g/cm}^3\text{)(CF)} = \frac{W_f\,(g)}{FL^3} \times 100$$

where, W_f = final body weight of fish (g/fish), W_i = initial body weight of fish (g/fish), T is the temperature in °C and d is feeding days, FL = Fork length of fish (cm).

Apparent Digestibility Coefficient (ADC) of nutrients and dry matter were calculated according to following equations:

$$ADC_{nutrient} = \left[1 - \left(\frac{Marker_{feed} \times Nutrient_{faeces}}{Marker_{faeces} \times Nutritent_{feed}}\right)\right] \times 100$$

$$ADC_{dry\ matter} = \left[1 - \left(\frac{Marker_{feed}}{Marker_{faeces}}\right)\right] \times 100$$

where $Marker_{feed}$ and $Marker_{faeces}$ represent the marker content (% dry matter) of the feed and faeces, respectively, and $Nutrient_{feed}$ and $Nutrient_{faeces}$ represent the nutrient contents (% dry matter) in the feed and faeces.

All statistical analyses were performed using SPSS 22.0 software package for Windows. The data were tested for normality (Shapiro–Wilk normality test) and equality of variance (Levene's test). For parametric data, one way analysis of variance (ANOVA) was performed. Thereafter, Tukey's multiple comparison test was used to identify the significant differences among the means of the experimental groups. For non-parametric data, Kruskal-Wallis test, followed by Dunn's multiple comparison test, was performed to decipher the significant differences between the groups. A significance level of $p < .05$ was chosen to indicate the differences.

3. Results

3.1. Experimental feeds

All the experimental feeds were nearly isoproteic, isocaloric and balanced for EPA + DHA. The content of amino acids (AA's) in the feeds were balanced to meet the AA requirements of Atlantic salmon (NRC 2011); through the dietary supplementation of crystalline amino acids, lysine, methionine, threonine and tryptophan. The content of lysine and methionine was 2.7–3.0% and 0.7–0.8% of feed (dry basis), respectively (Table 2). The polyunsaturated fatty acids, namely EPA + DHA were similar in the feeds (2.7–2.9% of dry basis; based on information from Table 3).

3.2. Apparent digestibility coefficients of feeds

Digestibility of DM, protein, lipid and ash differed significantly among the four feeds ($p < .05$; Table 4). The DM digestibility was significantly lower in CO-fed fish compared to fish fed NC and NZ, while that in ND-fed fish were ranked in between the CO and the other two algae-fed groups. Protein digestibility was higher ($p < .05$) in fish fed NC than those fed ND while the values of the CO and NZ groups were similar and lie between those of NC and ND. Lipid digestibility was highest in fish fed CO, while no differences were observed among the alga-fed groups. Digestibility values of ash in alga-fed fish were positive while the values of the fish fed CO were negative but no significant differences ($p < .05$) were detected among treatments.

3.3. Growth and feed utilization

The growth and feed utilization are given in Table 5. The fish grew from an initial average weight of 227.3 g to a final mean body weight of 419.6 g during the experimental period of 68 days. There were no significant differences in final weight, weight gain, specific growth rate, thermal growth coefficient, feed conversion ratio, feed intake or protein efficiency ratio of the different groups. There were no significant differences in condition factor or viscero-somatic indices (VSI) of the feed groups. Hepato-somatic indices (HSI) ranged between 1.10 and 1.19; the highest value was for the ND group compared ($p < .05$) to the lowest value of the NC group.

3.4. Proximate composition of whole body

The proximate composition of fish fed the four experimental feeds,

Table 4
Apparent digestibility coefficients (ADC %) of dry matter, lipid, protein and ash in Atlantic salmon fed the experimental feeds.

	CO	NC	ND	NZ	*p* value
Dry matter	63.3 ± 0.52^{b}	67.5 ± 0.41^{a}	65.3 ± 0.34^{ab}	66.1 ± 0.89^{a}	0.008
Protein	87.8 ± 0.11^{ab}	88.5 ± 0.07^{a}	86.5 ± 0.54^{b}	87.9 ± 0.60^{ab}	0.032
Lipid	94.3 ± 0.28^{a}	91.3 ± 0.04^{b}	91.1 ± 0.32^{b}	91.9 ± 0.52^{b}	0.002
Ash	−24.0 ± 2.05^{b}	12.9 ± 2.66^{a}	13.9 ± 1.06^{a}	7.7 ± 0.18^{a}	< 0.001

CO: Plant-based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed. Values are expressed as mean ± SD ($n = 5$ replicates). Values in the same row with different superscript letters indicate significant difference ($p < .05$).

Table 5
Growth performance, feed utilization and somatic indices of Atlantic salmon for experimental period.

	CO	NC	ND	NZ	p value
Growth parameter					
IBW(g)	227.94 ± 5.93	228.51 ± 1.82	225.27 ± 1.48	227.31 ± 4.24	0.628
FBW (g)	422.77 ± 22.16	415.05 ± 25.01	417.28 ± 21.08	423.26 ± 11.20	0.898
WG (%)	85.44 ± 7.80	81.61 ± 10.41	86.23 ± 4.74	85.21 ± 8.28	0.802
FI (% BW day^{-1})	0.83 ± 0.05	0.84 ± 0.05	0.82 ± 0.03	0.83 ± 0.02	0.836
SGR (% day^{-1})	0.91 ± 0.63	0.87 ± 0.08	0.90 ± 0.66	0.91 ± 0.38	0.774
FCR	0.90 ± 0.01	0.95 ± 0.05	0.89 ± 0.04	0.89 ± 0.02	0.109
PER	2.49 ± 0.05	2.39 ± 0.14	2.53 ± 0.12	2.52 ± 0.07	0.140
TGC	2.74 ± 0.21	2.64 ± 0.28	2.72 ± 0.22	2.76 ± 0.12	0.815
Somatic indices					
HSI	1.16 ± 0.03^{ab}	1.10 ± 0.59^{b}	1.19 ± 0.06^{a}	1.15 ± 0.02^{ab}	0.042
VSI	8.22 ± 2 0.27	8.30 ± 2.72	8.55 ± 0.50	8.38 ± 0.51	0.635
CF	1.41 ± 0.03	1.42 ± 0.03	1.44 ± 0.03	1.42 ± 0.03	0.332

CO: Plant-based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed. IBW, Initial body weight; FBW, Final body weight; WG, Weight gain; FI, Feed intake; SGR, Specific growth rate; FCR, Feed conversion ratio; PER, Protein efficiency ratio; TGC, Thermal growth coefficient; HSI, Hepato-somatic index; VSI, Viscero-somatic Index; CF, Condition factor.
Values are expressed as mean ± SD (n = 5 replicates). Values in the same row with different superscript letters show significant differences ($p < .05$).

Table 6
Proximate composition and energy of the whole fish on a dry matter basis (%).

	CO	NC	ND	NZ	p value
Protein	50.26 ± 0.35	50.72 ± 1.06	50.67 ± 0.64	50.65 ± 0.79	0.762
Lipid	41.94 ± 1.08	42.22 ± 1.65	39.26 ± 3.38	39.14 ± 2.14	0.075
Ash	5.40 ± 0.14	5.75 ± 0.38	5.60 ± 0.42	5.53 ± 0.15	0.366
Energy (KJ g^{-1})	29.05 ± 0.17^{ab}	28.82 ± 0.14^{b}	28.99 ± 0.10^{ab}	29.14 ± 0.23^{a}	0.048

CO: Plant based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed. Values are expressed as mean ± SD (n = 5 replicates). Values in the same row with different superscript letters indicate significant difference ($p < .05$).

sampled at the termination of the experiment, is provided in Table 6. No significant differences were observed in protein, lipid or ash content of the experimental groups. The energy content was significantly higher in NZ and lowest in fish fed NC ($p < .05$).

3.5. Fatty acid composition of fish whole body

The fatty acid composition of the whole body is given in Table 7. Significant differences were observed for saturated fatty acids (SFAs) and polyunsaturated fatty acids (PUFAs). The SFAs was significantly higher in fish fed CO compared with fish fed NZ ($p < .05$). The monounsaturated fatty acids (MUFAs) and n-3 PUFAs of the four groups were not significantly different. The n-6 PUFAs were significantly lower in fish fed CO compared to other groups ($p < .05$). Overall, the PUFAs were significantly higher in fish fed NZ compared to other groups ($p < .05$). As for the individual fatty acids, linoleic acid (LA), C18:2 n-6 dominated the n-6 fatty acids and it was lower in fish fed the CO feeds than in those fed the algal feeds ($p < .05$). The eicosapentaenoic acid (EPA, C20:5n-3) was found to be at the same level in fish fed the feeds with microalga even with a 50% reduction in fish meal and a 10% reduction in the fish oil compared to the CO-fed fish.

3.6. Histology of distal intestine

The morphology of distal intestine is shown in Supplementary Fig. 1. Villi height and width of fish fed CO, NC, ND and NZ is presented in Table 8. No significant differences were noted among the feeds. Proliferating cell nuclear antigen-positive cells were predominantly observed at the base of the distal intestinal villi and more diluted along the rest of the villi area (Fig. 2). Morphometric analysis of proliferating cells indicated a slight increase of CPI for all the microalgae incorporated feeds compared to the control group, but only the NC and NZ were significantly higher (Fig. 3).

4. Discussion

4.1. Apparent digestibility coefficients of feeds

The digestibility of protein, lipid and ash of the control feed used in the present trial were similar or even higher compared to fishmeal-based feed reported in our previous studies (Kiron et al., 2016; Sørensen et al., 2017). The digestibility of protein and lipid in the microalga-incorporated feeds in the present study were higher than those reported for 10% and 20% incorporation of *N. oceanica* in Atlantic salmon (Sørensen et al., 2017). These findings suggest that pre-processing of *N. oceanica* by extrusion, rendered intracellular nutrients more accessible for digestion. Effect of extrusion on cell disruption was not investigated in the present study. Other extrusion studies with *N. oceanica* have reported changes in the cell morphology characterized by wrinkled and shrunken cells; some cells with broken walls and others with emptied content (Wang et al., 2018). Extrusion may not have completely ruptured the cells; an even stronger treatment, i.e. a combination of enzymatic hydrolysis and high pressure homogenization could only achieve 95% disruption degree with another microalga *Neochloris oleoabundans* (Wang et al., 2015). Bead milling is an efficient mechanical method that increased the ADC of protein and lipid in tilapia fed the processed *Nannochloropsis gaditana* (Teuling et al., 2019). The efficiency of high-pressure homogenization was demonstrated using *Chlorella vulgaris*; it was reported that the process increased the ADC of protein, lipid, energy, total carbohydrate, starch and most essential amino acids and fatty acids in Atlantic salmon (Tibbetts et al., 2017).

Incorporation of the microalga (NC) even improved digestibility of dry matter and ash compared to the control group in the present study. Increased digestibility of ash was also observed in Nile tilapia and African catfish when they were fed *Nannochloropsis gaditana* (Teuling et al., 2017). Negative ash digestibility values are explained by drinking of sea water (Thodesen et al., 2001). Element analyses were not performed in the present experiment. However, for salmonids reared in

Table 7
Fatty acid composition (% of total fatty acids) of the whole fish.

Fatty acids	CO	NC	ND	NZ	P value
C14:0	2.78 ± 0.08^{a}	2.82 ± 0.04^{a}	2.80 ± 0.12^{a}	2.62 ± 0.04^{b}	0.005
C15:0	0.24 ± 0.05	0.22 ± 0.04	0.22 ± 0.04	0.20 ± 0.00	0.532
C16:0	10.86 ± 0.11^{a}	10.78 ± 0.11^{a}	10.70 ± 0.22^{ab}	10.52 ± 0.04^{b}	0.009
C17:0	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	1.000
C18:0	2.70 ± 0.07^{a}	2.58 ± 0.04^{b}	2.62 ± 0.04^{ab}	2.60 ± 0.70^{ab}	0.028
C20:0	0.30 ± 0.00	0.30 ± 0.00	0.30 ± 0.00	0.30 ± 0.00	1.000
C22:0	0.14 ± 0.05	0.14 ± 0.05	0.16 ± 0.05	0.18 ± 0.04	0.585
ΣSFAs	17.34 ± 0.19^{a}	17.14 ± 0.15^{ab}	17.08 ± 0.37^{ab}	16.78 ± 0.08^{b}	0.010
C16:1n-7	3.20 ± 0.00^{b}	3.32 ± 0.04^{a}	3.30 ± 0.70^{a}	3.20 ± 0.00^{b}	< 0.001
C18:1n-9	37.30 ± 0.22	37.36 ± 0.32	37.40 ± 0.29	37.58 ± 0.30	0.472
C20:1n-9	3.42 ± 0.10	3.38 ± 0.04	3.38 ± 0.13	3.42 ± 0.10	0.862
C22:1n-9	3.04 ± 0.15	2.98 ± 0.15	2.96 ± 0.20	2.96 ± 0.13	0.846
C24:1n-9	0.50 ± 0.00^{a}	0.42 ± 0.04^{b}	0.44 ± 0.05^{ab}	0.50 ± 0.00^{a}	0.004
ΣMUFAs	47.52 ± 0.16	47.60 ± 0.14	47.60 ± 0.14	47.72 ± 0.10	0.203
C18:2n-6	11.82 ± 0.11^{b}	12.12 ± 0.08^{a}	12.10 ± 0.21^{a}	12.22 ± 0.13^{a}	0.003
C18:3n-6	0.22 ± 0.04	0.24 ± 0.05	0.24 ± 0.05	0.22 ± 0.04	0.848
C20:2n-6	0.90 ± 0.00	0.90 ± 0.07	0.88 ± 0.04	0.92 ± 0.04	0.629
C20:3n-6	0.30 ± 0.00	0.30 ± 0.00	0.32 ± 0.04	0.30 ± 0.00	0.418
C20:4n-6	0.30 ± 0.00^{b}	0.40 ± 0.00^{a}	0.36 ± 0.05^{a}	0.40 ± 0.00^{a}	< 0.001
C22:5n-6	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	1.000
Σn-6 PUFAs	13.86 ± 0.13^{b}	14.20 ± 0.07^{a}	14.16 ± 0.19^{a}	14.30 ± 0.21^{a}	0.002
C18:3n-3	4.18 ± 0.08	4.26 ± 0.11	4.26 ± 0.11	4.30 ± 0.07	0.299
C18:4n-3	1.02 ± 0.04	1.04 ± 0.11	1.00 ± 0.07	1.00 ± 0.00	0.778
C20:3n-3	0.30 ± 0.00	0.32 ± 0.04	0.30 ± 0.00	0.30 ± 0.00	0.418
C20:4n-3	0.80 ± 0.00	0.76 ± 0.05	0.78 ± 0.04	0.76 ± 0.05	0.455
C20:5n-3	2.86 ± 0.05	2.94 ± 0.05	2.98 ± 0.08	3.02 ± 0.13	0.056
C22:5n-3	1.20 ± 0.00	1.20 ± 0.00	1.20 ± 0.00	1.24 ± 0.05	0.083
C22:6n-3	6.82 ± 0.13	6.60 ± 0.20	6.58 ± 0.22	6.64 ± 0.20	0.233
Σn-3 FUFAs	17.20 ± 0.00	17.08 ± 0.13	17.12 ± 0.16	17.26 ± 0.08	0.097
ΣPUFAs	31.06 ± 0.08^{b}	31.30 ± 0.07^{b}	31.28 ± 0.21^{b}	31.60 ± 0.18^{a}	< 0.001
n-3/n-6	1.24 ± 0.00^{a}	1.21 ± 0.01^{b}	1.21 ± 0.01^{b}	1.21 ± 0.01^{b}	0.011
EPA + DHA	9.68 ± 0.08	9.54 ± 0.20	9.56 ± 0.19	9.66 ± 0.13	0.449

CO: Plant based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed. Values are expressed as mean ± SD (n = 5 replicates). Values in the same row with different superscript letters indicate significant difference ($p < .05$).

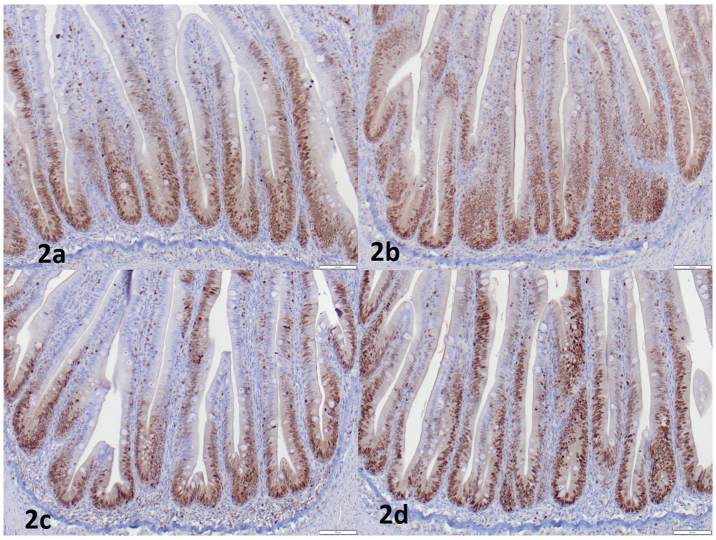

Fig. 2. Immunohistochemistry distal intestine of fish fed control feed (2a), or feeds with 10% extruded *N. oceanica* without additives (2b), or 10% extruded *N. oceanica* with Digestarom® (2c) or 10% extruded *N. oceanica* with ZEOFeed (2d). The bottom-right line is denoting 100 μm scale bar.

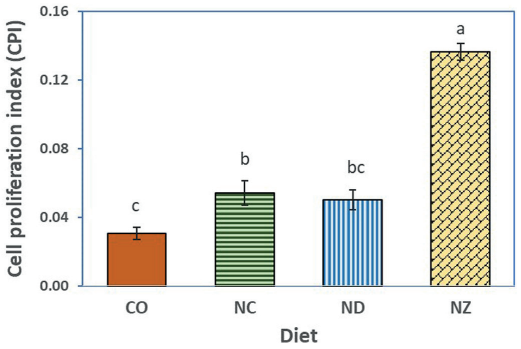

Fig. 3. Cell proliferating index in fish fed control feed, or feeds with extruded *N. oceanica* without (NC) or with Digestarom® (ND) or Zeofeed (NZ). Values are presented as means ± SEM, $n = 6$ fish per treatment group. Significant differences are denoted with different superscript ($p < .05$).

Table 8
Villi height and width (μm) in fish fed the different experimental feeds.

	CO	NC	ND	NZ
Villi height	610 ± 73.2	589 ± 50.4	552 ± 19.9	586 ± 56.2
Villi width	115 ± 4.7	101 ± 3.4	104 ± 4.5	103 ± 2.5

CO: Plant-based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed.
Values are expressed as mean ± SD ($n = 6$ fish per diet). No significant differences were observed among the feeds ($p > .05$).

seawater there is a high correlation between ADC of ash and absorption of Ca and Mg, some of the key minerals in seawater (Thodesen et al., 2001). Negative ADC of ash is thus a strong indication of high drinking rate. Differences in pellet quality can also alter the ash digestibility (Aas et al., 2011; Gong et al., 2019). Pellet quality was not analyzed in the present experiment, but is affected by incorporation of microalgae in the feeds (Gong et al., 2019. In the study of Gong et al., (2019) there were no differences between the pellet qualities of 10% *Scenedesmus* incorporated feed and those without the alga. A 20% incorporation of the alga resulted in a doubling of the hardness and 87% more negative ash value compared to the control feed.

Earlier studies have reported reduced digestibility of lipids in feeds with more SFAs (Kousoulaki et al., 2016; Kousoulaki et al., 2015). Salmonids have limited capacity to digest SFAs at low temperature when the SFA levels are high (Menoyo et al., 2003; Menoyo et al., 2007; Ng et al., 2004). The SFA levels were similar among feeds (Table 3) and are therefore not a likely explanation for the reduced lipid digestibility noted for the microalga-incorporated feeds. Lipid digestibility is also dependent on the position of the fatty acids on the triacylglycerol (TAG) (Mu and Høy, 2004; Nielsen et al., 2005). The location of the SFAs in the tested microalgal TAG are unknown, and the effect of the position on lipid digestibility warrants further investigation. Reduction in lipid digestibility with incorporation of *N. oceanica* can also be explained by the carbohydrate composition as well as the chemical and mechanical properties of the cell walls (Glencross et al., 2012; Teuling et al., 2017; Tibbetts et al., 2017). Microalgae have complex carbohydrates such as cellulose, pectins and hemicelluloses (Baudelet et al., 2017; Scholz et al., 2014). Carnivorous fishes do not have the capacity to digest non-starch polysaccharides (NSPs) and they are only non-nutritive fillers in feeds (Irvin et al., 2016; Krogdahl et al., 2005). Earlier studies have shown that NSPs have negative effects on lipid and energy digestibilities of fish feed (Aslaksen et al., 2007; Espinal-Ruiz et al., 2014; Irvin et al., 2016; Leenhouwers et al., 2006; Refstie et al., 1999). Aslaksen et al. (2007) and Lekva et al. (2010) found a linear reduction in digestibility of lipid with increasing cellulose level (0–18%) in feeds for Atlantic salmon and Atlantic cod (*Gadus morhua* L.). Insoluble fiber, such as cellulose, interfere with digestion by increasing the gastric emptying rate, i.e. by reducing the time for digestion and absorption. Soluble fibers of the NSP fraction from cereals and legumes, disturb fat micelle formation and increase viscosity of gut contents, leading to a reduced gastric emptying rate, which may affect fat digestion in farmed fish (Espinal-Ruiz et al., 2014; Leenhouwers et al., 2006; Øverland et al., 2009; Refstie et al., 1999; Sinha et al., 2011).

4.2. Growth performance and feed utilization of the fish

Atlantic salmon readily accepted the experimental feeds and there were no mortalities during the experiment. The overall growth performance and feed utilization were similar to earlier studies on Atlantic salmon (Austreng et al., 1987; Hatlen et al., 2012), or even better compared to Atlantic salmon of comparable size fed fishmeal-based feeds (Kiron et al., 2016; Sørensen et al., 2017). Feeding Atlantic salmon with 10% pre-extruded *N. oceanica* had no negative effect on feed intake, final mean body weight, weight gain, specific growth rate, and thermal growth coefficient. The present findings suggest that if the feeds are carefully balanced for essential amino acids and other essential nutrients, fishmeal incorporation can be reduced to 7.5% or even lower without compromising the growth (Kousoulaki et al., 2018; Kousoulaki et al., 2013). In contrast to Sørensen et al. (2017), who reported higher feed intake when salmon were fed defatted *N. oceanica*, there were no differences in feed intake in the present experiment. These findings are in line with Kiron et al. (2012) and Sprague et al. (2015) who reported no effect on feed intake when Atlantic salmon were fed *Nanofrustulum* sp. or *Tetraselmis* sp. at 10% inclusion rate, or *Schizochytrium* sp. at 11% inclusion level. In contrast, Atlantic salmon fed feeds containing 12% dried whole cells of the microalga *Phaeodactylum tricornutum* had reduced feed intake (Sørensen et al., 2016).

The growth of the fish in the present experiment was not impacted as noted for Atlantic salmon fed *Nanofrustulum* sp. or *Tetraselmis* sp. at 10% inclusion rate (Kiron et al., 2012). Other studies have reported negative effects on growth and/or feed conversion ratio when Atlantic salmon were fed feeds with *Desmodesmus* sp. (10/20%), *Schyzochrytrium* sp. (11%), or *P. tricornutum* (12%) (Kiron et al., 2016; Sørensen et al., 2016; Sprague et al., 2015). Taken together, the contrasting results suggest that direct comparison of microalgae varieties across experiments are difficult. The responses in the fish depend on the species and size, feed formulation, nutrient contents of feeds and their availability.

Improved growth, feed utilization and health effects have been reported in fish fed plant essential oils- supplemented feeds (Sutili et al., 2018). Giannenas et al. (2012) investigated the effect of supplementing two phytogenic feed additives containing either 1.2% carvacrol or 0.6% thymol on the performance of rainbow trout and found a significantly higher feed efficiency compared to the control group fed a basal diet. Nutrient digestibility were also improved in farmed land animals, e.g., broiler chickens, when their feeds were supplemented with Digestarom® (Murugesan et al., 2015). In line with our results, studies with channel catfish (*Ictalurus punctatus*) and gilthead seabream (*Sparus aurata*) also reported no effects on digestibility of dry matter and protein, growth performance and FCR when feeds were supplemented with 0.02% Digestarom® PEP MGE150 (Peterson et al., 2014; Rodrigues et al., 2018). The second additive tested in the present study is a clinoptilolite and a natural zeolite. Zeolites can be natural or synthetic materials with unique structure and physicochemical properties (e.g. detoxifying effects; antioxidant effect, effects on microbiota) (Ghasemi et al., 2016; Pavelić et al., 2018). It is used as a mycotoxin-binder in the feeds of terrestrial animals and it also improves gut health by preventing diarrhea in calves and pigs (Ghasemi et al., 2016; Papaioannou et al., 2005). Although the ability of clinoptilolite as health and growth

promoters in fish have not been studied much, there are reports suggesting improved growth rate and feed utilization in fish species such as gilthead sea bream and rainbow trout (Eya et al., 2008; Kanyılmaz et al., 2015). ZEOfeed did not have any significant effect on nutrient digestibility, FCR or growth of salmon in the present experiment. The dissimilar effects of these two feed additives noted in several studies may be attributed to the fish species, inclusion levels of the additives and duration of feeding period. Long-term feeding trials with species-specific optimal doses should confirm the benefits of the feed additives.

Histomorphological changes are not likely to explain the differences in nutrient digestibility as no clear differences were noted on villi length, width and gut health among the fish fed the different feeds. The immunohistochemistry analysis was performed to get an in depth understanding of the tissue homeostasis and the technique has earlier been used to study toxic mechanisms (Sanden and Olsvik, 2009) and intestinal inflammation (Bjørgen et al., 2018; Romarheim et al., 2010). The PCNA has a regulatory role in DNA replication and control of cell cycle. Although increased PCNA staining cannot be used as an independent indicator of cell activity (Maga and Hübscher, 2003), the increased staining in the ZEOfeed group is suggestive of greater cell proliferation in the intestine of this group. There were no other signs of ill-health to indicate the negative effect of the increased cell proliferation. Further in depth studies should gather more information about the effect of the increased cell proliferation on intestinal health.

4.3. Proximate composition of the fish

The whole body proximate composition of Atlantic salmon was not affected by either the intake of the microalgae or the feed additives. Whole body protein of fish in the present study was lower and lipid content of fish was higher than values (protein 55–58% of DM, lipid 29–37% of DM) reported for Atlantic salmon fed microalgae feed (Kiron et al., 2016; Sørensen et al., 2017). The proximate composition can vary with life stages of the fish and is also influenced by endogenous factors such as genetics, size and sex, as well as exogenous factors such as feed composition, feeding frequency and environment (Shearer, 1994). The ash content of the fish in the present study was in line with the values reported for fish fed microalgae feed (Kiron et al., 2016; Sørensen et al., 2016; Sørensen et al., 2017). It should be noted that because of the unavailability of the initial fish samples the nutrient retention values that would have given more valuable information cannot be discussed here. Additional studies are required to document nutrient retention efficiencies of fish fed these diets.

4.4. Fatty acid composition of the fish

In salmonid fish, the whole body fatty acid compositions are closely related to the fatty acid profile of the feed (Sissener, 2018; Sprague et al., 2016; Teimouri et al., 2016). The fatty acid composition in the experimental feeds used in the present experiment showed only minor differences and was also reflected in the whole body composition of the fish fed the different experimental feeds. The major differences observed for the n-6 PUFAs in whole body of fish fed algae feeds could be attributed to LA and arachidonic acid (C20:4n-6, ARA). The higher content of PUFA in fish fed NZ also can be explained by an increased content of LA, ARA and a trend towards increased EPA. The most noteworthy finding in this study was that the whole-body EPA + DHA levels of fish fed the algae diets were maintained at the same levels as the CO diet, even with a 50% reduction in fish meal and a 10% reduction in fish oil.

5. Conclusion

The present study showed that incorporation of 10% pre-extruded *Nannochloropsis oceanica* in plant-based commercial-like feeds reduced the lipid digestibility but did not affect the growth, feed utilization or body proximate composition. A slightly increased cell proliferation was observed for fish fed the microalga and was further increased by supplementation of feeds with ZEOfeed. Otherwise, the feed additives Digestarom® and ZEOfeed did not demonstrate any distinct advantage at their respective inclusion levels in salmon feed. The content of EPA and DHA was unaffected when fishmeal/fish oil was reduced from 15%/10% to 7.5%/9%, respectively.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735122.

CRediT authorship contribution statement

Yangyang Gong: Investigation, Formal analysis, Writing - review & editing. **Solveig L. Sørensen:** Investigation, Formal analysis, Writing - review & editing. **Dalia Dahle:** Investigation. **Nimalan Nadanasabesan:** Formal analysis. **Jorge Dias:** Investigation. **Luisa M.P. Valente:** Supervision, Funding acquisition. **Mette Sørensen:** Conceptualization, Methodology, Investigation. **Viswanath Kiron:** Conceptualization, Methodology, Investigation, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was funded by the Research Council of Norway (Project No. 260190, Alger-4laks) and is part by the COFASP ERA-NET project MARINALGAE4aqua. The authors would like to acknowledge Ghana K Vasanth for skillful technical assistance during the execution of the experiment. The support of the staff at the Research Station of Nord University is also highly appreciated. Yangyang Gong was financially supported by a fellowship from the China Scholarship Council as well as the funding from East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences. The funding agencies had no role in the design, analyses or writing of this article.

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Paper IV

Aquaculture 547 (2022) 737516

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Mucosal barrier status in Atlantic salmon fed marine or plant-based diets supplemented with probiotics

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ARTICLE INFO

Keywords:
Plant-based feed
Lactic acid bacteria
Mucous cell
Mucin and antimicrobial peptide genes
Short chain fatty acids

ABSTRACT

Feed ingredients and additives significantly affect the mucosal health of fish. A 3 × 2 factorial experiment was conducted to investigate the mucosal health of Atlantic salmon (*Salmo salar*) fed three basal feeds (namely, BG1, BG5 and BG2; marine-, plant-, and soybean meal-based feeds, respectively) or the basal feeds with (+) or without (÷) probiotics, *Lactobacillus fermentum* and *Lactobacillus plantarum*. Six diets were fed to fish distributed into 12 tanks (approximately 43 fish/tank). The average start weight of the experimental fish was about 122.6 g. After 38-days of feeding, the dorsal skin, gills and distal intestine were obtained for analysing histomorphometry and mucus-related genes. Digesta were also collected to study short chain fatty acids (SCFAs).

Fish fed BG2 had significantly higher number of mucous cells/μm^2 skin epithelium (SNE) than those fed BG1 and BG5. Addition of probiotics significantly increased SNE in BG5+ and BG2+ group compared to BG1÷ group. Similarly, the area and number of mucous cells/μm^2 gill epithelium (GME and GNE) were significantly higher in BG2 group, followed by BG5 and BG1 fish groups. Probiotics significantly increased GME and GNE in all feed types. Concerning intestine, villi height (VH) and enterocyte height (EH) were significantly higher for BG1 group, followed by BG5 and BG2 groups. Compared to fish offered BG2, fish fed BG1 had significantly wider villi (VW) and narrower lamina propria (LPW). The number of mucous cells (NM) and intraepithelial lymphocytes (IEL) in the intestine were significantly higher in BG2 fed fish than those offered BG5 and BG1. The indices VH, VW, EH, and IEL were not affected by probiotics. Although higher NM and IEL were observed in BG2÷ fish compared to those fed the other two diets, probiotics reduced NM and IEL. Fish fed BG2÷ showed symptoms of inflammation, including disappearance of supranuclear vacuoles (SNV). Probiotics improved VH, significantly reduced LPW and aided in the reappearance of SNV in BG2+ fed fish. Some of the gene expression data supported histological findings; notably, levels of *muc5ac1* in the skin and *defensin3* and *cathelcidin1* in the intestine were correlated with histology data. Moreover, the total SCFA concentration was significantly affected by feed ingredients. Only acetoacetic acid was affected by both factors.

Our findings suggest that feed ingredients can significantly alter the mucosal protective barrier of the organs. Supplementation of probiotics alleviated the inflammatory responses and activated selected innate immune defence molecules, without affecting growth. The positive effect of the probiotics was similar regardless of the feed ingredients, suggesting that these probiotics can be utilized as immune regulators to evoke favourable responses on the skin, gills and intestine.

1. Introduction

Fish feed accounts for a significant part of the variable costs in the farming of Atlantic salmon (*Salmo salar*). Efficient utilization of feeds by the fish is the key to keep the production cost low and the economic turnover high (Iversen et al., 2020). Since 1990, salmon feed industry

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https://doi.org/10.1016/j.aquaculture.2021.737516
Received 6 June 2021; Received in revised form 5 September 2021; Accepted 22 September 2021
Available online 27 September 2021

has gradually shifted its dependence from marine- to plant-derived ingredients (Aas et al., 2019); now soy protein concentrate is a key salmon feed ingredient, and pea protein concentrate, wheat gluten, corn gluten, fava beans, sunflower meal and sunflower protein are incorporated at lower levels (Aas et al., 2019; Øverland et al., 2009; Ytrestøyl et al., 2015). Regarding the fish oil, it has been replaced to a large extent by rapeseed oil in European salmon feeds (Aas et al., 2019; Sprague et al., 2016; Ytrestøyl et al., 2015).

Use of more plant-based ingredients has taken its toll on the health of fish (Sørensen et al., 2021). Less refined feed ingredients, in particular soybean meal (SBM) that contains various antinutritional factors such as saponins, cause enteritis in fish (Baeverfjord and Krogdahl, 1996; Booman et al., 2018; Knudsen et al., 2007; Krogdahl et al., 2015; Sanden et al., 2005; Sørensen et al., 2011; Vasanth et al., 2015). SBM derivatives-induced intestinal inflammation was characterized by abnormal intestinal villi and lamina propria, enterocytes lacking supranuclear vacuoles, immune cell-infiltrated lamina propria and submucosa, and intestinal ion and water transport disturbances (Baeverfjord and Krogdahl, 1996; Buttle et al., 2001; Kiron et al., 2020; Kortner et al., 2012; Krogdahl et al., 2000; Refstie et al., 2000; Urán et al., 2008a, 2008c, 2009; Van Den Ingh et al., 1996, 1991). These unfavourable conditions are known to affect the growth of the fish, shift the microbiota and weaken the local immune defences, thereby making the fish prone to diseases (Egerton et al., 2020; Gajardo et al., 2017; Krogdahl et al., 2000; Torrecillas et al., 2017).

Disease prevention and control coupled with reduced mortality during the grow-out phase of fishes is vital for sustainable development of aquaculture as well as to keep the production costs in check (Bang-Jensen et al., 2019; Iversen et al., 2020; Minniti et al., 2019). Fish body is exposed to various adverse environmental conditions, including many opportunistic pathogens that thrive in the rearing water. However, a rather complex immune system that encompasses innate and adaptive branches, exists to fight these threats and to maintain the health (Brunner et al., 2020; Magnadóttir, 2006). The first lines of defence include the mucosal barriers in the skin, gills and intestine (Cain and Swan, 2010; Kiron, 2012; Wang et al., 2011). The epithelial cells in these organs are covered by a mucus layer, which is mainly secreted by mucous cells. The mucus consists of several innate immune molecules such as mucins, protease, lysozyme, esterase, complement proteins, antibodies and antimicrobial peptides (AMPs) which could chemically inactivate the pathogens or arrest the formation of their colonies (Aranishi and Mano, 2000; Concha et al., 2003; Firth et al., 2000; Hatten et al., 2001; Johansson et al., 2008; Núñez-Acuña et al., 2018, 2016). In addition, the intestine mucus creates an optimum environment for the action of the digestive enzymes and aids in lubricating the digesta to ensure the integrity of intestinal mucosa during digestion (Kim and Ho, 2010). Therefore, a healthy mucosal surface with adequate number of mucous cells is essential to maintain the barrier functions and deploy appropriate molecules such as mucins and AMPs during defence (Pittman et al., 2011).

Commensal microbiota at the mucosal surfaces are also vital to maintain the mucosal barrier functions and to prevent colonization by potential pathogens (Lowrey et al., 2015). It is now known that microbiota can be modulated by feed ingredients (Hoseinifar et al., 2015; Nayak, 2010; Pérez-Sánchez et al., 2014) and additives such as probiotics (Gupta et al., 2019a) and pre-biotics (Gupta et al., 2019b). Lactic acid bacteria (LAB) such as *Lactobacillus* spp., *Lactococcus* spp., *Pediococcus* spp., *Carnobacterium* spp. and those belonging to the genus *Leuconostoc* can be considered as probiotics for aquaculture applications; for enhancement of gut health, to reduce the use of chemotherapeutics and to maintain fish welfare (Alonso et al., 2019; Andani et al., 2012; Hai, 2015; Merrifield et al., 2010; Ringø et al., 2018).

LABs have anti-inflammatory and antibacterial properties, and they are classified as GRAS (generally recognized as safe) (van Baarlen et al., 2013). Bacteria belonging to the genus, *Lactobacillus* are acid-tolerant facultative anaerobes, and they are either homo- or heterofermentative (Ringø et al., 2018). Kraus (1961) was the first to reveal the presence of lactobacilli in the gastrointestinal tract (GI) of a fish, herring (*Clupea harengus* L.). Since then, the existence of bacteria belonging to the genus *Lactobacillus* in the GI tract of several finfish species, including Atlantic salmon, has been reported by many authors (Gatesoupe, 2007; Hovda et al., 2007; Lauzon and Ringø, 2011; Merrifield et al., 2014; Ringø, 2004; Ringø et al., 2005; Ringø and Gatesoupe, 1998). Our group has reported the establishment of lactobacilli delivered through feeds in the intestinal mucus of Atlantic salmon (Gupta et al., 2019a).

It is now known that certain members of the microbiota can produce short chain fatty acids (SCFAs) by fermenting nondigestible carbohydrates in feeds (Adorian et al., 2020; Hoseinifar et al., 2017). The dominant SCFAs such as acetate, propionate and butyrate (Den Besten et al., 2013) act as energy providers, signalling molecules, gene expression regulators, inflammation suppressors and immune cell development regulators. Thus, they play a critical role in maintaining intestinal integrity and health (Koh et al., 2016; Louis et al., 2014; Morrison and Preston, 2016; Richards et al., 2016).

Important information on the fish mucosal health status can also be collected by studying the associated mucin and AMP related genes (Bridle et al., 2011; Broekman et al., 2013; Chang et al., 2006; Marcos-López et al., 2018). In Atlantic salmon, seven mucin secreting genes were reported previously; two *muc2* genes were mainly found in the intestine while five *muc5* were observed in other tissues such as pyloric caeca, gill or skin (Sveen et al., 2017). AMPs are a diverse group of defence molecules, and among them *cathelicidins* and *defensins* are powerful antimicrobials (Chang et al., 2006; Reyes-Becerril et al., 2013). Our previous study results indicated the ability of *muc2* to denote intestinal barrier status and the feed ingredient-induced alteration of AMP genes in the skin, gills and intestine (Sørensen et al., 2021). Furthermore, in mammals, SCFAs are suggested as biomarkers to assess the host health status (Farup et al., 2016). Hence, it is essential to gather more evidence on feed component-induced modulation of mucins and AMPs that are important gatekeepers of the mucosal barriers and SCFAs that support the health of the intestine.

The aim of the present short-term feeding study was to investigate the growth performance, the architecture of the mucosa of the first-line defence organs, expression of selected mucins and AMP genes in these organs and SCFAs in the digesta of Atlantic salmon post smolts fed plant-based or marine-based diets, with or without two lactic acid bacteria strains, *Lactobacillus fermentum* and *Lactobacillus plantarum* (1:1).

2. Materials and methods

The National Animal Research Authority (FDU: Forsøksdyrutvalget ID-5887) in Norway has approved the experiment, and the handling of the animals were in accordance with the approved protocols.

2.1. Experimental feed preparation

2.1.1. Feed preparation

For this trial, three basal feeds were prepared at the Feed Technology Center, Nofima, Bergen, Norway (Table 1). Extruded feeds were formulated based on the following ingredients: fish meal and fish oil (BG1), a mixture of plant and marine ingredients at a ratio of 70:30 (BG5) and SBM with marine ingredients (BG2). The ingredients of the experimental feeds were first homogenized (30 min) using a horizontal ribbon mixer and then they were subjected to a preconditioning step. During this step, water and steam were added into an atmospheric double differential preconditioner (DDC). The preconditioning step was followed by extrusion through a TX-52 co-rotating, fully intermeshing twin-screw extruder (Wenger Manufacturing Inc., Sabetha, KS, USA). While the temperature of the feed mash that was fed into the extruder was 86–88 °C, temperatures of the extruded feeds were different; 120, 128, and 137 °C for BG1, BG2 and BG5, respectively. Two of the feeds,

Table 1
Ingredient composition (%) of the three basal feeds employed in the study.

Ingredients	BG1	BG5	BG2
Fishmeal	50	10	30
Wheat meal	13.85	6.05	6.55
Wheat gluten	5	10	10
Soy protein concentrate	0	20	0
Soybean meal	0	0	20
Corn gluten	0	9	0
Pea protein concentrate	0	9	0
Fish oil	25	7.7	26.4
Rapeseed oil	0	19.8	0
Mineral premix	0.59	0.59	0.59
Vitamin premix	2	2	2
Monosodium phosphate	2.5	2.5	2.5
Carop. Pink (10% Astax)	0.05	0.05	0.05
Yttrium oxide	0.01	0.01	0.01
Choline	0.5	0.5	0.5
Methionine	0.3	0.9	0.6
Lysine	0	1.2	0.5
Threonine	0	0.4	0.1
Histidine	0.2	0.3	0.2

BG1, marine-based feed; BG5, plant-based feed; BG2, soybean meal-based feed. Three more experimental diets were prepared by coating two probiotic organisms to the three basal feeds.

BG2 and BG5 had lower wheat content; consequently, more moisture in the form of steam was added into the DDC to ensure good expansion of the feed pellets. The wet extrudates, expelled out of the 24 circular 2.5 mm dies at the extruder outlet, were cut with a rotating knife of the extruder. The extruded pellets were dried in a hot air dual layer carousel dryer (Paul Klockner, Nistertal, Germany) at constant air temperature (77 °C) to obtain pellets with approximately 7–8% moisture. Next, the feeds were coated with oil using a vacuum coater (Pegasus PG-10VC LAB, Dinnissen B.V., the Netherlands). Immediately after the oil coating, feeds were packed in sealed plastic buckets and shipped to the Research Station, Nord University, Bodø, Norway.

2.1.2. Probiotics coating on feed pellets

Two species of probiotics, *L. plantarum* R2 Biocenol™ (CCM 8674) and *L. fermentum* R3 Biocenol™ (CCM 8675) were isolated from the intestinal content of rainbow trout (*Oncorhynchus mykiss*) obtained from a fish farm, Rybárstvo – Požehy s.r.o. Dubové in the Slovak Republic (Fečkaninová et al., 2019). Pure cultures of probiotics were grown on de Man, Rogosa and Sharpe (MRS) agar plates (HiMedia Laboratories, Mumbai, India) under anaerobic condition (Oxoid Gas Pack Anaerobic system) at 37 °C for 48 h before they were inoculated into 1000 mL of MRS broth and incubated for 18 h at 37 °C on a shaker. The culture was centrifuged at 4500 rpm for 20 min at 4 °C in a cooling centrifuge (Universal 320 R, Hettich, Germany). The resulting cell pellets were washed twice and resuspended in 30 mL of 0.9% (w/v) sterile saline. The feeds (batches of 1800 g) were thoroughly coated with the bacterial suspensions using a vacuum coater (Rotating Vacuum Coater F-6-RVC, Forberg International AS, Norway) at 70 kPa at the feed laboratory of Nord University, Bodø, Norway. Post coating, the bacterial counts on diets were &10^8 cells/g as determined by spread plating on MRS agar plates and incubating anaerobically (Oxoid Gas Pack Anaerobic system) for 48 h at 37 °C. The feeds without probiotics were coated with 0.9% of sterile saline. The coated diets were stored at 4 °C until they were fed to the experimental fish.

2.1.3. Experimental feeds

In total, six experimental feeds were prepared for this study at the feed laboratory of Nord University, Bodø, Norway. The basal feeds without probiotics were named as BG1÷ (marine- based feed without probiotics), BG5÷ (plant-based feed without probiotics) and BG2÷ (SBM-based feed without probiotics). The basal feeds with probiotics were named as BG1+ (marine-based feed with probiotics), BG5+ (plant-based feed with probiotics) and BG2+ (SBM-based feed with probiotics). The nutrient and amino acid composition of the basal feeds is given in Table 2.

2.2. Fish, experimental design and feeding

Atlantic salmon post-smolts were obtained from Cermaq, Hopen, Bodø, Norway (Aquagen strain, Aquagen AS, Trondheim, Norway). The present experiment was the second phase of a large study (Sørensen et al., 2021) performed at the Research Station, Nord University, Bodø, Norway to test the effects of different combinations of plant and marine ingredients on the performance of Atlantic salmon. There were two replicate tanks for each treatment, and each tank contained 40–43 fish. The average initial weight of the fish was 122.6 ± 2.1 g (mean ± standard error of mean, SEM).

The feeding experiment was carried out in 12 circular fiberglass tanks (1100 L) that were connected to a flow-through system. Each tank was supplied with water pumped from Saltenfjorden, from a depth of 250 m. During the experiment, water flow rate was maintained at 1000 L per h, and the average temperature and salinity of the rearing water were 7.6 °C and 35 ‰, respectively. Oxygen saturation was always above 85%, measured at the water outlet. A 24 h photoperiod was maintained throughout the 38-day feeding trial. The fish were fed *ad libitum* using automatic feeders (Arvo Tech, Finland) during a 12-h period every day between 08:00 and 20:00 (7 feedings, 08:00–10:00, 10:00–12:00, 12:00–14:00, 14:00–16:00, 16:00–18:00, 18:00–19:00 and 19:00–20:00).

2.3. Sampling and data collection

At the beginning and end of the feeding experiment, all fish were individually weighed, and their fork lengths recorded. Fish were anesthetized using tricaine methanesulfonate (MS 222, 140 mg/L) before handling. Twelve fish per tank were sacrificed for obtaining the dorsal skin (left), gills (second arch) and intestine (approximately 2 cm of the anterior part of the distal intestine) (Sanden and Olsvik, 2009; Sundell

Table 2
Analyzed proximate composition (% as is) and amino acid composition (% as is) of the three experimental feeds.

Composition	BG1	BG5	BG2
Moisture	5.3	6.3	4.9
Protein	42.5	42.8	42.2
Lipid	29.0	26.0	28.6
Ash	11.2	7.02	9.45
Energy (KJ/100 g)	2000	1994	2029
Amino acids			
Alanine	2.44	2.04	2.03
Arginine	2.35	2.35	2.33
Aspartic acid	3.50	3.51	3.43
Glutamic acid	6.92	9.04	8.03
Glycine	2.61	1.75	2.18
Histidine	1.01	1.17	1.02
Hydroxyproline	0.31	0.16	0.22
Isoleucine	1.66	1.66	1.64
Leucine	3.01	3.54	2.93
Lysine	2.89	3.05	2.85
Phenylalanine	1.67	2.10	1.79
Proline	2.19	2.88	2.47
Serine	1.81	2.04	1.91
Threonine	1.64	1.9	1.64
Tyrosine	1.25	1.50	1.35
Valine	1.96	1.88	1.86
Tryptophan	0.43	0.41	0.44
Cysteine	0.41	0.53	0.50
Methionine	1.37	1.68	1.67
[a]Σ EPA/DHA	5.90	1.7	5.8

BG1, marine-based feed; BG5, plant-based feed; BG2, soybean meal-based feed.
[a] Σ EPA/DHA was calculated based on the content in the fish oil.

and Sundh, 2012). Tissues from 6 fish were immediately placed in 10% neutral buffered formalin (NBF) for 24 h at room temperature for the histological evaluation, and tissues from remaining 6 fish were transferred to tubes filled with RNA later® (Ambion Inc., Austin, Texas, United States), and stored at −20 °C for gene expression analysis. Another 5 fish per tank were stripped for digesta and stored at −20 °C for analysing SCFA composition.

2.4. Growth performance calculations

Fish growth performance was analysed using the following equations.

$$\text{Weight gain (WG\%)} = ((\text{FW} - \text{IW})/\text{IW}) \times 100$$

$$\text{Specific growth rate (SGR)} = ((\text{Ln (FW)} - \text{Ln (IW)})/\text{D}) \times 100$$

$$\text{Thermal growth coefficient (TGC)} = \left(\left((\text{FW})^{(1/3)} - (\text{IW})^{(1/3)}\right)\Big/(\text{T} \times \text{D})\right) \times 1000$$

$$\text{Condition factor (CF)} = \left(\text{FW}/\text{FL}^3\right) \times 100$$

Where, FW = mean final body weight of fish (g), IW = mean initial body weight of fish (g), T is the water temperature in °C, D is feeding duration in days. IL and FL are the initial and final fork length (cm) of fish, respectively.

2.5. Histomorphometry

Standard histological procedures were adopted, and the analyses were performed at the histology laboratory of the Research station, Nord University, Bodø, Norway. Fixed tissues were dehydrated with increasing concentrations of ethanol, followed by immersion in xylene and paraffin (Sørensen et al., 2011). Next, tissue sections of 4 μm were prepared using microtome and mounted onto a glass slide, after which they were stained with Alcian blue - periodic acid–Schiff (pH 2.5). Stained slides (one section per fish) were covered with a coverslip after adding a drop of glue, Pertex® (Histolab Products AB, Askim, Sweden). Thereafter, microphotographs were captured at 40× magnification by a camera (Leica MC170HD, Heersbrugg, Switzerland) fitted on a light microscope (Leica DM1000, Wetzlar, Germany), and using a software, Leica Application Suite (LAS V4.12.INK, Heerbrugg, Switzerland). All the images were examined with ImageJ 1.52a (Schneider et al., 2012).

2.5.1. Collection of skin samples from the dorsal area

Tissues (approximately 2 cm) were sliced transversely into 3 equal parts after removing most of the muscles that were attached to the skin and decalcified with 10% formic acid (25 blocks per L) for 5 h. The tissues were rinsed with phosphate-buffered saline (PBS) prior to standard histological procedure. Approximately 600–900 μm (length) skin microphotographs (9 per fish) were generated to investigate the skin mucous cells.

2.5.2. Collection of gill samples

To measure the area or count the number of mucous cells in the gills, 10 secondary lamellae from 5 different filaments per fish were chosen. Thus, in this study 50 secondary lamellae per fish were examined to understand the effect of the diets.

2.5.3. Histomorphometric analysis of the dorsal skin and gills

First, 'Freehand selections' tool of ImageJ was employed to demarcate the total area of skin epithelium (SE) and then 'Brightness and Hue' under 'Colour threshold' of the 'Image' menu was adjusted, while keeping 'Thresholding method' as 'Default', 'Threshold colour' set to red and 'Colour space' to HSB (hue, saturation and brightness). Next, using the measure option under the 'Analyze' menu SE was calculated (Gong et al., 2020). Thereafter, the 'Wand tool' was used to select individual mucous cells. Next, the background was cleared using 'Edit' and then the image was converted to 8 bits to retain only the mucous cells. The total area of skin mucous cells (SM) and number of skin mucous cells (SN) were determined by selecting 'Threshold' under 'Image' menu, and by setting 'Analyze particles' to '30 to infinity' under the 'Analyze' menu in ImageJ (Supplementary Fig. 1). SE, SM and SN were used to calculate 2 indices: SME (SM per SE) and SNE (SN per SE). The same image analysis procedure that is described for skin was employed for gills to examine the total area of gill epithelium (GE), the total area of gill mucous cells (GM) and number of gill mucous cells (GN). The obtained values were used to calculate 2 indices: GME (GM per GE) and GNE (GN per GE) (Supplementary Fig. 2).

2.5.4. Collection of intestine samples

The intestine contents were first rinsed off with 10% NBF prior to fixation. After trimming off the excess tissues, the intestine segment was processed and embedded longitudinally. For the histomorphometric analysis, 10 simple, long, well-oriented and intact villi per fish were selected from 3 to 5 different locations. Approximately, 10 microphotographs per fish were generated.

2.5.5. Histomorphometric analysis of distal intestine

The evaluation of the intestine histomorphology included a quantitative and a semi-quantitative assessment. For the quantitative assessment, height (VH) and width (VW) of villi, height of enterocytes (EH), and width of the associated lamina propria (LPW) were measured; these parameters helped us to evaluate the diet-induced alterations in the intestinal microscopic structure. Width of a villus varies along its height, and hence to measure VW, each villus was partitioned into 6 equal parts from the base to tip (Supplementary Fig. 3). From these 5 points, VW, EH and LPW were gauged employing the analysing tools ('straight' and 'segmented lines') of the ImageJ, and the average of the 5 values was registered. The semi-quantitative assessment included the evaluation of the number of intestinal mucous cells (NM), number of intraepithelial lymphocytes (IEL), and presence of supra nuclear vacuoles (SNV) in enterocytes of intestinal villi. A scoring system was developed (Supplementary Table 1) based on previous articles (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2007; Knudsen et al., 2008; Silva et al., 2015; Urán et al., 2008a). Each index of interest received a score from 1 to 5, and these scores were used for the downstream analyses.

2.6. Gene expression analysis

For the present study, relative mRNA levels of mucin genes (*muc2*, *muc5ac1*, *muc5ac2,* and *muc5b*) in the skin, gills and distal intestine, and AMP genes (*defensin 1 - def1, defensin 2 - def2, defensin 3 - def3, defensin 4 - def4,* and *cathelicidin 1 - cathl1*) in the skin and distal intestine were studied. Primers were purchased from Eurofins Genomics (Luxembourg, Luxembourg) and the sequences and details of all target and reference genes are described in Sørensen et al. (2021). The RNA extraction, cDNA synthesis and qPCR were performed as described by Sørensen et al. (2021).

2.7. Quantification of short chain fatty acids by isotachophoresis

Approximately 1 g of digesta per fish was homogenized with deionized water (50 ml). The solution was filtered through normal filter paper. The filtrates (5 ml per fish) were kept in cryotubes at −20 °C until further analysis. The produced short chain fatty acids (formic, acetoacetic, lactic, succinic, acetic, propionic, valeric and butyric acids) were determined by capillary isotachophoresis (Electrophoretic analyzer EA 202 M, VILLA LABECO spol. s.r.o., Spisska Nova Ves, Slovakia) as described by Gancarcikova et al. (2020).

2.8. Statistical analysis

In the current study, tank was used as the experimental unit for growth performance calculations (Kiron et al., 2016). However, individual fish was considered as the experimental unit for histological evaluation (Bansemer et al., 2015; Cerezuela et al., 2013; Urán et al., 2008b), gene expression and SCFAs composition analyses. All statistical analyses were executed using R (version 3.6.3) and R studio (version 1.2.5033) for windows. Normality of the data was checked with Shapiro–Wilk test and the homogeneity of variance was assessed by Levene's test. In this experiment, feed type (based on ingredients composition) was taken as the first factor (FeedIn: BG1, BG5 and BG2) and probiotic treatment as the second factor (ProbTr: "÷" (without probiotics) and "+" (with probiotics)). The effects of the factors and their interaction (FeedIn×ProbTr) were assessed by analysing the data using two-way analysis of variance (two-way ANOVA). Hereafter, the term 'BG1' shows or represents the average value of both 'BG1÷' and 'BG1+' (marine-based feed with and without probiotics). We have adopted the same strategy for 'BG5' and 'BG2'. For the groups with (+) and without (÷) probiotics, the average values of the different FeedIn are shown in the tables. Parametric two-way ANOVA was employed for data (IW, IL, FW, FL, WG, SGR, TGC, CF, SME, SNE, GME, GNE, VH, VW, EH, LPW, *muc2*, *muc5ac1*, *muc5ac2*, *muc5b*, *def1*, *def3*, *def4*, and *cathl1*) that followed gaussian distribution and had equal variance. When necessary, data were log transformed (gene expression and SCFAs data). Significant differences among the means of the experimental groups were revealed by Tukey's honestly significant difference (HSD) test. Two-way aligned rank transform analysis of variance (ART ANOVA) from ARTool package (version 0.10.7) was used for non-parametric semi-quantitative data (NM, IEL and SNV). Here, post-hoc comparisons, based on estimated marginal means (emmeans), were performed on a linear model for the response aligned and ranked data (Feys, 2016; Wobbrock et al., 2011). Spearman correlations for all the combinations of histologically evaluated mucous cell indices and the selected mucus-related genes were evaluated using the function from the package "psych" in R software. Statistical differences are reported at a significance level of $p < 0.05$. Means ± SEM of parameters are presented in all tables and figures, except Table 4, Figs. 3 and 7.

3. Results

3.1. Growth performance

There were no mortalities during the experiment. The growth performance parameters are presented in Table 3. The fish weight increased from an average range of 116–127 g to a range of 186–200 g during the experiment. There were no significant differences in FW, FL, SGR, TGC and WG of the diet groups. On the other hand, CF was significantly affected by feed type (factor FeedIn, Table 3); fish fed BG2 had lower CF compared to BG1 and BG5. None of the growth performance parameters was affected by feeding the probiotics (factor ProbTr). Furthermore, we did not find any interaction (FeedIn×ProbTr) effect on the parameters.

3.2. Histomorphometry

The results of the two-way ANOVA for the main factors, FeedIn and ProbTr are presented in Table 4 and Figs. 1–3; employing the data on dorsal skin, gill and distal intestine morphometric indices from the histology study.

3.2.1. Mucous cells in the dorsal skin

The results did not reveal any significant differences in SME of the diet groups (Fig. 1A). However, SNE was significantly influenced by both factors, feed type (FeedIn) and probiotics (ProbTr). Fish fed BG2 had significantly more SNE compared to the fish fed BG1 and BG5 (Table 4 and Fig. 1B). Note that the probiotic groups (+) had significantly more SNE compared to groups without probiotics (÷) (Table 4; the main factor effect). Diet groups BG2÷, BG2+ and BG5+ had significantly more SNE compared to the diet group BG1÷ (Fig. 1B). We did not find any significant interaction of the two factors (FeedIn×ProbTr). However, all the probiotic fed groups showed similar increasing tendency for SNE (Table 4 and Fig. 1B).

3.2.2. Mucous cells in the gills

Histological evaluation of mucous cells in the gills revealed significant effects of feed type (factor FeedIn) and probiotics (factor ProbTr) on GME (Fig. 1C and Table 4) and GNE (Fig. 1D and Table 4). Fish fed BG2 had significantly more GME and GNE compared to BG5 followed by those fed BG1. Addition of probiotics to all the feed type significantly increased GME and GNE (Fig. 1C and D). We did not detect a significant

Table 3
Growth performance indicators of Atlantic salmon offered feeds with different combination of marine and plant ingredients, and with or without probiotic supplementation.

Parameters:		IW (g/fish)	IL (cm)	FW (g/fish)	FL (cm)	CF (g/cm^3)	SGR	TGC	WG (%)
Means of main effect:									
FeedIn	BG1	126.91 ± 1.56	21.51 ± 0.10	196.92 ± 5.45	24.67 ± 0.25	1.31 ± 0.01^B	1.15 ± 0.05	2.74 ± 0.14	55.11 ± 2.98
	BG5	124.58 ± 2.22	21.33 ± 0.09	199.90 ± 7.51	24.64 ± 0.19	1.34 ± 0.02^B	1.24 ± 0.09	2.94 ± 0.23	60.47 ± 5.29
	BG2	116.34 ± 2.59	21.16 ± 0.11	186.36 ± 3.37	24.78 ± 0.15	1.22 ± 0.00^A	1.24 ± 0.03	2.88 ± 0.06	60.25 ± 1.71
ProbTr	÷	121.83 ± 2.85	21.30 ± 0.01	195.78 ± 5.01	24.74 ± 0.15	1.29 ± 0.02	1.25 ± 0.05	2.94 ± 0.13	60.82 ± 3.13
	+	123.38 ± 2.35	21.37 ± 0.01	193.01 ± 5.16	24.65 ± 0.17	1.29 ± 0.03	1.18 ± 0.04	2.77 ± 0.11	56.41 ± 2.57
Means of interaction effect:									
BG1	÷	126.99 ± 2.03	21.44 ± 0.14	194.30 ± 11.47	24.58 ± 0.47	1.31 ± 0.01	1.12 ± 0.11	2.64 ± 0.30	52.90 ± 6.58
	+	126.82 ± 3.23	21.57 ± 0.19	199.54 ± 5.74	24.76 ± 0.36	1.32 ± 0.02	1.19 ± 0.01	2.84 ± 0.05	57.33 ± 0.51
BG5	÷	123.43 ± 4.85	21.34 ± 0.22	205.56 ± 7.12	24.85 ± 0.15	1.34 ± 0.02	1.34 ± 0.01	3.20 ± 0.01	66.58 ± 0.78
	+	125.72 ± 1.86	21.31 ± 0.08	194.24 ± 14.96	24.43 ± 0.34	1.33 ± 0.05	1.14 ± 0.16	2.69 ± 0.43	54.40 ± 9.61
BG2	÷	115.08 ± 4.36	21.10 ± 0.16	187.48 ± 5.30	24.80 ± 0.19	1.23 ± 0.01	1.29 ± 0.03	2.98 ± 0.03	62.97 ± 1.57
	+	117.61 ± 4.26	21.22 ± 0.20	185.25 ± 6.15	24.76 ± 0.33	1.22 ± 0.01	1.20 ± 0.01	2.77 ± 0.01	57.53 ± 0.48
p-values	FeedIn (F)	0.286	0.207	0.364	0.900	0.008	0.518	0.648	0.495
	ProbTr (P)	0.977	0.619	0.724	0.726	0.882	0.323	0.371	0.305
	F × P	0.948	0.849	0.681	0.665	0.913	0.298	0.338	0.295

BG1, marine-based feed; BG5, plant-based feed; BG2, soybean meal-based feed; FeedIn, factor feed ingredients; ProbTr, factor probiotics; ÷, without probiotics; +, with probiotics. F × P, Interaction between feed type and probiotics. IW, initial weight; IL, initial length; FW, final weight; FL, final length; CF, condition factor; SGR, specific growth rate; TGC, thermal growth coefficient; WG%, weight gain in percentage. Values are expressed as means ± SEM of two replicates. The uppercase letters A, B and C (based on post-hoc results) represent significant differences ($p < 0.05$) among feed groups (BG1, BG5 and BG2). Interaction effect was not detected for any of the growth parameters.

Table 4
Atlantic salmon skin, gills and intestinal histomorphometric indices.

Tissues:		Skin		Gills		Intestine						
Parameters:		SME (ratio)	SNE (number /μm)	GME (ratio)	GNE (number /μm)	VH (μm)	VW (μm)	EH (μm)	LPW (μm)	NM (score)	IEL (score)	SNV (score)
Means of main effect:												
FeedIn	BG1	0.1573 ± 0.01	0.0010 ± 0.00^{A}	0.0327 ± 0.00^{A}	0.0005 ± 0.00^{A}	1144.59 ± 46.97^{C}	116.03 ± 3.73^{B}	57.35 ± 1.93^{C}	8.10 ± 0.46^{A}	$3\ (1.8)^{B}$	$5\ (1.0)^{B}$	$5\ (0.0)^{B}$
	BG5	0.1774 ± 0.01	0.0012 ± 0.00^{AB}	0.0481 ± 0.00^{B}	0.0007 ± 0.00^{B}	999.19 ± 31.57^{B}	103.52 ± 2.61^{A}	48.40 ± 1.23^{B}	6.64 ± 0.38^{A}	$4\ (1.3)^{C}$	$4\ (1.0)^{B}$	$5\ (0.0)^{B}$
	BG2	0.1712 ± 0.01	0.0013 ± 0.00^{B}	0.0612 ± 0.00^{C}	0.0010 ± 0.00^{C}	877.21 ± 50.65^{A}	105.03 ± 4.18^{A}	43.87 ± 1.79^{A}	18.31 ± 1.22^{B}	$2\ (1.0)^{A}$	$2\ (2.0)^{A}$	$1\ (1.0)^{A}$
ProbTr	÷	0.1660 ± 0.01	0.0011 ± 0.00^{X}	0.0375 ± 0.00^{X}	0.0006 ± 0.00^{X}	979.98 ± 37.83	107.96 ± 3.32	49.22 ± 1.43	12.38 ± 0.74^{Y}	$4\ (2.3)^{Y}$	$4\ (3.0)$	$5\ (1.8)^{X}$
	+	0.1713 ± 0.01	0.0012 ± 0.00^{Y}	0.0571 ± 0.00^{Y}	0.0009 ± 0.00^{Y}	1034.02 ± 48.30	108.43 ± 3.69	50.52 ± 1.87	9.66 ± 0.64^{X}	$2\ (1.0)^{X}$	$3\ (1.0)$	$5\ (3.0)^{Y}$
p-values	FeedIn (F)	0.149	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	ProbTr (P)	0.534	0.008	<0.001	<0.001	0.249	0.752	0.27	<0.001	<0.001	0.888	0.002
	F × P	0.789	0.452	0.366	0.263	0.083	0.603	0.448	0.003	0.008	<0.001	<0.001

BG1, marine-based feed; BG5, plant-based feed; BG2, soybean meal-based feed; FeedIn, factor feed ingredients; ProbTr, factor probiotics; ÷, without probiotics; +, with probiotics. SME, total area of skin mucous cells per total area of skin epithelium; SNE, number of skin mucous cells per total area of skin epithelium; GME, total area of gill mucous cells per total area of gill epithelium; GNE, number of gill mucous cells per total area of gill epithelium; VH, height of villi; VW, width of villi; EH, height of enterocyte; LPW, width of lamina propria; NM, number of intestinal mucous cells; IEL, number of intraepithelial lymphocytes; SNV, supra nuclear vacuoles. Significant differences ($p < 0.05$) among feed groups (BG1, BG5 and BG2) are indicated by uppercase superscripts A, B and C (based on the post-hoc tests for the group) and those between probiotic groups (without, ÷ and with, +) are indicated by X and Y in each column (based on the probiotic main effect). For interaction effects (F × P) and post-hoc results for each feed type, please refer to corresponding figures. Values for SME, SNE, GME, GNE, VH, VW, EH and LPW are presented as means ±SEM, n = 12 per treatment group. Parametric data were analysed by two-way ANOVA followed by Tukey's HSD test. The nonparametric score data (NM, IEL and SNV) were analysed with functions from ARTool package (nonparametric two-way ANOVA) followed by post-hoc tests using functions from emmeans package. Median, interquartile range (IQR) is reported for score data (NM, IEL, SNV).

interaction effect between feed ingredients and probiotics for the indices, GME or GNE.

3.2.3. Distal intestine histomorphometry

The morphological indices of the distal intestine, VH, VW, EH, LPW, NM, IEL and SNV, were differently affected by feed type (factor FeedIn) and probiotic treatment (factor ProbTr). In addition, the interaction (FeedIn×ProbTr) effects also depended on the indices. The marine-based feed group (BG1) had higher value for most of the indices except LPW (Table 4). The values for fish fed the plant-based feed (BG5) showed the same trend but ranked in between the values of the other two feed groups. Fish fed the feed with SBM (BG2) had the lowest values for most indices and had all the signs of enteritis (Table 4).

3.2.3.1. Height of villi (VH). The VHs of the feed groups were significantly different (Fig. 2A). Fish fed BG1 had longest villi, followed by fish fed BG5 and shortest villi was observed for fish fed BG2. Addition of probiotics did not alter the VH in fish fed BG1 and BG5, but VH tended to increase in the diet group, BG2+ (Fig. 2A). The interaction between feed ingredients and probiotics was not significant.

3.2.3.2. Width of villi (VW). Average VW for the fish fed BG1 was significantly higher compared to BG5 and BG2 (Table 4). Addition of probiotics did not alter the average VW in any of the feed groups (Fig. 2B). However, the probiotics tended to increase the VW in fish fed diets BG1+ and BG5+. The interaction FeedIn×ProbTr was not significant.

3.2.3.3. Height of enterocyte (EH). Feed type had a significant effect on the EH. Significantly shortest enterocytes were observed in fish fed BG2. The average EH in the fish fed BG1 was significantly 1.18- and 1.31-fold higher than BG5 and BG2, respectively (Fig. 2C). The probiotic treatment or interaction (FeedIn×ProbTr) did not have a significant effect on EH.

3.2.3.4. Width of lamina propria (LPW). The LPW was significantly affected by feed type and supplementation of probiotics. In addition, the two factors were found to interact with each other. Significantly wider lamina propria was observed in fish fed BG2 compared to BG1 and BG5. The LPW was significantly reduced in fish fed BG1+ and BG2+ while no changes were observed for fish fed the BG5+ (Fig. 2D).

3.2.3.5. Number of distal intestinal mucous cells (NM). The score for NM was significantly affected by feed ingredients (factor FeedIn) and probiotics (factor ProbTr) (Table 4 and Fig. 3A). Furthermore, the two factors were found to interact significantly (Table 4 and Fig. 3A). The scores for NM were significantly lower (more mucous cells, Supplementary Table 1) for fish fed BG2 compared to the other two feed groups. The NM per villus of fish fed diet groups BG1+ and BG5+ were significantly increased compared to the respective groups (BG1÷ and BG5÷) without probiotics. However, fish fed the BG2+ showed a decreased NM (higher score; less mucous cells). The percentage of the score for NM is shown in Fig. 3A.

3.2.3.6. Number of intraepithelial lymphocytes (IEL). The IELs were also significantly affected by feed ingredient composition (Table 4 and Fig. 3B). Although the factor ProbTr did not have an effect on IEL, the interaction of the factors was significantly different. The score for IEL was significantly lower (more IEL per simple villi, Supplementary table 1) for fish fed BG2 compared to those fed the other two feeds. The IEL score for fish fed diet groups BG1+ and BG5+ were significantly reduced compared to those without probiotics. However, the BG2+ group had a higher score (less IELs) compared to BG2÷ (Fig. 3B).

3.2.3.7. Supranuclear vacuoles (SNV). Fish fed BG1 and BG5 feeds had

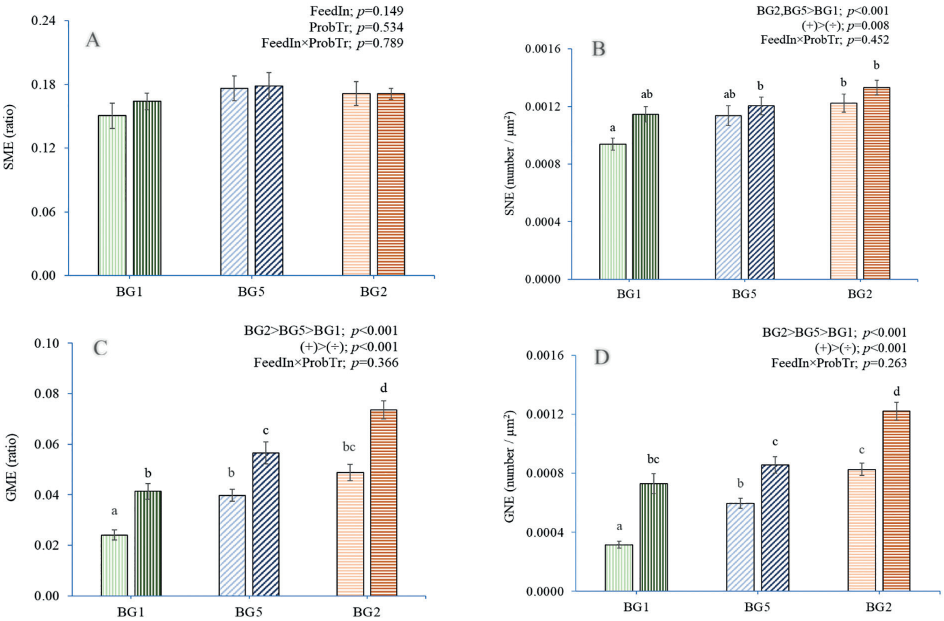

Fig. 1. Mucous cell-based indices associated with the skin and gills of Atlantic salmon offered different experimental feeds. (A) SME - the total area of mucous cells per total area of epithelium in the dorsal skin, (B) SNE - the number of mucous cells per total area of epithelium in the dorsal skin, (C) GME - the total area of mucous cells per total area of epithelium in the gills and (D) GNE - the number of mucous cells per total area of epithelium in the gills. BG1, BG5 and BG2 are marine-, plant- and soybean meal-based feeds, respectively. For each feed, light colour bar (left side) represents diet without probiotics (÷) and dark colour (right side) represents diet with probiotics (+). The effects of main factors (FeedIn and ProbTr) and their interaction (FeedIn×ProbTr) were determined by two-way ANOVA and *p* values are indicated in the upper right corner. Different lowercase letters denote significant difference ($p < 0.05$) among all diet groups; based on post-hoc (Tukey's HSD) tests. Values are presented as mean ± SEM.

larger SNVs along the entire apical part of the enterocytes in the villi. The SNV score (almost 5) of these two groups were not affected by probiotics (Table 4). On the other hand, the BG2÷ fed fish had the lowest score of 1 (almost no SNV in the enterocytes) and when the fish were fed probiotics we observed a significant increase in SNV. Scattered small SNV seemed to reappear in some enterocytes of the fish fed the diet BG2+ and it had received an average score of 1.7 out of 5 (Table 4 and Fig. 3C).

3.3. Gene expression

Relative expression of mucin genes in the skin, gills and intestine were found to be tissue specific (Table 5). The skin expressed *muc5ac1*, *muc5ac2* and *muc5b*. The gills expressed *muc5ac2* and *muc5b*. The distal intestine expressed only *muc2*. Relative expression of AMP genes in the skin and distal intestine of Atlantic salmon were also tissue specific. The skin expressed *def1* and *cathl1*. The distal intestine expressed *def3, def4* and *cathl1*. Results showed that feed ingredients (factor FeedIn), probiotics (factor ProbTr) and their interaction (FeedIn×ProbTr) affected the expression patterns of the genes in the 3 tissues differently. The results of the two-way ANOVA are presented in Table 5, and the effect of the interaction could be deciphered from Figs. 4–6.

3.3.1. Dorsal skin

The transcription of mucin and AMP genes in the dorsal skin were significantly affected either by feed ingredients or probiotics (either with or without an interaction effect), the exception was the expression of *def1* (Fig. 4D). Feed ingredient composition (main effect of the factor FeedIn) significantly altered the transcription of *muc5ac2* (Fig. 4B) and *cathl1* (Fig. 4E), but not those of the other two mucin genes. Fish fed BG1 and BG5 had significantly higher expression of *muc5ac2* and *cathl1*, respectively (Table 5, Fig. 4B and E). Probiotics significantly upregulated the expression of mucin genes in BG5 and BG2; *muc5ac1* (Fig. 4A, factor ProbTr) and *muc5b* (Fig. 4C, factor ProbTr). On the other hand, the expression of these two genes were downregulated in BG1+ fed fish. As for *cathl1*, fish fed diet BG2+ showed upregulation, while other diets tended to downregulate the expression of the AMP gene. The interaction (FeedIn×ProbTr) was significant for *muc5ac1* and *muc5b* as well as for the AMP gene, *cathl1*.

3.3.2. Gills

Expression of the mucin gene, *muc5ac2* in the gills was not significantly altered by either feed ingredient composition or probiotics (Fig. 5A). The mucin gene, *muc5b*, by contrast, was significantly affected by feed composition, and significantly higher expression was observed for the fish fed BG5 compared to BG2 (Table 5, factor FeedIn). When compared to fish fed plant-based feed (BG5), expression of *muc5b* was downregulated by 1.8-fold in fish fed SBM-based feed (BG2). Addition of probiotics to feed did not significantly affect the expression of *muc5b*. However, there was an upregulation (1.3-fold) and downregulation (1.4-fold) tendency in fish fed BG5+ and BG2+, respectively compared to BG5÷ and BG2÷ (Fig. 5B).

3.3.3. Distal intestine

Expression of the mucin gene, *muc2* was affected only by the feed ingredient composition (factor FeedIn). Fish fed BG2 had significantly lower mucin mRNA levels compared to the other two feed groups. Probiotics did not influence the mucin expression in any of the feed

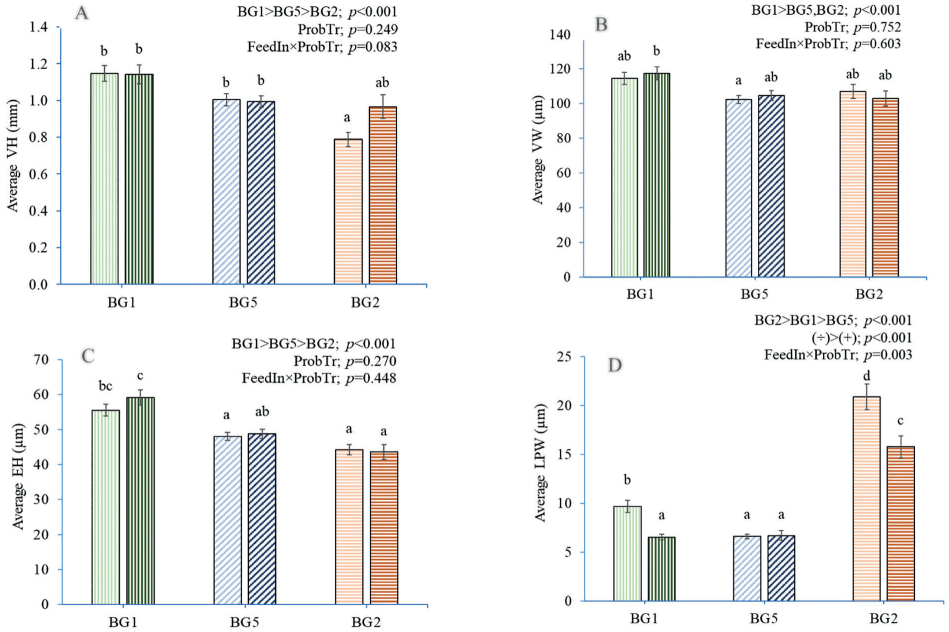

Fig. 2. Mucosa-based indices associated with the distal intestine of Atlantic salmon offered different feeds. (A) VH - height of villi, (B) VW - width of villi, (C) EH - height of enterocytes and (D) LPW - width of lamina propria. BG1, BG5 and BG2 are marine-, plant- and soybean meal-based feeds, respectively. For each feed, light colour bar (left side) represents diet without probiotics (÷) and dark colour (right side) represents diet with probiotics (+). The effects of main factors (FeedIn and ProbTr) and their interaction (FeedIn×ProbTr) were determined by two-way ANOVA and *p* values are indicated in the upper right corner. Different lowercase letters denote significant difference ($p < 0.05$) among all diet groups; based on post-hoc (Tukey's HSD) tests. Values are presented as mean ± SEM.

groups (Fig. 6A). Feed ingredient composition affected the expression of all AMP genes. Compared to other feed groups, fish fed BG2 had lower mRNA levels of *def3* (Fig. 6B) and *def4* (Fig. 6C, factor FeedIn). We observed an increasing trend in *def4* expression in the BG5+ fed fish (factor ProbTr; $p = 0.052$). However, fish fed BG1 had lower mRNA levels of *cathl1* (Fig. 6D). Supplementation of probiotics to the diet groups significantly influenced the AMP genes, especially *cathl1*. All probiotics-incorporated diet groups had significantly increased the expression of *cathl1* compared to their respective groups without probiotics. A significant interaction ($p = 0.056$) between feed ingredients and probiotics was observed for *def3*. The relative mRNA level of *def3* was upregulated in fish fed BG1+ and BG5+ compared to BG1÷ and BG5÷, while such a change was not observed for BG2. The mRNA level of *def4* was downregulated in fish fed BG1, while the mRNA levels in fish fed BG5 and BG2 were upregulated (not significantly; after probiotic feeding). The interaction between feed ingredients and probiotics (FeedIn×ProbTr) was not statistically significant.

3.4. Correlation between mucous cell indices and mucus-related gene expression data

Analysis of the data using Spearman correlation test revealed significant correlation between most of the histologically analysed mucous cell indices (Fig. 7). Significant positive correlations were observed for the following pairs: between SME and SNE ($r = 0.45$, $p < 0.001$), between GME and GNE ($r = 0.90$, $p < 0.001$). NM was positively correlated with GME ($r = 0.43$, $p < 0.001$) and GNE ($r = 0.50$, $p < 0.001$). Likewise, SNE was positively correlated with GME ($r = 0.43$, $p < 0.001$) and GNE ($r = 0.52$, $p < 0.001$). The correlations or the interactions among mucus-related genes from the skin, gills and intestine are also reported in Fig. 7. Significant correlation was also detected between histologically analysed mucous cells indices and most of the mucus-related gene data. SNE was positively correlated with skin *cathl1* ($r = 0.32$, $p = 0.007$) and negatively with skin *muc5ac2* ($r = -0.30$, $p = 0.015$). NM was positively correlated with intestinal *cathl1* ($r = 0.45$, $p = 0.001$) and negatively with intestinal *muc2* ($r = -0.38$, $p = 0.004$).

3.5. Short chain fatty acid composition

In total 7 short chain fatty acids were detected in the digesta and the sum of these SCFAs varied from 31 to 60 mmol/L, based on values from fish fed the different diets (Table 6). The total SCFAs were significantly affected by feed ingredient composition. Fish fed BG1 had significantly higher total SCFAs, followed by BG5 and BG2. Fish fed feeds without probiotics had significantly more total SCFAs than those with probiotics. The interaction between the two main factors (FeedIn×ProbTr) was not significantly different for the total SCFAs. Most of the individual SCFAs, except acetoacetic acids were significantly affected only by feed ingredients. Feeding with probiotics resulted in a significant reduction in acetoacetic acid and a tendency towards reduction in succinic acid ($p = 0.051$, Table 6) in digesta compared to those of fish fed diets without probiotics. Fish fed BG1 had more lactic acids, while BG5 feed groups had more acetoacetic acids in the digesta. Irrespective of feed groups, the concentration of butyric acid was the lowest among the determined SCFAs.

4. Discussion

Mucosal surfaces of the skin, gills and intestine with their inherent protecting capacity and arsenal of immune molecules are vital for the

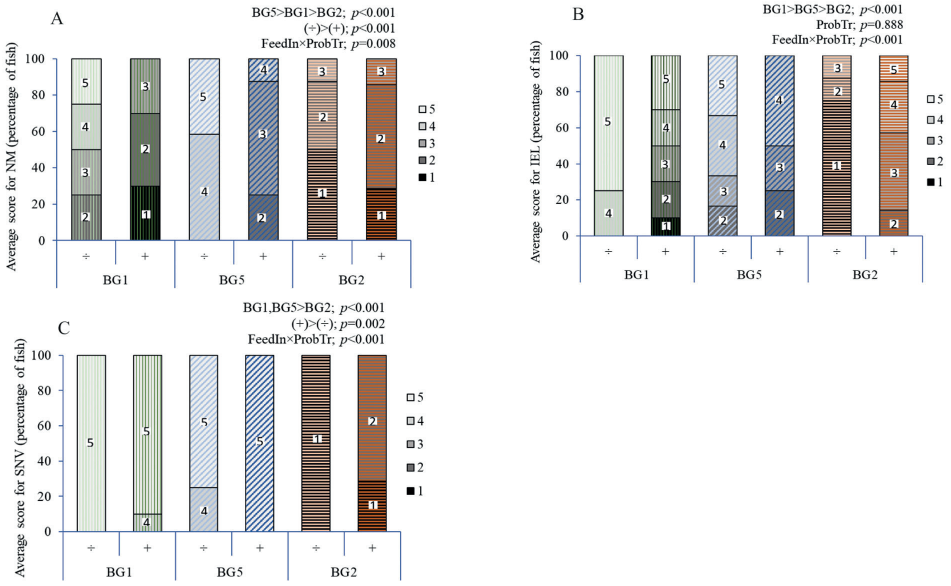

Fig. 3. Scores associated with the cells and cell feature of the distal intestine of Atlantic salmon offered different feeds. (A) NM - number of mucous cells, (B) IEL - number of intraepithelial lymphocytes and (C) SNV - supranuclear vacuoles per villi. BG1, BG5 and BG2 are marine-, plant- and soybean meal-based feeds, respectively. For each feed, light colour bar (left side) represents diet without probiotics (÷) and dark colour (right side) represents diet with probiotics (+). The effects of main factors (FeedIn and ProbTr) and their interaction (FeedIn×ProbTr) were determined by non-parametric two-way ANOVA and *p* values are indicated in the upper right corner. Scores are presented as percentage and legends indicate the scores. The labels on the stacked bar plots are the scores of a particular group.

health of fishes. A damaged mucosal surface in fish fails to effectively carry out its function, thereby making the fish susceptible to infectious diseases. The present study was designed to reveal the efficacy of both plant- or marine-based feeds and probiotics, by assessing the growth performance, morphology of the mucosal surfaces of the skin, gills and distal intestine, mucus-related gene expression in the aforementioned organs and SCFA composition in the digesta of Atlantic salmon. Overall, the present study showed that feed ingredient composition (FeedIn), probiotic treatment (ProbTr) and interaction between feed ingredients and probiotics (FeedIn×ProbTr) significantly affected the parameters of interest.

Use of probiotics isolated either from the GI (Ramesh et al., 2015) or mucus (Tapia-Paniagua et al., 2012) of aquatic animals could be considered as an efficient strategy to ensure sustainable aquaculture. In the present study, a mix of *Lactobacillus plantarum* R2 Biocenol™ (CCM 8674) and *Lactobacillus fermentum* R3 Biocenol™ (CCM 8675) were coated on the feeds. Earlier studies have indicated that a combination of two or more probiotic bacteria, including species from *Lactobacillus,* may improve growth and immune performance of the host aquatic animals (Alishahi et al., 2018; Beck et al., 2015; Foysal et al., 2020; Wang and Gu, 2010; Xu et al., 2012).

The LAB strains used in the present study were isolated from the intestinal content of rainbow trout (*Oncorhynchus mykiss*) and the bacteria were considered as probiotics based on the features, namely tolerance to different pH values, bile, temperature, antagonistic activity against salmonid pathogens such as *Aeromonas salmonicida* subsp. *salmonicida* CCM 1307 and *Yersinia ruckeri* CCM 6093 and the best growth properties *in vitro* (Fečkaninová et al., 2019). These probiotic strains have the potential for use in prevention, intervention or therapy of infections in aquaculture. Our previous study indicated that dietary supplementation with the two LAB strains modulated the composition and interaction of the intestinal microbiota of Atlantic salmon. *L. fermentum* feeding increased the bacterial diversity in the intestinal mucus of the fish (Gupta et al., 2019a). Among the LAB strains isolated from Chinese pickles, *L. fermentum* showed the most effective antibacterial activity against *Staphylococcus aureus* (Song et al., 2021). In a study with common carp, a diet supplemented with *L. fermentum* URLP18 at 2×10^8 CFU/g improved growth performance, non-specific immunity and health status and survival rate during a *Aeromonas hydrophila* challenge (Krishnaveni et al., 2021). Improved disease resistance was also demonstrated in tilapia fed *L. plantarum* prior to infecting the fish with the bacterial fish pathogen *Edwardsiella tarda* (Sherif et al., 2021). In the latter study, there was no difference in mortality between groups fed *L. plantarum* for 2 and 4 weeks prior to the infection with *E. tarda*. *L. plantarum* has also demonstrated a protective role in tilapia exposed to waterborne aluminum (Al) (Yu et al., 2017); the bacteria significantly increased feed utilization and growth performance, decreased the mortality of Al-exposed fish, reduced pathological conditions as well as Al accumulation in tissues. We did not include a challenge experiment as part of this study because our design was intended to investigate if there was any effect of supplementation of the two probiotics *L. fermentum and L. plantarum* in marine- or plant- derived feeds.

4.1. Effect of feed ingredients and probiotics on the growth performance

The 38-day long feeding study did not reveal any significant differences in most of the performance indices of the study groups. The plant-derived ingredients are approximately 3–6 times cheaper than fishmeal (The World Bank, 2021). Hence, our nonsignificant differences in the growth data indicate that cheaper non-marine source derived ingredients can impart the same growth in Atlantic salmon compared to marine-based ingredients. The lower condition factor of BG2 fed fish after 38 days of feeding can be in line with other studies that employed SBM in the diets of Atlantic salmon (Baeverfjord and Krogdahl, 1996;

Table 5
Gene expression in the skin, gills and intestine of Atlantic salmon.

Tissues:		Skin				Gills		Intestine			
Gene type:		AMPs	Mucins			Mucins		AMPs			Mucins
Parameters:		*def1*	*cath11*	*muc5ac1*	*muc5ac2*	*muc5b*	*muc5ac2*	*muc5b*	*def3*	*def4*	*cath11*	*muc2*
Means of main effect:												
FeedIn	BG1	0.63 ± 0.07	0.45 ± 0.06^{A}	0.43 ± 0.05	0.53 ± 0.07^{B}	0.30 ± 0.04	1.08 ± 0.09	0.12 ± 0.02^{AB}	1.05 ± 0.25^{AB}	1.10 ± 0.11^{B}	0.08 ± 0.03^{A}	2.75 ± 0.20^{B}
	BG5	0.79 ± 0.09	0.65 ± 0.06^{B}	0.48 ± 0.06	0.35 ± 0.06^{A}	0.38 ± 0.04	1.02 ± 0.08	0.18 ± 0.03^{B}	1.38 ± 0.34^{B}	0.87 ± 0.14^{A}	0.10 ± 0.04^{AB}	2.53 ± 0.24^{B}
	BG2	0.75 ± 0.06	0.44 ± 0.04^{A}	0.58 ± 0.10	0.35 ± 0.05^{A}	0.32 ± 0.05	0.91 ± 0.09	0.10 ± 0.02^{A}	0.40 ± 0.11^{A}	0.77 ± 0.08^{A}	0.12 ± 0.03^{B}	0.71 ± 0.05^{A}
ProbTr	÷	0.73 ± 0.07	0.50 ± 0.05	0.42 ± 0.06^{X}	0.37 ± 0.05	0.29 ± 0.04^{X}	1.01 ± 0.09	0.13 ± 0.02	0.59 ± 0.12	0.85 ± 0.11	0.04 ± 0.01^{X}	1.99 ± 0.15
	+	0.71 ± 0.07	0.53 ± 0.05	0.58 ± 0.08^{Y}	0.44 ± 0.06	0.37 ± 0.04^{Y}	0.99 ± 0.09	0.13 ± 0.02	1.30 ± 0.34	0.98 ± 0.12	0.16 ± 0.06^{Y}	2.00 ± 0.18
p-values	FeedIn (F)	0.089	<0.001	0.627	0.006	0.169	0.165	0.005	0.001	0.002	0.028	<0.001
	ProbTr (P)	0.803	0.283	0.014	0.159	0.021	0.927	0.458	0.065	0.052	<0.001	0.939
	F × P	0.827	0.005	<0.001	0.636	0.001	0.342	0.353	0.056	0.201	0.311	0.907

BG1, marine-based feed; BG5, plant-based feed; BG2, soybean meal-based feed; FeedIn, factor feed ingredients; ProbTr, factor probiotics; ÷, without probiotics; +, with probiotics. AMPs, Antimicrobial peptides; *def1, defensin1; def3, defensin3; def4, defensin4; cath11, cathelicidin1*; F × P, Interaction between feed type and probiotics. Significant differences ($p < 0.05$) among feed groups (BG1, BG5 and BG2) are indicated by uppercase superscripts A, B and C (based on post-hoc tests for the group), and between probiotic groups (without, ÷ and with, +) are indicated by the uppercase letters X and Y in each column (based on the probiotic main effect). For interaction effects and post-hoc results for each feed type, please refer to the corresponding figure. Values are presented as means ± SEM, n = 12 per treatment group. Data were analysed by two-way ANOVA followed by Tukey's HSD test.

Knudsen et al., 2007; Krogdahl et al., 2015; Sørensen et al., 2021; Urán et al., 2008b). A previous study showed that fish fed BG2 had morphological changes consistent with soybean meal-induced enteritis (Sørensen et al., 2021). This condition is associated with saponins in full fat soybean meal (Knudsen et al., 2007; Krogdahl et al., 2015). Heat treatment can dampen the action of heat stable antinutritional factors (ANFs) such as saponins, phytate, tannins, oligosaccharides, phytoestrogens (Drew et al., 2007; Liener, 1994). On the other hand, heat labile ANFs, typical proteins such as lectins and protease inhibitors are easily inactivated to safe levels during extrusion (Romarheim et al., 2006). All the experimental feeds were extruded prior to the LAB coating. Hence, we expect that heat labile ANFs in soybean meal were inactivated during extrusion, while the process did not remove the heat stable ANFs in the BG2 diet. Phytate is usually reduced through enzymatic treatment (Storebakken et al., 1998), and was most likely present in BG5. The duration of the experiment was too short to reveal effects of feed composition or probiotics on growth performance, in contrast to other studies that reported the ability of probiotics to improve growth performance, survival rate and health status of fish (Ramos et al., 2017; Wuertz et al., 2021; Xia et al., 2020).

4.2. Effect of feed ingredients and probiotics on histology and gene expression

4.2.1. Dorsal skin mucous cells, mucin and AMP genes

Histological evaluation of salmon skin indicated that the feed ingredients and probiotics evoked changes in the microscopic structure of the epidermis. We observed an increase in the number of skin mucous cells per unit skin epithelium area (SNE) when the fish were fed plant- (BG5) and SBM-based (BG2) feeds. The fish fed marine-based feeds (BG1) had a significantly lower SNE. Based on the findings from the study of the intestine, antinutritional factors present in the plant and SBM-based feeds can cause intestinal inflammation (Krogdahl et al., 2015), which in turn can activate the mucosal immune system of skin, as described in Sørensen et al. (2021). Such a connection between local immune systems has been shown in many cases. In humans, for example, intestinal bowel disease is known to cause disturbances in the host defence system and overstimulate certain immune pathways, and this response can lead to cutaneous disorders such as sub-epidermal blisters (Huang et al., 2012). The increased SNE by probiotics indicate an activation of skin mucosal response as described in Hernandez et al. (2010).

The area of skin mucous cells per unit area of skin epithelium (SME) was not significantly affected by feed ingredients or the probiotic treatment. Nevertheless, we observed a positive correlation between SNE and SME. Marine-based feed groups had less SNE, but apparently larger mucous cells. On the other hand, SBM-based feed groups had higher SNE, so apparently, smaller mucous cells. Mucous cells in the epidermis are essential for the production of mucus (Pittman et al., 2013), and mucus contains mainly mucins, which either bind to outer layer of epidermis and provide additional layer of defence to protect epithelial cells, or create viscous gel that prevents microbial penetration (Dang et al., 2020; Dash et al., 2018). Hyperplasia of skin mucous cells seems to be a general response to unfavourable physiological factors, like stress and low pH (Zuchelkowski et al., 1985, 1981), chemical factors, like high water aluminium levels (Ledy et al., 2003), high water nitrate levels and low dissolved oxygen (Vatsos et al., 2010), or biological factors, like pathogens (van der Marel et al., 2010).

Fishes are constantly in contact with pathogens (opportunistic or obligatory), and when pathogens invade the skin, the mucous cells that are located in the epidermis, will continuously secrete mucus to physically remove the pathogens from the surface (Karlsen et al., 2018; Peatman et al., 2015). In addition, AMPs present in fish mucus kill pathogens by interacting directly and disturbing the osmotic pressure in microbial cells (Mahlapuu et al., 2016; Raju et al., 2020). Administration of probiotics to the plant-based feed tended to downregulate the mRNA levels of AMPs while the SBM-fed group had higher expression of

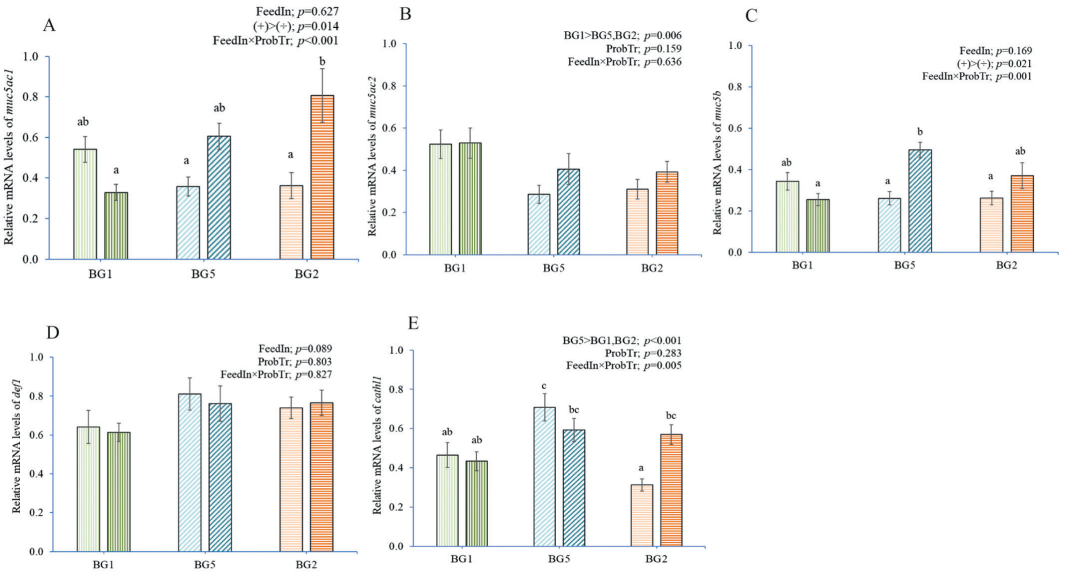

Fig. 4. Relative mRNA levels of mucin and antimicrobial peptide genes in the skin of Atlantic salmon fed different diets. (A) *muc5ac1*, (B) *muc5ac2*, (C) *muc5b*, (D) *def1* (*defensin1*) and (E) *cathl1* (*cathelicidin1*). BG1, BG5 and BG2 are marine-, plant- and soybean meal-based feeds, respectively. For each feed, light colour bar (left side) represents diet without probiotics (÷) and dark colour (right side) represents diet with probiotics (+). The effects of main factors (FeedIn and ProbTr) and their interaction (FeedIn×ProbTr) were determined by two-way ANOVA and *p* values are indicated in the upper right corner. Different lowercase letters denote significant difference ($p < 0.05$) among all diet groups; based on post-hoc (Tukey's HSD) tests. Values are presented as mean ± SEM.

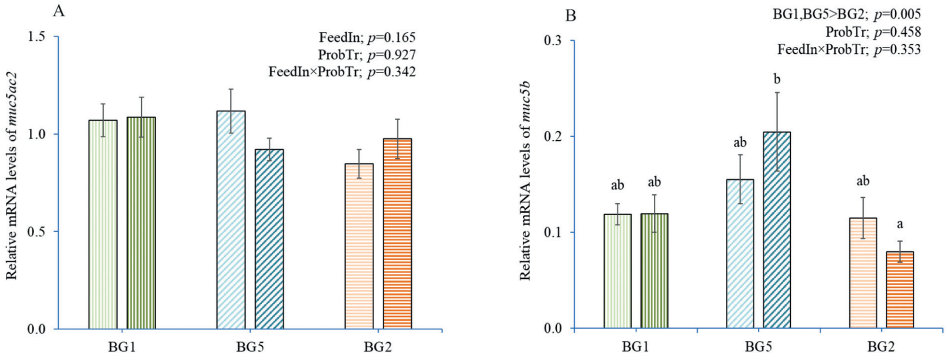

Fig. 5. Relative mRNA levels of mucin genes in the gills of Atlantic salmon fed different diets. (A) *muc5ac2* and (B) *muc5b*. BG1, BG5 and BG2 are marine-, plant- and soybean meal-based feeds, respectively. For each feed, light colour bar (left side) represents diet without probiotics (÷) and dark colour (right side) represents diet with probiotics (+). The effects of main factors (FeedIn and ProbTr) and their interaction (FeedIn×ProbTr) were determined by two-way ANOVA and *p* values are indicated in the upper right corner. Different lowercase letters denote significant difference ($p < 0.05$) among all diet groups; based on post-hoc (Tukey's HSD) tests. Values are presented as mean ± SEM.

AMP genes. Furthermore, the positive correlation between SNE and the skin *cathl1* suggests that probiotics might have influenced both the AMP gene expression and the number of skin mucous cells.

As for the mucin gene, *muc5ac2* it was downregulated in fish fed plant- and SBM-based feeds; this result is not in line with the histological observation of increased number of mucous cells in the skin. Although the probiotic supplementation did not influence the expression of a gel-forming mucin gene, *muc5ac2* in any of the diet groups, the combination of plant-based, or SBM-based feeds and probiotics caused an upregulation of two other gel-forming mucin genes, namely *muc5ac1* and *muc5b*. This observation agrees with the result on increased number of mucous cells observed in skin epidermis.

4.2.2. Gills mucous cells and mucin genes

Pathogens can increase the gill mucous cell number and mucus production (Andrews et al., 2010; Lødemel et al., 2001). Hyperplasia and hypertrophy of gill mucous cells are general responses to external stimuli (Dang et al., 2020, 2019; Haddeland et al., 2020). A correlation

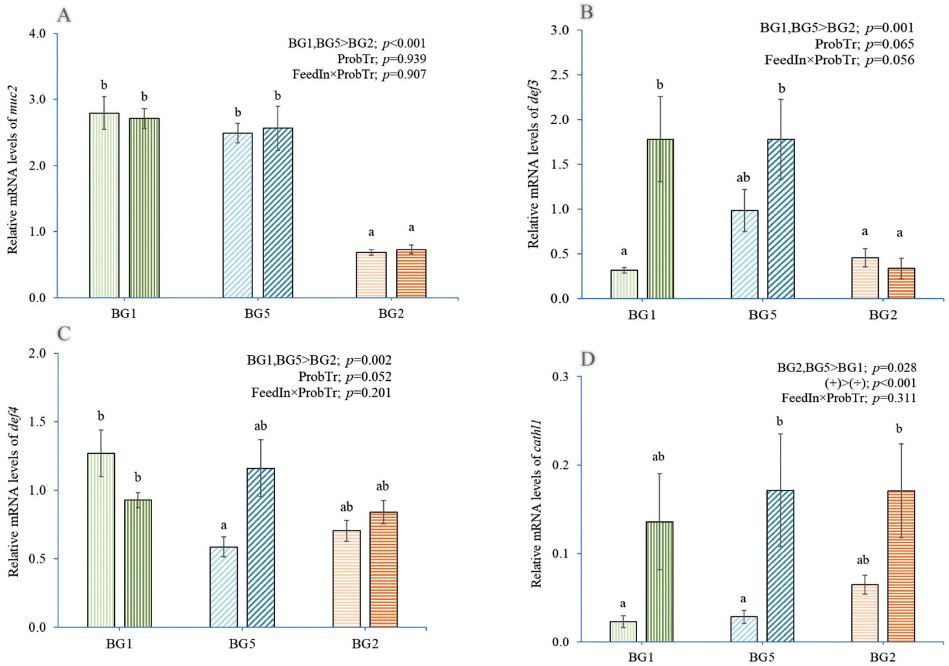

Fig. 6. Relative mRNA levels of mucin and antimicrobial peptides genes in the distal intestine of Atlantic salmon fed different diets. (A) *muc2*, (B) *def3* (*defensin3*), (C) *def4* (*defensin4*) and (D) *cathl1* (*cathelicidin1*). BG1, BG5 and BG2 are marine-, plant- and soybean meal-based feeds, respectively. For each feed, light colour bar (left side) represents diet without probiotics (÷) and dark colour (right side) represents diet with probiotics (+). The effects of main factors (FeedIn and ProbTr) and their interaction (FeedIn×ProbTr) were determined by two-way ANOVA and *p* values are indicated in the upper right corner. Different lowercase letters denote significant difference ($p < 0.05$) among all diet groups; based on post-hoc (Tukey's HSD) tests. Values are presented as mean ± SEM.

between number of mucous cells and the mucus secretion was documented by Bosi et al. (2005).

In the present study, our analyses detected significant effects of both feed ingredients and probiotics on the gill mucous cells. The parameter GME was used to assess the total area of gill mucous cells that cover unit area of gill epithelium. For fish fed marine-based feed without probiotics (BG1÷), it was 0.024, indicating that 100 μm^2 of gill epithelium is covered by 2.4 μm^2 of mucous cells. For the plant-based (BG5÷) and SBM (BG2÷)- based groups, GME values were 0.039 and 0.049, respectively. Probiotic incorporation in all the three feeds - marine-, plant- and SBM-based - significantly increased the GME by 1.7, 1.4 and 1.5 times than their corresponding groups without probiotics (BG1÷, BG5÷ and BG2÷), respectively. Having more mucous cells is linked to better disease resistance in the case of amoebic gill disease (Roberts and Powell, 2005). Therefore, probiotics used in our study have shown their potential to be included among the candidates that can be utilized in aquaculture disease prevention.

Histomorphometric analysis of fish gills showed a similar trend for GNE and GME. Furthermore, there was a positive correlation between GME and GNE, indicating that the GME might have increased due to increased GNE. GNE indicates the number of mucous cells per unit area of gill epithelium. Fish fed feeds without probiotics (÷) had lower value for GNE compared to feed groups with probiotics (+). Fish fed the marine-based feed (BG1÷) had on average 300 mucous cells per mm^2. For fish fed the plant (BG5÷)- and SBM-based feed (BG2÷), the GNE were 2 and 3 times higher compared to marine-based feed (BG1÷) groups, respectively. The feeds BG1+, BG5+ and BG2+ increased the number of gill mucous cells per unit area of gill epithelium (GNE) by 2.3, 1.4 and 1.5 times, respectively, compared to the respective fish groups fed feeds without probiotics. The dietary administration of probiotics might have altered the metabolism in the intestine and the metabolites (bile acids, lipoproteins, amino acids and SCFAs) might have translocated through blood to the gills, thus the increased response (Martin et al., 2007).

The relative mRNA levels of *muc5ac2* in the gills were unaffected by feed ingredients and probiotics. The lower expression of *muc5b* in fish fed BG2 indicates the gill health marker potential of the gene. Dietary administration of probiotics to BG2÷ feed groups further downregulated the expression of the mucin gene *muc5b*. However, an upregulation pattern was observed for fish fed BG5. A study has revealed that the number of goblet cells in the airway epithelium of rats increased and there was a subsequent increase in the expression of the mucin genes *muc5a* and *muc5b* (Kim et al., 2019). The significant positive correlation between gill mucous cell indices (GME and GNE) and other two mucous cell indices in the skin (SNE and SME) indicates the relationship between mucosal tissues in different organs and their response to different feed ingredients and probiotics. However, gill mucin gene expression results did not significantly correlate with histological observations related to gills (Fig. 7).

4.2.3. Distal intestinal morphology, mucin and AMP genes

The height of the simple villi differed among the feed groups and this observation is in line with earlier studies (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2015; Moldal et al., 2014; Sohrabnezhad et al., 2017; Urán et al., 2009). Intact and longer villi are associated with more enterocytes, higher enzyme production, and improved absorption of

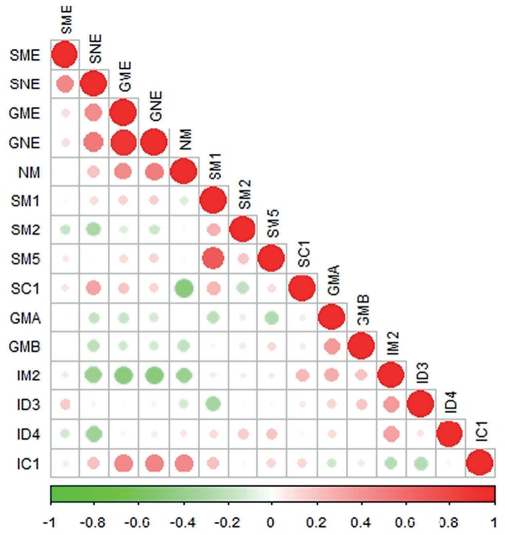

Fig. 7. Plot showing the correlation for all the combinations of histologically evaluated mucous cell parameters and the selected mucus-related genes. Skin *defensin1* is not shown because the correlation is not significant. Significant correlations ($p < 0.05$; Spearman rank correlation test) are shown using circles. Positive correlations are indicated by shades of red, and negative correlations are shown by shades of green. Blank cells indicate non-significant correlations between the variables. SME, the ratio between total area of mucous cells and total area of epithelium in the dorsal skin. SNE, the ratio between number of mucous cells and total area of epithelium in the dorsal skin. GME, the ratio between total area of mucous cells and total area of epithelium in the gills. GNE, the ratio between number of mucous cells and total area of epithelium in the gills. NM, number of intestinal mucous cells. SM1, skin *muc5ac1*. SM2, skin *muc5ac2*. SM5, skin *muc5b*. SC1, skin *cathelicidin1*. GMA, gill *muc5ac2*. GMB, gills *muc5b*. IM2, intestine *muc2*. ID3, intestine *defensin3*. ID4, intestine *defensin4*. IC1, intestine *cathelicidin1*. *(For interpretation of the references to colour in this figure legend, the reader* should*referthe web version of this article.)*

nutrients (Caspary, 1992). Wild caught post-smolt Atlantic salmon weighing 120 g was reported to have villi of height 0.7 mm (Løkka et al., 2013). In the present study, fish fed the marine-based diet had the longest villi (on average 1.1 mm), but SBM-based feed reduced the height to 0.87, due to inflammation. Feeding the fish with probiotics increased the villi height in the SBM fed group. This result is in line with earlier studies that reported improved intestinal structure and immunity in tilapia fed lactic acid bacteria (Pirarat et al., 2011) and increased villi height in rainbow trout fingerlings fed probiotic-supplemented (*Bacillus cereus*) diet (Gisbert et al., 2013). Even in piglets, probiotic feeding increased villi height (Gāliņa et al., 2020). The lactic acid bacteria, *Pediococcus acidilactici* in combination with short chain fructooligosaccharides increased villi height in the anterior intestine of Atlantic salmon reared in sea cages (Abid et al., 2013). The two probiotic strains used in the present experiment might have colonized the intestine, as noted in a previous study (Gupta et al., 2019a) and fermented the oligosaccharides to produce more total SCFAs, thus improving the villi structure. The fish fed marine-based feed had significantly wider villi compared to the other two feed groups, and the administered probiotics did not affect the villi width in Atlantic salmon, as observed in rainbow trout fed probiotic-supplemented (*Bacillus cereus*) feed (Gisbert et al., 2013). Average width of villus was calculated based on measurements taken at 5 different locations of a villus (Supplementary Fig. 3). Although the fish fed SBM-based feed had widened villi, quantitative measurements revealed that the villi width of plant-based feed group was similar to that of SBM-based feed group. Width of the villi was calculated considering both height of enterocytes (two sides) and width of lamina propria. It should be noted that in the fish fed the SBM-based feed, the height of enterocytes decreased while the width of the lamina propria increased. Therefore, we did not observe any significant differences in the overall width in fish fed the plant- and the SBM-based feeds. Nonetheless, studies that assessed SBM-induced enteritis reported widening of villi width (Moldal et al., 2014); based on semi-quantitative scoring. Our findings suggest that width of villi cannot solely be used as an index to quantify the morphological changes in the distal intestine. Other indices like height of the enterocytes and width of the lamina propria should also be included.

The present study has also evaluated the height of the enterocytes (columnar epithelium) in the distal intestine of Atlantic salmon. Marine-based feed in the present study provided essential nutrients including amino acids to the fish so that the columnar epithelium can develop

Table 6
Short-chain fatty acid concentration (mmol/L) in the digesta of the study groups.

Parameters:		Formic acids	Acetoacetic acids	Lactic acids	Succinic acids	Acetic acids	Propionic acids	Butyric acids	Total acids
Means of main effect:									
FeedIn	BG1	4.83 ± 0.27^{C}	10.99 ± 0.59^{A}	16.73 ± 0.65^{C}	9.63 ± 0.52^{B}	12.50 ± 0.72^{B}	3.83 ± 0.42^{B}	2.56 ± 0.60^{B}	59.93 ± 1.55^{C}
	BG5	3.14 ± 0.31^{B}	15.04 ± 0.78^{B}	6.73 ± 0.45^{B}	5.99 ± 0.34^{A}	8.09 ± 0.64^{A}	2.56 ± 0.26^{A}	0.86 ± 0.07^{A}	41.92 ± 1.14^{B}
	BG2	2.42 ± 0.20^{A}	10.04 ± 0.46^{A}	4.59 ± 0.36^{A}	5.52 ± 0.24^{A}	9.40 ± 0.90^{A}	2.32 ± 0.27^{A}	1.12 ± 0.14^{A}	34.56 ± 1.95^{A}
ProbTr	÷	3.58 ± 0.30	12.45 ± 0.79^{Y}	9.09 ± 0.43	7.39 ± 0.38	10.52 ± 0.86	3.06 ± 0.36	1.94 ± 0.40	47.21 ± 1.56^{Y}
	+	3.35 ± 0.22	11.59 ± 0.43^{X}	9.75 ± 0.55	6.71 ± 0.35	9.48 ± 0.65	2.75 ± 0.27	1.08 ± 0.15	43.73 ± 1.53^{X}
Means of interaction effect:									
BG1	÷	5.14 ± 0.43^{d}	10.23 ± 0.62^{b}	16.12 ± 0.68^{d}	10.00 ± 0.40^{b}	12.70 ± 0.87^{b}	4.24 ± 0.52^{b}	3.01 ± 0.89	60.24 ± 1.35^{d}
	+	4.51 ± 0.11^{cd}	11.75 ± 0.55^{b}	17.78 ± 0.63^{d}	9.27 ± 0.64^{b}	12.30 ± 0.57^{b}	3.42 ± 0.32^{ab}	2.10 ± 0.32	59.61 ± 1.75^{d}
BG5	÷	3.53 ± 0.36^{bc}	14.90 ± 1.1^{cd}	6.65 ± 0.36^{bc}	6.19 ± 0.40^{a}	9.03 ± 0.69^{a}	2.59 ± 0.30^{a}	1.72 ± 0.14	44.10 ± 1.41^{c}
	+	2.76 ± 0.27^{ab}	15.18 ± 0.47^{d}	6.82 ± 0.53^{c}	5.80 ± 0.27^{a}	7.15 ± 0.58^{a}	2.54 ± 0.22^{a}	NA	39.74 ± 0.86^{bc}
BG2	÷	2.08 ± 0.12^{a}	12.23 ± 0.66^{bc}	4.51 ± 0.24^{a}	5.99 ± 0.33^{a}	9.81 ± 1.02^{ab}	2.36 ± 0.26^{a}	1.11 ± 0.16	37.29 ± 1.92^{ab}
	+	2.77 ± 0.28^{ab}	7.85 ± 0.26^{a}	4.66 ± 0.48^{ab}	5.06 ± 0.15^{a}	8.99 ± 0.79^{a}	2.29 ± 0.28^{a}	1.14 ± 0.12	31.83 ± 1.98^{a}
p-values	FeedIn(F)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.002
	ProbTr (P)	0.472	0.048	0.240	0.051	0.106	0.276	0.492	0.010
	F × P	0.013	<0.001	0.460	0.214	0.616	0.458	0.383	0.291

BG1, marine-based feed; BG5, plant-based feed; BG2, soybean meal-based feed; FeedIn, factor feed ingredients; ProbTr, factor probiotics; ÷, without probiotics; +, with probiotics. F × P, Interaction between feed type and probiotics. NA, No data available. Valeric acid was tested, but not detected in any of the diet groups. The uppercase letters A, B and C (based on post-hoc tests) represent significant differences ($p < 0.05$) among feed groups (BG1, BG5 and BG2); and the uppercase letters X and Y (based on the probiotic main effect) represent significant differences between the probiotic groups (without, ÷ and with, +). Significant differences ($p < 0.05$) among all groups are indicated by different superscripts (a, b, c, or d; post hoc results for each feed type) in each column. Values are mean ± SEM, n = 10 per treatment group. Data were analysed by two-way ANOVA followed by Tukey's HSD test.

properly without any height-associated defects. When compared to marine-based feed, on average, a nine and 13.5 μm reduction in the height of intestinal epithelium was observed in the fish fed plant- and SBM-based feed, respectively. Indeed, enterocytes of the distal intestine of Atlantic salmon are the first cells that are affected when the fish are fed SBM-based diets (Urán et al., 2008a, 2008c). The present study quantitatively confirmed that the intestinal epithelium height was reduced in fish fed plant- and SBM-based ingredients.

Previous studies have assessed the width of the lamina propria by semi-quantitative scoring (Knudsen et al., 2007). The present study has used a quantitative approach and found a widening of lamina propria in fish fed SBM-incorporated feed. SBM-induced enteritis causes widening of the central stroma of the mucosal folds (Baeverfjord and Krogdahl, 1996). The present study showed that administration of probiotics significantly reduced the width of the lamina propria in fish fed BG1+ and BG2+. Probiotics also reduced the number of intraepithelial lymphocytes in the group fed BG2+ that had intestinal inflammation. In contrast, feeding *P. acidilactici* was found to increase the number of intraepithelial lymphocytes in Atlantic salmon (Vasanth et al., 2015). The present study also suggests that probiotics alleviate the progression of inflammation caused by SBM. A possible mechanism could be that probiotics reduce the lamina propria width possibly by suppressing the influx of the inflammatory cells. Other studies with mammals have also shown improved intestinal tight-junction and barrier function via modulation of protein components (Sultana et al., 2013; Yang et al., 2016). However, further research is needed to assess the inflammatory response markers.

In the present study, acid and neutral goblet cells were found scattered among the intestinal epithelial cells of fish fed BG1 and BG5. Administration of probiotics to these groups further increased the number of mucous cells. This is in line with other studies in fish that reported increased proliferation and differentiation of goblet cells and a consequent increase in mucus secretion in seabream fed *L. fermentum* (Dawood et al., 2015). Furthermore, *L. rhamnosus* or *P. acidilactici* feeding was found to increase the number of mucous cells in tilapia intestine (Pirarat et al., 2011; Standen et al., 2013). Moreover, higher goblet cell density was reported in rainbow trout fed *Spirulina platensis* (Sheikhzadeh et al., 2019). Dietary and oral administration of probiotic was also found to increase the number of goblet cells in the intestine of mice (El Aidy et al., 2013), piglets (Gāliņa et al., 2020; Zhang et al., 2017) and pigs (Desantis et al., 2019).

A possible mode of action of probiotics is that they colonize the mucus and make use of the mucin molecules as carbon, nitrogen and energy sources (Meslin et al., 1999). They release end-products of mucus fermentation, different secretory metabolites, and bioactive factors, which activate diverse signalling cascades and secretory elements that affect goblet cells. Members of microbiota can release proteolytic enzymes like meprin β from the apical membrane of enterocyte. Meprin β helps in the detachment of mucus from goblet cells and the metalloprotease cleaves the N-terminal region of the MUC2 mucin (Derrien et al., 2010; Schütte et al., 2014). Moreover, probiotic structural elements, such as lipopolysaccharides, flagellin A, and lipoteichoic acids or several metabolites (adenosine triphosphate) can regulate mucin gene expression by affecting the host immune responses (Dharmani et al., 2009).

The increased number and aggregated mucous cells in fish fed SBM-based feed (BG2) may be a general response to inflammation. Interestingly and in contrast to the observations in the skin and gill histomorphometry, the group fed the SBM-based diet supplemented with the probiotics (BG2+), did not show a further increase in the number of mucous cells, compared to BG2÷ but a decrease. A possible explanation for this observation, could be that feeding fish with SBM and probiotics, both factors that tend to increase the production of mucus, for a prolonged period of time, could have led to a depletion of the mucous cells. This has been observed in many cases of chronic intestinal inflammation, wherein the initial increased mucus production was markedly decreased after a while (Dharmani et al., 2009; Kim and Ho, 2010). However, both BG5+ and BG2+ had almost similar scores linked to mucous cells. This indicates a potential interaction of the probiotics and the different feed ingredients on the number of mucous cells.

In the present study, the *muc2* expression in the distal intestine was not altered by the administration of probiotics. The upregulation of the AMP genes *def3*, *def4* and *cathl1* with the administration of probiotics suggests increased immune responses (Rakers et al., 2013). Moreover, significant positive correlation between *cathl1* and NM indicates that feed ingredients or probiotics influenced the AMP gene, *cathl1* and increased the mucous cells number in the intestine of Atlantic salmon. Intestinal mucin gene *muc2* was positively correlated with skin *cathl1* ($r = 0.24$, $p = 0.051$) and gill *muc5ac2* ($r = 0.28$, $p = 0.018$), indicating the association of the mucosal areas in different mucosal tissues (Fig. 7). In colonic murine mucosa, *cathelicidin* gene was upregulated by bacterial DNA through Toll-like receptor-mediated pathway (Koon et al., 2011). Intestinal inflammation breaks the mucosal barrier, which in turn gives way for opportunistic bacteria to translocate into the intestinal layers (Vrakas et al., 2017). *Cathelicidin*s were upregulated in the inflamed intestine of Atlantic salmon fed soy saponin (Kiron et al., 2020). It is also stated that gastrointestinal tract disorders can be treated through supplementation of *cathelicidin* peptides (Chow et al., 2013). Hence, probiotic-induced *cathl1* can be considered as a strategy to counteract intestinal inflammation.

Supranuclear vacuoles in the distal intestinal enterocytes of Atlantic salmon appear approximately 54 days post hatch (Sahlmann et al., 2015). Macromolecules like proteins are taken up via pinocytosis in epithelial cells, and some intracellular proteins like ferritin ends up in supranuclear vacuoles (Elbal et al., 2004; He et al., 2012; Rombout et al., 1985). Endocytic vesicles and lysosomes fuse, and subsequently ferritin digestion occurs in the SNVs. Accumulation of SNV in distal intestinal enterocytes of Atlantic salmon has also been reported previously (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003; Sanden et al., 2005; Urán et al., 2008a). The marine-based feed group in the present study had large SNVs along the entire apical part of the distal intestinal enterocytes. In the present study, fish fed the plant-based feed also had similar characteristics. However, the fish fed SBM-based feed developed enteritis and lacked SNVs. Such anomalies have also been reported by other authors (Bakke-McKellep et al., 2000; Krogdahl et al., 2003; Krogdahl and Bakke-McKellep, 2005; Nordrum et al., 2000; Urán et al., 2009). Disappearance of SNVs is associated with reduced endocytosis or uptake block (Urán et al., 2008c), corroborating with the results on the absence of small SNVs. Probiotic feeding in the present study helped in the reappearance of SNVs in the enterocytes of the fish. Thus, we suggest new modes of action of probiotics on the host health; enhancing endocytosis and aiding in subsequent reappearance of the SNVs.

4.3. Effect of feed ingredients and probiotics on short chain fatty acids

Intestinal microbiota utilizes dietary ingredients especially certain fibres, and by fermenting them they produce SCFAs which are absorbed by the intestinal epithelium of fish. Of these SCFAs, butyrate is utilized by the intestinal epithelial cells as an energy source, propionate is taken up by liver and high levels of acetate can be detected in blood (Louis and Flint, 2017). The SCFAs improve growth and health of the fish because they reduce the luminal pH, avoid infections, strengthen immune system and maintain mucosal integrity (Adorian et al., 2020; Guillon and Champ, 2000; Hoseinifar et al., 2017; Park and Floch, 2007). Supplementation of dietary fibres, which can be utilized by microbiota to produce SCFAs (e.g. acetic and butyric acid) in the digesta, was shown to influence the intestinal mucous cells as well as skin mucus production in fish (Adorian et al., 2020).

In the present study, plant- and SBM-based feeds were expected to provide more fibres than marine-based feed but did not result in more SCFAs. It has been reported that there is no linear correlation between dietary fibre and SCFA concentration in rat cecum (Den Besten et al.,

2013; Levrat et al., 1991). Moreover, administration of probiotics was also expected to influence the microbiota because one of the probiotics (*Lactobacillus*) was found to be a core member in Atlantic salmon (Gupta et al., 2019a). Thus, feed ingredient composition and probiotics were expected to alter the SCFA profile. The level of SCFAs in salmon faeces was in the same range as reported for rats (Campbell et al., 1997). In the rat study, the SCFAs ranged from 36 to 61 mmol/L depending on the intake of fibre, but the ratio between the acetate:propionate:butyrate remained the same. We also observed a significant reduction in total SCFAs in the plant- (BG5) and SBM (BG2)-based feed consumed fish compared to the marine-based feed group. The ratio between acetic: propionic acids was in the range 3 to 4.16; lower in the BG1 fed fish and higher for the BG2 fed group. The ratio between propionic:butyric acids was between 1.41 and 2.1. Marine-based and plant or SBM-based diet can shape the SCFA profile differently; we observed a shift in dominant SCFA (from lactic acid to acetoacetic acid). The plant or SBM-based feeds, BG5 and BG2 contained more long fibres rather than oligosaccharides and type of fibre is known to affect the formation and profile of SCFAs. Fish fed plant or SBM-based ingredients had lower faecal dry matter content (Sørensen et al., 2021), which could explain the lower concentration of SCFAs.

In humans, acetate, propionate and butyrate account for 85–95% of the SCFAs and acetic acid alone accounts for more than 50% (Markowiak-Kopeć and Śliżewska, 2020). Studies of human microbiota have revealed the relationship between SCFAs and microbiota, and intestinal microbiome balance maintenance and microbial metabolite production stimulation by probiotic microorganisms (Markowiak-Kopeć and Śliżewska, 2020; Tsukuda et al., 2021). Acetic acid was a dominant SCFA in the present experiment also, but lactic acid and acetoacetic acid were higher in fish fed BG1 and BG5, respectively. Research with rats has also shown that SCFAs are involved in MUC gene transcription and thickness of mucous layer; feeds that provide more SCFAs, but low proportion of butyrate, help in forming thicker mucous layer in the colon (Hedemann et al., 2009). In line with this, our experiment showed the best gut health in fish fed BG1, producing the highest concentration of total SCFAs, with a rather high concentration of acetic acid. The SCFAs provide energy to the intestinal epithelium cells and stimulates the release of gastrointestinal peptide or growth factors which may affect cell proliferation, thereby increasing villi height (Blottiere et al., 2003; Pelicano et al., 2005). Although we observed only lower concentration of butyric acid in the digesta, the SCFA may still have an important role for intestinal health in Atlantic salmon. In the present study, administration of probiotics had significantly reduced the total SCFAs as well as the acetoacetic acid concentration in digesta. Campbell et al. (1997) observed the lowest concentration of SCFAs in the intestine of rats fed cellulose instead of short chain fibres. A noteworthy observation in the present study was that the probiotic administration tended to reduce the total SCFAs ($p = 0.010$), mainly because of a significant reduction in acetoacetic acids in fish fed the BG2 diet. However, further research should confirm how probiotics could shift the microbiota profile in the host intestine.

5. Conclusion

The present study has shown that number of mucous cells in the dorsal skin, gills and distal intestine were affected by feed ingredient composition and probiotics. Appearance of many mucous cells can be interpreted as an overall immune response to intestinal inflammation. The distal intestinal histomorphology of fish was influenced by the feed ingredient composition. Intestinal indices of fish fed plant-based feed was almost similar to that of fish fed marine-based feed. However, fish fed SBM-based feed developed enteritis. Addition of probiotics to SBM-based feed groups did not completely prevent the development of enteritis. However, positive responses like increased villi height, reduced width of lamina propria, reduced number of intraepithelial lymphocytes and reappearance of supra nuclear vacuoles were observed in Atlantic salmon post-smolts. Expression of mucin and AMP genes were tissue specific and the mRNA levels were affected by feed ingredient composition and probiotics. Correlation between mucous cell histomorphometric indices and gene expression data suggests that feed ingredients or probiotics influence both the mucus cell counts and mucus-related gene expression. Moreover, short chain fatty acid composition was also altered. In order to boost innate immune response and enhance intestinal health, the probiotics employed in the present study can be incorporated in marine- and plant-based feed without compromising fish growth. Although probiotics tended to alleviate the feed induced inflammation, further knowledge should be acquired if these probiotics are to be used as supplements in SBM-based salmon feed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was funded by Nord University, Faculty of Biosciences and Aquaculture and the Slovak Research and Development Agency under the contract No. APVV-19-0234. The research project was a continuation of the project “Blodanalyser av laks som metode for vurdering av tarmhelse” partially sponsored by MABIT with grant number AF0082. The authors would like to acknowledge the skilful technical assistance of Ghana K Vasanth and Dalia Dahle during the execution of the study. The support of the staff at the Research Station of Nord University is also highly appreciated.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.737516.

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Yousri Abdelmutalab Ahmed Abdelhafiz
Insights into the bacterial communities of Nile tilapia – core members and intergenerational transfer
ISBN: 978-82-93165-41-5

No. 43 (2022)
PhD in Aquatic Biosciences
Fredrik Ribsskog Staven
Interaction studies on lumpfish exposed to Atlantic salmon: behavioural observations and the underlying physiological and neurobiological mechanisms
ISBN: 978-82-93165-42-2

Atlantic salmon is a globally important aquaculture species, and the feed ingredients and diets used to farm salmon have been slowly changing over the past decades. Much of the marine ingredients have been replaced with plant protein concentrates and plant lipids. Novel ingredients such as microalgae, and feed additives such as probiotics, have been studied for possible fish health benefits. The mucosal surfaces of salmon, including the skin, gills, and intestine tract, are potentially affected by the fish diet. These mucosal surfaces have important barrier and immune functions that are vital for fish health and growth. In the present thesis, we performed feeding experiments wherein Atlantic salmon were fed diets containing various combinations of marine and plant ingredients, microalgae, and probiotics, and measured the fishes' mucosal health by several parameters and methods, including histology and gene expression analysis. In addition, two different approaches towards improving microalgae utilization were tested. Our results show that some plant ingredients negatively affect the intestinal health of Atlantic salmon, and that probiotics can improve the fishes' mucosal health. This thesis contributes important knowledge towards understanding the connections between fish diets and fish mucosal health, which will promote the continuous improvement of fish health in salmon aquaculture.

NORD University

ISBN: 978-82-93165-43-9
Trykk: Trykkeriet, Nord universitet
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