Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Science

Philosophiae Doctor (PhD)
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Gut health and microbiota during the life cycle of Atlantic salmon (*Salmo salar*) produced under commercial Arctic conditions: Modulation by functional feed ingredients investigated *in vivo* and *in vitro*

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SUMMARY

A consequence of the foreseen increase in demand for Atlantic salmon (*Salmo salar*) is search of new production sites. In Norway, new production sites are available in the Arctic areas, such as Finnmark. As most studies of biology and requirements of Atlantic salmon have been conducted in more southerly areas, the present knowledge on how extreme variation in photoperiod, very low average temperature and specific pathogens occurring in the Arctic areas are influencing fish in general and, in this context gut health and microbiota in particular, is limited. Functional feed ingredients are commonly used in diets for fish produced to improve fish health and disease resistance in particular during stressful farming conditions. They are claimed to have positive effects on gut functions and health, at least under certain conditions, via direct or indirect actions on the function of the intestinal mucosa and/or via modulation of gut microbiota. However, documentation of their effects under commercial conditions is limited in general, and absent for the Arctic region.

The goal of this thesis was to fill some of the knowledge gaps regarding gut health and microbiota of Atlantic salmon in the life period from late freshwater to seawater in the Arctic region for which two strategies were chosen: Firstly, to observe biomarkers of important, health-related gut functions in fish in a commercial size experimental farm fed diets without and with functional feed ingredients over a year from the last weeks before seawater transfer, and secondly, to use an *in vitro* approach to study effects of functional feed ingredients in a gut cell line model to reveal mechanisms underlying possible *in vivo* effects of functional feed ingredients.

Observation from the commercial size experimental farm

Atlantic salmon were observed at four sampling time points from late freshwater stage until about one year in seawater: in May, two weeks before seawater transfer (i.e. FW); in June, four weeks after seawater transfer (SW1); in November (SW2), and in April the following year (SW3) (**Paper I** and **II**). Two series of diets were fed, varying throughout the observation time in nutrient composition according to the requirements of fish, one with functional feed ingredients (Test diet), and the other without (Ref diet). The functional feed ingredients, i.e. nucleotides, yeast cell walls, a prebiotic and essential fatty acids, were supplemented to the diets either as single ingredient or as a mixture based on a strategy

developed by the feed producer for commercial production in the Arctic region.

Overall, the growth performance of the fish, gut health and gut microbiota varied greatly between the sampling time points, whereas the inclusion of functional feed ingredients in the diet affected the fish only marginally.

Compared to fish observed at FW, fish at SW1, i.e. four weeks after seawater transfer, showed substantially lower plasma cholesterol and triglycerides levels. The same was the case for gene expression levels related to immune and barrier functions, i.e. cytokines, T-cell markers and tight junction proteins, indicating suppressed status of some key physiological functions after seawater transfer. This suppression is most likely a cause of the increased vulnerability to diseases observed in Atlantic salmon just after seawater transfer. During the period SW1 to SW3 the plasma nutrients and most gene expression biomarkers returned to the levels observed at FW.

Hyper-vacuolization was observed in the pyloric caeca enterocytes, particularly the fish at SW1 and SW2, indicating a situation of gut mucosa lipid malabsorption. The increasing symptoms of lipid malabsorption were corresponded with the up-regulated perilipin-2 (*plin2*) expression levels.

Microbiota in digesta from the distal intestine at FW was dominated by phylum Firmicutes (e.g. genera *Lactobacillus*, *Weissella*, *Peptostreptococcus* and family *Ruminococcaceae*), Proteobacteria (e.g. genera *Photobacterium*, *Deefgea* and *Pseudomonas*) and Bacteroidetes (mainly genus *Flavobacterium*). The gut microbiota also showed slightly alteration just after seawater transfer, i.e. at SW1. Specifically, it was strongly dominated by the phylum Firmicutes (mainly genus *Lactobacillus*) and Proteobacteria (mainly genus *Photobacterium*). As fish progressed towards SW2 and SW3, the genera *Lactobacillus* and *Mycoplasma* became more prominent with a corresponding decline in genus *Photobacterium*.

Multivariate association analysis identified the expression levels of gut barrier function genes to be negatively correlated with 26 taxa including genera *Megasphaera*, *Photobacterium* and certain lactic acid bacteria (LAB). Also, the relative abundance of *Megasphaera* was positively correlated with the levels of gut immune gene expressions and plasma nutrients.

Mostly, the functional feed ingredients did not significantly affect the observed indicators of production or gut functions of the fish. However, the mix of functional feed ingredients used at SW2, nucleotides, yeast cell walls and essential fatty acids, seemed to represent a metabolic cost for the fish, as indicated by a tendency to slower growth, and a reduction in condition factor. Plasma triglyceride levels showed a corresponding significant decrease. At the same sampling time point, decreased microbial richness and diversity and low relative abundance of LAB were observed in Test-fed fish.

Observation from the *in vitro* study

The nucleotides, mannanoligosaccharides (MOS) and β-glucans, were selected for evaluation of effects on intestinal epithelial functions at cellular level using the rainbow trout intestinal epithelial cell line, RTgutGC (**Paper III**). The MOS treatment seemed to be the most potent modulator of immune and barrier functions in RTgutGC, and strongly suppressed reactive oxygen species (ROS) production and cell proliferation. Treatment with β-glucans induced high gene expression levels of immune and barrier functions, as well as possibly enhanced barrier functions via increasing transepithelial electrical resistance (TEER) levels and F-actin content. Compared to MOS and β-glucans, nucleotide treatment induced minor effects, i.e. only on expression levels of a few genes, i.e. interleukin 1 β (*il1b*), interleukin 8 (*il8*) and E-cadherin (*cdh1*).

Altogether, this thesis work fills knowledge gaps regarding dynamics of gut health and microbiota in Atlantic salmon from late freshwater stage until about one year in seawater in the Arctic region of Norway, effects of strategic use of selected functional feed ingredients throughout the production cycle, as well as mechanisms underlying effects of these functional feed ingredients.

SUMMARY IN NORWEGIAN

En konsekvens av den forventede økningen i etterspørselen etter atlantisk laks (*Salmo salar*) er søk etter nye lokaliteter for produksjon. I arktiske områder i Norge finnes muligheter for økt produksjon. Men, ettersom de fleste studier av effekter av variasjon i miljø og fôr på laksens produksjonsbiologi og helse er utført i mer sydlige områder, mangler vi kunnskaper om slike forhold for fisk produsert under arktiske forhold så som virkninger av ekstreme variasjoner i fotoperiode, lav gjennomsnittstemperatur og spesifikke patogener på fiskevelferd og helse. Arbeidet som presenteres i denne avhandlingen hadde som må å fylle kunnskapshull som gjelder tarmfunksjon og helse gjennom kritiske perioder av produksjonssyklusen hos fisk produsert under arktiske forhold. Funksjonelle ingredienser brukes i fiskefôr for å forbedre fiskehelse og sykdomsresistens, spesielt under stressende oppdrettsforhold. Bruken begrunnes med at de kan ha positive effekter på tarmfunksjon og helse, via direkte virkning på tarmslimhinnen og/eller via modulering av tarmmikrobiota. Dokumentasjonen for slike virkninger er imidlertid, svært begrenset, særling under kommersielle forhold. Målet med dette doktorgradsarbeidet var å fylle noen av kunnskapshullene som gjelder produksjon av laks under arktiske strøk. To strategier ble valgt. I den første ble forhold i en kommersiell produksjon indersøkt. I den andre ble effekter av funksjonelle ingredienser på tarmceller studert under *in vitro* betingelser.

Observasjoner under kommersielle forhold

Atlantisk laks ble undersøkt på fire tidspunkter, fra sent i ferskvannsfasen til etter ett år i sjøvann: i mai, to uker før overføring til sjøvann (FW); i juni, fire uker etter overføring til sjøvann (SW1); i november (SW2), og i april året etter (SW3) (**Artikkel I og II**). Fisken ble gitt kommersielt fôr gjennom hele forsøksperioden. Det varierte i næringsstoffsammensetning gjennom livsstadiene i henhold til variasjoner i laksens næringsstoffbehov. Fisken i forsøket ble delt i to populasjoner. Den ene fikk en serie av fôr som var uten tilsettinger av funksjonelle ingredienser (Ref diet), den andre en tilsvarende serie fôr som var supplert med funksjonelle ingredienser (Test diet). De funksjonelle fôringrediensene var nukleotider, et gjærcelleveggprodukt, et prebiotisk produkt og essensielle fettsyrer. De ble tilsatt fôrene enten som en enkelt ingrediens eller i blandinger i henhold til en strategi som var utviklet av produsenten.

Forsøket viste at fiskens vekst tarmhelse og tarmmikrobiota varierte sterkt mellom prøvetakingstidspunktene, mens strategien som var valgt for bruk av funksjonelle fôringredienser i dietten, påvirket fisken marginalt.

Sammenlignet med fisk observert ved FW viste fisk ved SW1, dvs. fire uker etter overføring av sjøvann, vesentlig lavere nivåer av en rekke biomarkører, som plasmakolesterol og triglyserider. Det samme var tilfelle for genekspresjonsnivåer relatert til immun- og barrierefunksjoner: dvs. cytokiner, T-cellemarkører og tight junction proteiner, noe som indikerer undertrykking av noen svært viktige fysiologiske funksjoner etter overføring til sjøvann. Disse endringene har trolig nær sammenheng med den økte mottageligheten for sykdom hos fisken etter utsett. I løpet av perioden SW1 til SW3 kom imidlertid plasma næringsstoffene og de fleste genuttrykksmarkørene tilbake til nivåene som ble observert ved FW.

Hypervakuolisering ble observert i enterocytter i blindsekkene, spesielt fisken ved SW1 og SW2, noe som indikerer lipid malabsorpsjon. De økende symptomene på lipidmalabsorpsjon korrelerte med oppreguleringen av perilipin-2 (*plin2*) som er en indikator på lipidakkumulering.

Når det gjelder mikrobiota i tarminnholdet i baktarmen ved FW, ble fisken dominert av fylum Firmicutes (f.eks. Slektene *Lactobacillus*, *Weissella*, *Peptostreptococcus* og familien *Ruminococcaceae*), Proteobacteria (f.eks. Slektene *Photobacterium*, *Deefgea* og *Pseudomonas*) og Bacteroidetes (hovedsakelig slekten *Flavobacterium*). Tarmmikrobiotaen viste også endring etter overføring til sjøvann. Ved SW1 ble tarmmikrobiotaen sterkt dominert av fylum Firmicutes (hovedsakelig slekten *Lactobacillus*) og Proteobacteria (hovedsakelig slekten *Photobacterium*). Etter hvert som fisken utviklet seg mot SW2 og SW3, ble slektene *Lactobacillus* og *Mycoplasma* mer fremtredende med en tilsvarende nedgang i slekten *Photobacterium*.

Multivariate assosiasjonsanalyser identifiserte ekspresjonsnivåene av sentrale gener i tarmens barrierefunksjon til å være negativt korrelert med mikrober i 26 taxa inkludert slektene *Megasphaera*, *Photobacterium* og visse melkesyrebakterier (LAB). Også relativ forekomst av *Megasphaera* var positivt korrelert med uttrykksnivåene av immune relaterte gener og næringsstoffer i plasma.

Tilsetting av de funksjonelle ingrediensene til det kommersielle fôret som ble brukt i dette prosjektet så ikke ut til å ha en positiv effekt på noen av de observerte funksjons- og helseindikatorene i tarmen hos fisken. Ved SW2 så de ut til å representere en metabolsk kostnad for fisken, uttrykt som redusert vekst, lavere kondisjonsfaktor og lavere nivå av triglyserider i plasma. På det samme prøvetidspunktet ble det også observert redusert mikrobiell «richness» og «diversitet» og lavt relativt innhold av melkesyrebakterier hos fisk som fikk Test Diet. På dette tidspunktet, men ikke ved de andre, var gjærcelleveggproduktet inkludert i Test Diet.

Observasjoner fra *in vitro* studiet

De funksjonelle ingrediensene nukleotider, mannanoligosakkarider (MOS) og ß-glukaner ble valgt til studier av virkningsmekanismer av slike ved bruk av en tarmepitelcellelinje fra regnbueørret (RTgutGC) (**Papir III**). MOS så ut til å være den mest potente modulatoren av immun- og barrierefunksjoner i RTgutGC. Samtidig undertrykte MOS sterkt ROS-produksjon og celleproliferasjon. Eksponering for ß-glukaner induserte høye genuttrykksnivåer av immun- og barrierefunksjoner, samt økte, dvs forbedret, transepitel-elektrisk motstandsnivå (TEER) og F-aktininnhold. Sammenlignet med MOS og ß-glukaner induserte nukleotider ekspresjonsnivåer av bare noen få gener, dvs. interleukin 1 ß (*il1b*), interleukin 8 (*il8*) og E-cadherin (*cdh1*).

Totalt sett bidrar arbeidet i denne avhandlingen med ny kunnskap om dynamikken i tarmhelse og tarmmikrobiota hos atlantisk i laks fra sent ferskvannsfase til ett år i sjøvannsfasen under arktiske forhold i Norge og hvordan funksjonelle fôringredienser påvirker tarmfunksjon og helse in *vivo* og in *vitro*.

LIST OF ABBREVIATIONS

Aqps	Aquaporins
ASVs	Amplicon sequence variants
BBM	Brush border membrane
DI	Distal intestine
EC	Epithelial cells
FAO	Food and Agricultural Organization of the United Nations
GALT	Gut-associated lymphoid tissue
Igs	Immunoglobulins
IEC	Intestinal epithelium cells
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
LP	Lamina propria
LU	Lumen
MI	Mid intestine
ML	Mucus layer
MOS	Mannanoligosaccharides
OTU	Operational taxonomic unit
PAMPs	Prototype pathogen-associated molecular patterns
PC	Pyloric caeca
PI	Proximal intestine
PRRs	Pattern recognition receptors
ROS	Reactive oxygen species
RTgutGC	Rainbow trout intestinal derived cell line
RTgutF	intestinal fibroblasts
TEER	Transepithelial electrical resistance
TLR	Toll-like-receptor

LIST OF ARTICLES

Paper I

Gut immune functions and health in Atlantic salmon (*Salmo salar*) from late freshwater stage until one year in seawater and effects of functional ingredients: A case study from a commercial sized research site in the Arctic region

Jie Wang, Trond M. Kortner, Elvis M. Chikwati, Yanxian Li, Alexander Jaramillo-Torres, Jan Vidar Jakobsen, Jarle Ravndal, Øyvind Jakobsen Brevik, Olai Einen, Åshild Krogdahl. Fish and Shellfish Immunology 106 (2020): 1106-1119

Paper II

Gut microbiota of Atlantic salmon (*Salmo salar*), observed from late freshwater stage until one year in seawater, and effects of functional ingredients: A case study from a commercial sized research site in the Arctic region

Jie Wang, Alexander Jaramillo-Torres, Yanxian Li, Trond M. Kortner, Karina Gajardo, Øyvind Jakobsen Brevik, Jan Vidar Jakobsen, Åshild Krogdahl. Under revision in ***Animal Microbiome***

Paper III

Rainbow trout (*Oncorhynchus mykiss*) intestinal epithelial cells as a model for studying gut immune function and effects of functional feed ingredients

Jie Wang, Peng Lei, Amr Ahmed Abdelrahim Gamil, Leidy Lagos, Yang Yue, Kristin Schirmer, Liv Torunn Mydland, Margareth Øverland, Åshild Krogdahl, Trond M. Kortner. Frontiers in immunology 10 (2019): 152

INTRODUCTION

In Norway, Atlantic salmon production in the northernmost areas, such as Finnmark, contributed with 8.9% of total aquaculture production in 2019 (*https://www.fiskeridir.no*). Lack of information on the consequences of the extreme changes in harsh Arctic conditions of these areas, e.g. low average temperature, longer winter period and extreme variation in photoperiod, which may challenge the physiology of the fish, hinders optimization of the production management compared to those conducted in more southerly areas. Also, many of the pathogens in these waters are unique, some causing severe disease outbreaks. It is highly likely that, due to environmental effects on fish biology, the influence of diet composition on gut health and microbiota may differ in fish produced in the northerly areas from that of fish grown more southerly. The general lack of information on how fish in the north may differ in biology and interaction with the environment, has stimulated the feed producers to recommend using functional feed ingredients with the aim to strengthen the fish' capacity to manage harsh environmental conditions and resist specific pathogens.

Functional feed ingredients are widely used in aquatic feed and are claimed to improve immunity during stressful farming situations (Tacchi et al., 2011;Kiron, 2012). A number of studies support this argument and show functional feed ingredients have positive effects on gut immune responses (Carbone and Faggio, 2016) and barrier functions (Torrecillas et al., 2013;Torrecillas et al., 2014). The mechanism of action of functional feed ingredients may be directly or indirectly via the intestinal mucosa and/or via modulation of gut microbiota (Ringø et al., 2012;Merrifield and Ringø, 2014;Ringø et al., 2016;Guerreiro et al., 2018). However, the detailed knowledge on their mechanisms of action is still limited.

The present thesis work aimed to strengthen knowledge on gut health and microbiota of Atlantic salmon by observing fish under commercial conditions over a one-year period starting from the late freshwater stage. The observations in the seawater stage started just after seawater transfer and were followed by observations in the autumn, and the spring the following year. In this study, effects of two diet series were compared, one without and one with functional feed ingredients to understand how they might modulate gut health status and microbiota under commercial conditions in the Arctic region. A complementary *in vivo* study of mechanisms underlying effects of functional feed ingredients was also conducted

employing a cell line developed from the intestine of rainbow trout, the RTgutGC. *In vivo*, fish gut health and function were evaluated observing various endpoints including intestinal somatic indices, digesta trypsin activity and total bile acid level, brush border membrane enzyme activity, histo-morphological appearances, gut function related gene expressions, as well as the microbiota in digesta of the distal intestine. *In vitro*, the endpoint analysis included the cell viability, brush border digestive enzyme activity, barrier functions, cell migration, reactive oxygen species (ROS) production, morphology, as well as gene and protein expression.

The chapters below give background information and knowledge relevant for this thesis.

Atlantic salmon farming in Norway

Aquaculture production is currently the fastest-growing sectors for animal food production and is considered to be so in the near future. The world aquaculture production reached 82 million tons fish in 2018, with Atlantic salmon contributing with about 3% of total aquaculture production (FAO, 2020). In Norway, Atlantic salmon is an economically important species with about 1.4 million tons in 2018 (FAO, 2020), and has the potential for further development to reach a level 4-5 times higher than today by 2050 (Almås and Ratvik, 2017).

A condition for fulfilling such an increase in Atlantic salmon production is expansion of the aquaculture production into areas with the potential for growth in this industry. One area of current interest for salmon expansion is the northernmost areas in Norway, such as Finnmark. However, literature searches reveal that knowledge relevant for fish production under Arctic conditions regarding nutrition, digestive functions, gut health and microbiota is very limited. Most studies have been carried out under more southerly conditions. Environmental differences, such as low average temperature and extreme variation in photoperiod, affect feed intake and growth rate. Also, disease challenges in these regions are different, some causing severe disease outbreaks. For example, two pathogens are of particular concern in these Arctic areas, the bacteria *Tenacibaculum finnmarkense* and the micro parasite *Parvicapsula pseudobranchicola*. The former causes ulcers in the unscaled parts of the

salmon skin, usually presented as erosions around the mouth and fins. The latter infects smolts shortly after seawater entry and disease outbreaks occur after 3-4 months (Nylund et al., 2005;Nylund et al., 2018).

As the salmon production in the northernmost region of Norway is increasing, it becomes more and more urgent to increase knowledge on the fluctuations of fish health biomarkers and microbiota through the production cycle, to be able to develop feed and management strategies for host health and welfare in the aquaculture industry in these areas.

The Atlantic salmon intestine

The intestine is a multifunctional organ responsible for digesting and absorbing the nutrients from the ingested food, as well as transporting nutrients, ions and water from intestinal lumen into systemic circulation. Among other functions, the intestine is responsible for osmoregulation via both transcellular and paracellular routes. Moreover, the intestine is acting as a barrier towards the environment by physical, chemical and immunological functions, vital for the fish health and protection against pathogens and potential harmful components. Lastly, the intestine hosts a great number of bacteria with potential effects on most intestinal digestive, absorptive, barrier and immune functions.

Morphology and functions

The intestine of Atlantic salmon can be divided into three macroscopically distinguishable parts (**Figure 1**): proximal intestine (PI) with pyloric caeca (PC), mid intestine (MI) and distal intestine (DI).

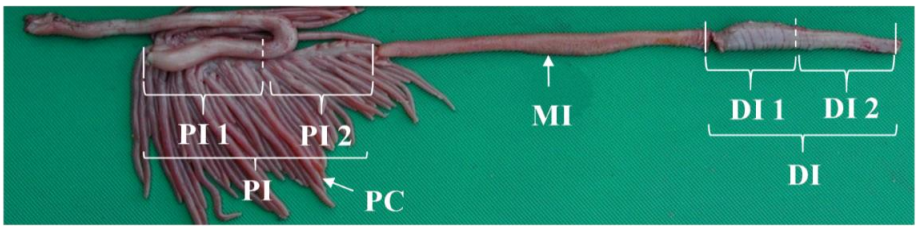

Figure 1: *Anatomy of the intestine of Atlantic salmon. Abbreviations: PI, proximal intestine divided in two (PI 1 and PI 2); PC, pyloric caeca; MI, mid intestine; DI, distal intestine divided in two (DI 1 and DI 2). Modified from Bjørgen et al. (2020).*

A wide range of digestive enzymes, secreted into the intestine from pancreatic tissue embedded in the mesentery fat depot between the PC or located on the brush border of enterocytes, break down complex nutrients, such as proteins, lipids, and carbohydrates, into small fragments that can be absorbed by the enterocytes. The digestive capacities and nutrient absorptive functions vary along the digestive track in Atlantic salmon (Bakke-McKellep et al., 2000;Denstadli et al., 2004;Krogdahl et al., 2015b). The proximal part of the intestine is the primary site for digestion and absorption of feed nutrients in salmon, accounting for about 70% of the total nutrient absorption (Bakke-McKellep et al., 2000;Denstadli et al., 2004). For example, trypsin is one of the main proteolytic enzymes secreted by the pancreatic tissue (Krogdahl and Sundby, 1999) and becomes active after activation in the intestinal lumen. The trypsin activities in the chyme decrease gradually from PI to DI in Atlantic salmon (Lilleeng et al., 2007;Krogdahl et al., 2015a). Bile acids from the gallbladder, mainly composed by taurocholic acid (Kortner et al., 2014), mixed in digesta play an important role in solubilization of lipid and stabilizing digestive enzyme activates (Buchinger et al., 2014). As the chyme moves along the digestive tract, the levels of bile acids decreased, as observed in Atlantic salmon (Kortner et al., 2013;Gu et al., 2014) suggesting efficient reabsorption and recycling.

Intestinal mucosa

The Atlantic salmon intestinal mucosa comprises three layers (**Figure 2**): a monolayer of epithelial cells (EC) directly facing the intestinal lumen (LU) and a mucus layer (ML) protecting the monolayer. The monolayer of cells is attached to the basement membrane surrounding the third layer, the lamina propria (LP). The LP is a structure of loose connective tissue in which blood vessels, as well as nerves are important structures. In land animals, lymphatic vessels are also draining nutrients and other components from the tissue. A lymphatic system has not yet been identified in Atlantic salmon. The blood vessels transport nutrients to the rest of the body. The LP also contains a complex population of immune cells protecting the organism against pathogens and alien components which might enter the mucosa.

Digestive functions

The epithelial layer of the intestinal mucosa is located on a highly folded membrane. The apical side of the absorptive cells is also highly folded, called the brush border membrane

(BBM). Together, these folds make an enormous surface for absorption securing high digestive and absorptive capacity. On the other hand, this surface exposes the organisms to the environment. A wide range of digestive enzymes are harbored by intestinal absorptive cells, such as leucine aminopeptidase, maltase and alkaline phosphatases, localized on the BBM. Nutrients released from the digestive processes in the intestinal lumen can be further digested into smaller components under the effects of these BBM enzymes. Transporters and channels in the BBM convey the nutrients from the intestinal LU into the systemic circulation (Bakke-McKellep et al., 2000;Bakke et al., 2010).

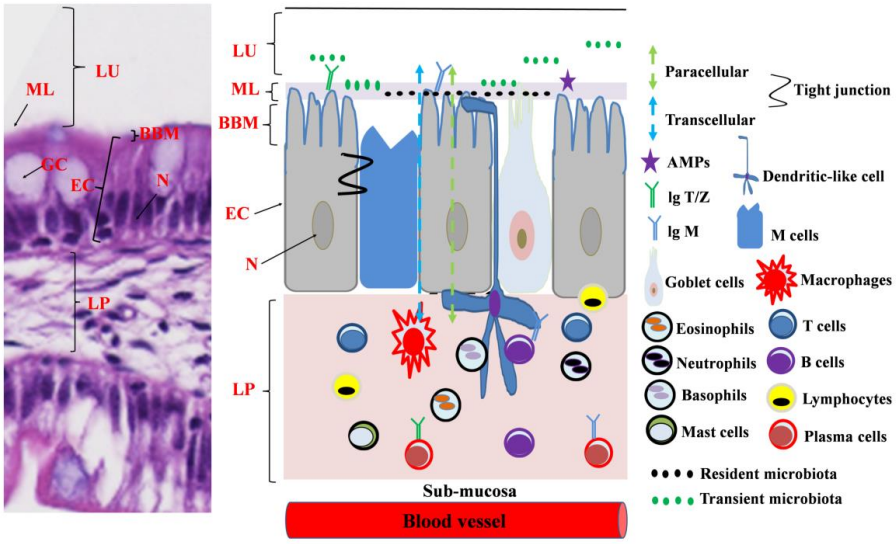

***Figure 2**: Structure of the intestinal epithelium. Left: Histological section of the distal intestinal epithelium (Source: Elvis Chikwati). Right: Schematic drawing of intestinal epithelium. The intestinal mucosa in teleost is mainly composed by microbiota, mucus layer, intestinal epithelium cells and lamina propria (mainly epithelial cells, macrophages, goblet cells, B cells, T cells, granulocytes, lymphocytes and plasma cells). M cells and dendritic cells are shown, although their presence in fish intestine is not demonstrated yet. Abbreviation: LU: Lumen; ML: Mucus layer; BBM: Brush border membrane; EC: Epithelial cells; GC: Goblet cells; N: Nucleus; LP: Lamina propria. AMPs, antimicrobial peptides; Ig, immunoglobulin. Modified from (Gomez et al., 2013;Hu et al., 2016).*

Barrier and osmotic functions

The ML covering the intestinal mucosa acts as the first physical and chemical barrier against

potential harmful components from dietary or endogenous origin. The mucus is produced by goblet cells and enriched with a great number of defense components, including mucins, lysozymes, complement components, lectins, antimicrobial peptides and immunoglobulins, which plays critical roles in maintaining the intestinal homeostasis and forming defense against pathogens (Ellis, 2001;Dongarra et al., 2013;Beck and Peatman, 2015;Martin et al., 2016). Besides defense functions, mucins have important roles in structural forming and lubrication of the mucosal wall (Beck and Peatman, 2015). Two type of mucin genes, annotated as *muc2* and *muc5*, have been widely reported in fish (van der Marel et al., 2012;Salinas, 2015;Sveen et al., 2017). In Atlantic salmon, *muc2* is highly expressed in the intestinal regions, whereas *muc5* is mainly expressed in skin, gill and PC (Krogdahl et al., 2015a;Sveen et al., 2017).

The intestinal epithelial cells and their cell-to-cell connections, i.e. tight and adherens junctions, form the intestinal epithelial barrier defending pathogens and regulating paracellular pathway and osmotic pressure in the gut (Sundell and Sundh, 2012). The claudin family, zonula occludens family and occludin are the best-known types of tight junction proteins in intestinal epithelial cells of Atlantic salmon. Regarding the claudin family, mRNA expression of *claudin-15* and *-25b* seems to be predominant in the intestine of Atlantic salmon (Tipsmark et al., 2010a), while three *claudin-3* isoforms are highly expressed in kidney tissue (Tipsmark and Madsen, 2012). Regarding the zonula occludens family, zonula occludens 1 (ZO-1) has been widely reported to involve in paracellular signaling pathways (Kosinska and Andlauer, 2013). The main function of occludin has been found to be regulation of water and large macromolecule flux possibly via the “leak” pathway, and its gene expression order is gill > intestine > kidney in Atlantic salmon (Tipsmark and Madsen, 2012;Kosinska and Andlauer, 2013). The adherens junctions are located below the tight junctions, also playing crucial roles in the formation of the intestinal epithelial barrier. The most extensively studied adherens junction is the E-cadherin, which is responsible for forming intestinal barrier via regulation of actin cytoskeleton and paracellular pathways (Kosinska and Andlauer, 2013). Among these tight and adherens junction proteins, some can increase paracellular permeability, whereas others have the opposite functions (Sundell and Sundh, 2012). Previous studies have claimed that occludin and claudin family were involved in the reorganization of intestinal epithelium and altered paracellular permeability in Atlantic salmon during seawater acclimation (Tipsmark et al., 2010a;Tipsmark and Madsen, 2012).

In addition to permeable tight junctions, specific water transporters, specifically aquaporins (aqps), located in the BBM are involved in osmoregulation via the regulation of water permeability (Sundell and Sundh, 2012). In Atlantic salmon, aqps, i.e. *aqp-1a*, *aqp-1b*, *aqp-8ab* and *aqp-10*, are the main water channel protein genes in the intestine. These genes show marked increase in expressions during the smoltification period (Tipsmark et al., 2010b;Sundell and Sundh, 2012). These aqps are located both in the BBM and the sub-apical of intestinal regions (Madsen et al., 2011). Compared to other water channel genes, the *aqp-8ab* is suggested to play the most important role in water uptake in the intestine of Atlantic salmon after seawater transfer due to its high expression (Tipsmark et al., 2010b).

Immune functions

The mucus layer and epithelial cells together with tight junctions form the first physical barriers to block the entry of pathogen and protect the host from external environment. In addition to these physical barriers, the immune system inside the intestinal mucosa is also a vital defense mechanism when harmful stimuli succeed in bypassing the physical barriers. The immune system is coordinated by the gut associated lymphoid tissue (GALT) in the intestinal mucosa. The GALT comprises various protective and antimicrobial molecules, as well as immune cells, such as leukocytes in LP (mainly macrophages, granulocytes, lymphocytes and plasma cells) and intraepithelial lymphocytes (mainly T cells and B cells), that are involved in a complex network of innate and adaptive immune responses (**Figure 2**) (Gomez et al., 2013;Lazado and Caipang, 2014).

Like in humans and land-animals, the protective components of the innate immune system in fish represent the first immune barrier. The innate immune system is mainly formed by humoral innate immunity and cellular innate immunity. The humoral innate immunity contains a wide variety of defense components, such as complements, lysozyme, proteases and antimicrobial peptides. Regarding the cellular innate immunity, in mammals the main components are natural killer cells, mast cells, dendritic cells, macrophages and granulocytes including eosinophils, basophils and neutrophils, while in fish the main cellular defense components are mast cells, macrophages and granulocytes (reviewed by (Gomez et al., 2013)). As part of the innate immune system, the presence of innate pattern recognition receptors (PRRs) that are expressed by the GALT and intestinal epithelial cells could recognize exogenous molecules via the pathogen-associated molecular patterns (PAMPs).

Some PAMPs are part of cell walls of bacteria (e.g. lipopolysaccharide (LPS)) and fungi (e.g. β-glucans). The PRRs could enhance innate immune responses through activation of the signals of related immune and inflammatory cells, which will increase expression of soluble mediators. In fish, most studies on the PRRs are the toll-like-receptors (TLR) that play a pivotal role for the innate immune response (Tao et al., 2012). So far, about 20 different TLRs have been identified in fish (Li et al., 2017). These specific TLRs are induced by immune cells, such as macrophages and neutrophils, against different PAMPs, which make the immune system able to distinguish between self and non-self antigens. The TLRs could induce immune responses via recruiting different adaptors, such as myeloid differentiating factor 88 (MyD88), and thereby regulate diverse downstream signaling proteins (Tao et al., 2012). Moreover, the activation of TLR2 has been reported to maintain gut barrier function, such as ZO-1, via the MyD88-independent pathway (Resta-Lenert and Barrett, 2009).

The adaptive/specific immune system in fish intestine is mainly related to humoral adaptive immunity (i.e. immunoglobulins (Igs)) and cellular adaptive immunity (i.e. B cells and T cells). The Igs, antibodies secreted by B cells, could recognize specific molecule of pathogens, called antigens. Two types of Igs have been identified in the fish intestine to date, i.e. IgM^+ and IgT^+/IgZ^+ (Zhao et al., 2008). T cells are abundant in the intestine, and the expression of a number of T cell marker genes, including cluster of differentiation 3γδ (*cd3γδ*), 4α (*cd4α*) and 8β (*cd8β*), have been widely investigated in Atlantic salmon (Bakke-McKellep et al., 2007;Lilleeng et al., 2009;Marjara et al., 2012). Moreover, T cells marker genes could be induced by anti-inflammatory cytokines (Beck and Peatman, 2015) and suppressed by gamma-interferon-inducible lysosomal thiol reductase (*gilt*) that plays an important role in antigen processing (Lilleeng et al., 2009).

The common functions for both innate and specific immunity are the production and release of cytokines (Secombes et al., 2001;Secombes, 2016). The pro-inflammatory and anti-inflammatory cytokines are signaling substances involved in regulating different immune cells to mount an appropriate immune responses, and thereby secure maintenance of immune homeostasis to inhibit excessive activation of the inflammatory responses during intestinal infections (Fast et al., 2007;Gomez and Balcazar, 2008;Rombout et al., 2011;Marjara et al., 2012). Moreover, the pro-inflammatory cytokines genes can be modulated by the MyD88-

independent pathway (Zhou et al., 2014).

Gut Microbiota

The microbial communities that inhabit the intestine, termed as gut microbiota, play important roles in various host-related functions (Bengmark, 2013;Kamada and Nunez, 2013;Wang et al., 2017;Egerton et al., 2018). The gut microbiota comprises bacteria, archaea, fungi and viruses. It can be divided into digesta- (called transient or allochthonous) and mucosa-associated (called resident or autochthonous) microbiota based on their location in the intestine. The digesta-associated microbiota includes the microorganisms living in the digesta, while the mucosa-associated microbiota refers to microorganisms inhabiting the mucosal layer and is in close contact with enterocytes.

Over the last few years, since this thesis work started, results of many studies regarding functional roles of gut microbiota in fish and effects of variation in diet and environmental conditions have been published. Most of them are the results of experiments conducted under controlled conditions, or in farms in the more southerly areas. It is reported that the gut microbiota has potential relationships with host responses in fish, for better or worse, such as growth performance (Ringø et al., 2016;Wang et al., 2017), endogenous enzyme activities (Ray et al., 2012), nutrient digestion and utilization (Semova et al., 2012;Falcinelli et al., 2015), development and maturation of gut-associated lymphoid tissue (Austin, 2006;Gomez and Balcazar, 2008;Merrifield and Ringø, 2014) immune responses (Rawls et al., 2004;Rolig et al., 2015) and energy homeostasis (Butt and Volkoff, 2019).

Regarding Atlantic salmon, the previous advances in understanding gut microbiota cover various factors affecting the taxonomic composition, including diet composition (Zarkasi et al., 2016;Gajardo et al., 2017;Catalan et al., 2018), various environmental factors (separate or synergistic effect, e.g. daylight, temperature, salinity, dissolved oxygen, water currents, etc.,) (Hovda et al., 2012;Zarkasi et al., 2014;Zarkasi et al., 2016;Dehler et al., 2017;Rud et al., 2017;Fogarty et al., 2019;Lokesh et al., 2019), location within the digestive tract (Gajardo et al., 2016), disease (Karlsen et al., 2017) and developmental stages (Zarkasi et al., 2014;Lokesh et al., 2019). A few very recent studies have described the associations between gut microbiota and host responses in Atlantic salmon, and have found differentially abundant taxa closely related to flesh pigmentation (Nguyen et al., 2020a;Nguyen et al.,

2020b), lipid metabolism (Dvergedal et al., 2020), immunostimulation (Parra et al., 2020), and gut immune and barrier gene expressions (Li et al., 2020). However, the studies on associations between gut microbiota and host responses are still a largely unexplored area.

Functional feed ingredients

There is no common and accepted definition for what functional feed ingredients are so far. The general understanding is that such diet additives induce beneficial effects, beyond the basic nutritional requirement, on growth performance and health of fish (Tacchi et al., 2011;Kiron, 2012). For growth benefits, functional feed ingredients may reduce energy expenditure, and then increase growth performance via decreasing body metabolic demands and inhibition of protein turnover and oxygen demand (Tacchi et al., 2011). Improvement of health status of fish fed functional feed ingredients have been suggested to be related to the direct or indirect regulation of innate and adaptive immune responses, and/or the enhancement of beneficial gut bacteria, such as lactic acid bacteria (LAB) (Kiron, 2012;Guerreiro et al., 2018;Hossain et al., 2019). Prebiotics and nucleotides, the most common functional feed ingredients used in aquaculture production (Kiron, 2012;Ringø et al., 2012;Akhter et al., 2015;Guerreiro et al., 2018;Hossain et al., 2019), were used in the *in vivo* work of this thesis according to a strategy developed by the feed producer.

Prebiotics

Prebiotics could be defined as "the feed ingredients that allow specific changes in the composition and/or activity of gut microbiota for the benefits of the host" (Gibson et al., 2010). The most commonly used prebiotics include the extracts from yeast, bacteria, fungi or plants (Song et al., 2014). Previous studies have indicated that these prebiotics could improve growth performance (Li, 2004;Staykov et al., 2007;Munir et al., 2016), alter the intestinal morphology (Dimitroglou et al., 2009), regulate gut microbiota (Ringo et al., 2006;Dimitroglou et al., 2009), enhance cellular and humoral immune responses of epithelial tissues and thereby increase disease resistance, as demonstrated against *Streptococcus iniae* infection (Li, 2004;Staykov et al., 2007;Akhter et al., 2015;Munir et al., 2016;Guerreiro et al., 2018). The extracted products from yeast cell walls, such as oligosaccharides (e.g. mannan oligosaccharide (MOS)), and polysaccharides (e.g. β-1, 3, 1, 6-glucan), are widely used as prebiotics in aquatic feed. These yeast cell wall products were used in the *in vitro*

work of this thesis. Atlantic salmon fed a diet with inclusion of these yeast cell wall products can increase feed efficiency, alter gut morphology, improve immune responses, modulate gut microbiota and enhance disease resistance against sea lice infestation (**Table 1**).

Table 1 Overview of yeast cell wall products used in Atlantic salmon diets.

Types	Results	Study
MOS	Serum lysozyme activity↓	Grisdale-Helland et al. (2008)
	Oxygen consumption↓	
	Body protein level ↓	
	Body energy level ↑	
	Microvilli density↑	Sweetman et al. (2008)
	Microvilli height↑	
	Feed efficiency ratio↑	Refstie et al. (2010)
	SBM-induced enteritis↓	
	Sea lice infestation↓	Dimitroglou et al. (2011)
	Carcass protein content↑	
	Liver glycogen deposition↑	
	Modulate intestinal microbiome	Green et al. (2013)
Yeast extracts	Calreticulin-like protein↑	Micallef et al. (2017)
β-1,3,1,6-glucan	Sea lice infestation↓	Refstie et al. (2010)
	Number of immune cells↑	Kiron et al. (2016)
	Innate immune responses↑	Carolina (2018)

Nucleotides

Nucleotides are low-molecular weight intracellular compounds of crucial importance for many physiological and biochemical functions, such as cell division and differentiation, tissue growth, repair and development, nutrient metabolism and immune functions (Gil, 2002). Nucleotides may be supplied through three pathways: de novo synthesis, the salvage pathway and the diet. They are not considered essential nutrients, but de novo synthesis and the salvage are energy consuming processes. Dietary supply may therefore save energy (Tacchi et al., 2011;Ringø et al., 2012). Several different commercial nucleotide supplements are available and have gained attention as potentially beneficial ingredients in aquaculture feed, being reported to promote growth performance, increase quality of brood-fish and larvae, modulate intestinal morphology, enhance immune functions and defend sea lice infection, as well as modulate gut microbiota (reviewed by (Hossain et al., 2019). These benefits may be associated with the regulation of expression of growth factors and immune related genes, as well as increased colonization of beneficial intestinal bacteria (Wei et al., 2015;Xu et al., 2015).

What is optimum supply seems to vary between the fish species. For example, positive effects on growth performance were observed in juvenile red sea bream fed diets supplemented with mixed nucleotide in the range 1.0 - 1.5 g kg^{-1} (Hossain et al., 2016a). With somewhat higher levels, also rainbow trout have shown improved growth when fed diets supplemented with nucleotides, i.e. in the range 1.5 - 2 g kg^{-1} (Tahmasebi-Kohyani et al., 2011). For Atlantic salmon, earlier studies (**Table 2**) have observed that fish fed diet with 2 g kg^{-1} Optimûn®, containing 0.03% nucleotides, promoted growth performance, increased intestinal fold height and antibody titers. Also, they can reduce mortality (Burrells et al., 2001c) and sea lice infection rate (Burrells et al., 2001a). In addition, salmon fed a diet supplemented with nucleotides have been found to show an increase in Na+, K+-ATPase activity in PC (Oulad et al., 2009) and enhance disease resistance against *Caligus rogercresseyi* infestation (González and Troncoso, 2009).

Table 2 Overview of types of nucleotides used in Atlantic salmon diets.

Types	Results	Study
Optimûn®	Sea lice infection↓	Burrells et al. (2001a)
Nucleotides	Sea lice infection↓	Burrells et al. (2001b)
Optimûn®	Mortality ↓ Plasma chloride↓ Growth rate↑ Antibody titre↑ Intestinal fold height↑	Burrells et al. (2001c)
Nucleotides	Immunofluorescence of Na+, K+-ATPase↑	Oulad et al. (2009)
Optimûn® and Sanictum®	Disease resistance against *Caligus rogercresseyi* ↑	González and Troncoso (2009)

Of note, there are still many gaps in the present knowledge on dietary inclusion of functional feed ingredients in fish, such as various aspects regarding their digestion, absorption, metabolism and how their effects vary depending on variation in characteristics of the functional feed ingredient itself, timing and duration of administration, environmental factors, fish species and life stages, as stated in previous reviews (Ringø et al., 2016;Guerreiro et al., 2018;Hossain et al., 2019).

Impacts of commercial conditions on gut immune functions and microbiota

Through the freshwater to seawater production periods under commercial conditions, the salmon may sense and respond to a range of changeable factors, including daylight, temperature, water salinity, water dissolved oxygen, water alkalinity, aquaculture practices (e.g. handling stress), as well as diets (e.g. adjustments of macronutrient composition). All

these factors may alone or together influence gut health and microbiota, and are hard to control under the commercial conditions (Kononova et al., 2019;Legrand et al., 2020). Most previous studies did not aim to highlight the weight of every single factor separately but explore the extent of the effect of all these potential factors together. Of note, the most prominent alterations of immune functions take place in the period of freshwater-to-seawater transition supposedly due to the physiological alterations taking place in the fish, tremendous handling stress, and the new environmental challenges (Sissener et al., 2009;Tipsmark et al., 2010b;Sundh et al., 2014;Johansson et al., 2016). Available information shows clear alterations in immune functions, and reports from the industry document great losses due to transportation stress and diseases after seawater transfer (Karlsen et al., 2018). A study found that the expression of about 300 immune-related genes were severely suppressed without recovery for at least three weeks after seawater transfer (Johansson et al., 2016). Another study also found inflammatory gene expression, i.e. NOD-like receptors C5, clearly depressed after seawater transfer (Pontigo et al., 2016). Moreover, regarding microbiota, the freshwater-to-seawater transition have been found to alter bacterial communities significantly (Lokesh and Kiron, 2016;Rudi et al., 2018;Jaramillo-Torres et al., 2019).

Taken together, it is important to increase the knowledge on fluctuations of gut health and microbiota through freshwater to seawater production periods under commercial conditions. However, most available information were conducted in laboratory conditions or in more southerly areas. For Atlantic salmon in the northernmost areas under commercial conditions, the knowledge is still limited.

The characterizations of rainbow trout intestinal epithelial cell line (RTgutGC)

Although functional feed ingredients are widely used in aquafeed and claimed to improve growth and health, knowledge on their mechanisms of action is limited due to lack of targeted research tools. The use of cell lines may offer well-controlled experimental environment and reproducible tools for the investigation of the mechanisms underlying effects of functional feed ingredients on intestinal functions and health at the molecular and cellular levels. Moreover, the cell lines will help reduce the current dependence on feeding trials and use of experimental fish, and thus contribute to a development in line with the 3R principles. In mammals, the extensively studied and used intestinal epithelial cell lines, such as Caco-2, show many characteristics which are similar to mucosal cells *in vivo*. They have

been a valuable tool for exploration of basic mechanisms of intestinal epithelial functions and the interactions with dietary additives. For example, nucleotide treatment has been observed to increase intestinal maturation using the human Caco-2 and rat IEC-6 cell lines (He et al., 1993). Treatment with β-glucans has been shown to affect intestinal immune and barrier functions using the human Caco-2 cells (Maren Amasheh and Michael Fromm, 2008;Amasheh et al., 2009;Volman et al., 2010).

Over the last 10 years, the fish gut cell line RTgutGC, the first of its kind from fish, has been established based on cultures developed from rainbow trout distal intestine (Kawano et al., 2011). Since the initial isolation of the RTgutGC cells, some of their structural and functional features have been documented when grown on conventional culture plates without (**Figure 3 A**) or with permeable membrane insert **(Figure 3 B**). In the latter, the cells grow on a permeable membrane forming a two-compartment intestinal barrier model consisting of a polarized epithelium, which is divided into an upper (apical) and a lower (basolateral) compartment to mimic gut lumen and portal blood, respectively.

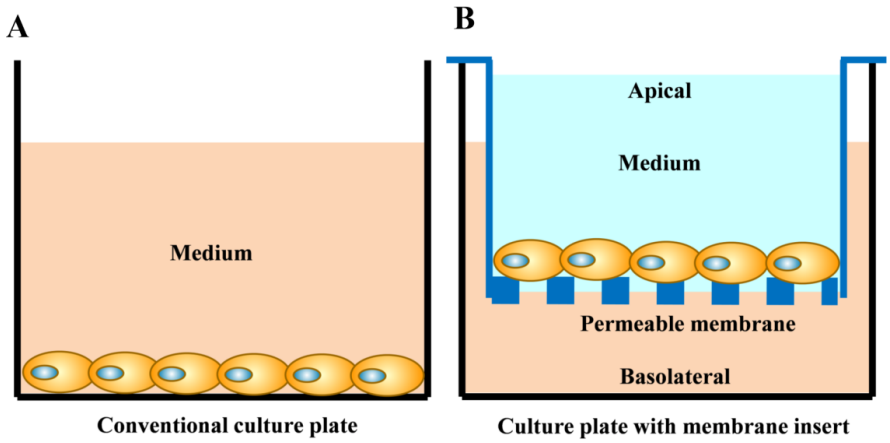

Figure 3: *Schematic representation of RTgutGC cells grown on conventional culture plates without (**A**) or with **(B)** permeable membrane insert.*

To be useful for the study of mechanisms underlying *in vivo* effects of functional feed ingredients, it is important that a gut cell line has characteristic which resembles those of *in vivo* gut cell. Regarding the characteristics of RTgutGC, in comparison to gut cells *in vivo*, studies have shown that their expression levels of the mucus related genes were similar levels as found in *in vivo* tissue (Schug et al., 2019). For brush border membrane, specific enzymes,

such as Na+/K+ -ATPase (Minghetti et al., 2017) and alkaline phosphatase activity (Kawano et al., 2011) have been observed. Two other important characterizations, the existence of microvillus structures and mucins, have been suspected (Langan et al., 2017;Langan et al., 2018), but are not conclusively confirmed.

The cytoskeleton is essential for maintaining cell interactions in the intestinal epithelium and is suggested to be a major target of intestinal injury. Actin filaments, including filamentous actin (F-actin), are key cytoskeletal elements involved in stabilizing cell-to-cell connections, cellular shape changes and cellular migration. The F-actin has been observed in RTgutGC cells (Drieschner et al., 2017;Minghetti et al., 2017). Their intensity and distribution of stress fibers could be altered after nutrient deprivation (Pumputis, 2017) and phytochemical naringenin treatment (Pumputis et al., 2020).

When cells grow on permeable membrane insert, measurement of the transepithelial electrical resistance (TEER) is used to assess the tightness of cellular epithelium, i.e. the higher TEER values suggesting higher tightness and existing barrier functions (Geppert et al., 2016). In addition, an epithelium is formed shown as polarized epithelial cells which grow as monolayers and are tightly connected via tight junction proteins. A characteristic of such monolayers is the prevention of passage of nanoparticles from the apical to the basolateral chamber (Geppert et al., 2016). Junction proteins, such as claudin 3, ZO-1 and e-cadherin, have been observed in RTgutGC using confocal fluorescence microscopy (Geppert et al., 2016;Langan et al., 2017;Minghetti et al., 2017;Pumputis, 2017). Also, ZO-1 has been observed using scanning and transmission electron microscopy (Minghetti et al., 2017), and its barrier related gene expression level is similar to tissue *in vivo*, but downregulated after immune stimuli exposure with LPS and Poly (I:C) (Schug et al., 2019). Altogether, these observed barrier functions of RTgutGC cells exhibit epithelial cell characteristics as these *in vivo*.

Regarding immune functions, for some immune related genes in RTgutGC cells, compared to *in vivo* tissue, their expression level are similar, for others, however, lower or higher levels have been reported (Schug et al., 2019). Moreover, the immune stimuli (LPS and Poly (I:C)) can activate the *myd88* and Ticam-dependent signaling pathways, which resulted in downstream activation of pro-inflammatory cytokines and interferon (Schug et al., 2019).

Based on these structural and functional characteristics, this cell line has been proposed as a physiologically adequate fish intestinal epithelial model, equivalent to the Caco-2 cell line for human intestinal epithelium (Kawano, 2009;Minghetti et al., 2017). In our work of **Paper III,** the RTgutGC was used to further explore their features and functions as a fish intestinal epithelial model and to assess effects of functional feed ingredients on intestinal epithelial immune, barrier and digestive functions.

AIMS OF THE STUDY

The work presented in this thesis was conducted firstly to strengthen knowledge on gut health and microbiota of Atlantic salmon in commercial production in the Arctic region of Norway, in the period from a late freshwater stage, through the transfer to seawater and onward until the fish had reached about 2 kg. Other goals were to study and reveal mechanisms underlying effects of functional feed ingredients, *in vivo* as well as *in vitro*. In order to achieve the goals, three aims were formulated:

Aim 1: To gain knowledge on fluctuations in performance and gut health and assess the effects of functional feed ingredients in Atlantic salmon in the period from late freshwater stage until one year in seawater under commercial conditions in Arctic region (work presented in **Paper I**).

Aim 2: To strengthen knowledge on the dynamics of gut microbiota assemblage and its potential association with host responses, and to gain understanding of the effects of functional feed ingredients on the microbiota in Atlantic salmon from freshwater to seawater life stages under large scale, commercial conditions in Arctic region (work presented in **Paper II**).

Aim 3: To strengthen knowledge on the characteristics of the RTgutGC cell line and its suitability as *in vitro* model for studies of intestinal epithelial functions in fish, and to better understand the mechanism of action of functional feed ingredients on intestinal epithelial cells by employing this *in vitro* model (work presented in **Paper III**).

HYPOTHESES

The following hypotheses were based on the aims of the current thesis work:

1: Performance, gut health and gut microbiota profile in Atlantic salmon vary substantially during the period from freshwater to one year in seawater, in particular during the seawater transfer period.

2: Functional feed ingredients improve production, fish gut health and stimulate growth of beneficial bacteria in the gut.

3: Specific intestinal bacterial communities in Atlantic salmon show clear associations with host gut functions.

4: The RTgutGC cell model can be used for evaluation of mechanisms underlying effects of functional feed ingredients on intestinal epithelial functions.

SUMMARY OF RESULTS

Paper I and II present the work to gain knowledge on fluctuations in growth performance, gut health and microbiota, as well as potential association of microbiota with host responses, and assessing the effects of functional feed ingredients in Atlantic salmon from late freshwater stage until one year in seawater under commercial conditions in Arctic regions. In these studies, fish were observed from late freshwater stage until one year in seawater at four sampling time points: in May, two weeks before seawater transfer (i.e. FW); in June, four weeks after seawater transfer (SW1); in November (SW2) and in April (SW3) the following year. Two series of diets were fed, varying throughout the observation time in nutrient composition according to the requirements of fish, one without (Ref diet), and the other with functional ingredients (Test diet). The functional feed ingredients, i.e. essential fatty acids, nucleotides, yeast cell walls and a prebiotic, were supplemented to the diets either as single ingredient or as a mixture according to recommendations from the producers.

In the work of **Paper I,** the fish gut health was assessed based on histopathological indicators of lipid malabsorption and gut inflammation, expression of gut immune, barrier and other health related genes, plasma biomarkers, somatic indices of intestinal sections, growth performance as well as biomarkers of digestive functions. For the work of **Paper II**, the microbiota was characterized in samples of the distal intestinal digesta of fish using 16S rRNA gene sequencing.

Effects of sampling time points

The results presented in **Paper I** overall revealed that fish from seawater sampling time points showed higher intestine-somatic indices, plasma ions concentration, as well as expression levels of *aqp8ab* compared to those from FW throughout the observation period. Four weeks after seawater transfer, i.e. at SW1, the fish showed lower levels of plasma cholesterol and triglycerides, as well as lower gene expression levels of some barrier and immune-functions in the DI, i.e. inflammation cytokines (*il1β, il10, tgfβ, ifnγ*), T-cell markers (*cd3γδ*), *myd88* and tight junction proteins (*zo-1, claudin-15, claudin-25b*), than those before seawater transfer (FW). At SW2 and SW3, most of these gut immune biomarkers, as well as plasma cholesterol and triglycerides increased to the levels observed at FW. Hyper-vacuolization was observed in the PC enterocytes indicating a situation of gut mucosa lipid

malabsorption. The severity of lipid malabsorption increased gradually from FW through SW1, culminated at SW2 and decreased at SW3. The increasing symptoms of lipid malabsorption were also corresponded with the up-regulated expression levels of *plin2* that a marker for lipid accumulation.

The work of **Paper II** comprises the characterization of microbiota in distal intestinal digesta throughout the observation period from freshwater to seawater. Analyses of alpha diversity revealed that fish from seawater sampling time points had higher microbial richness, as indicated by Observed species index, while microbial diversity tended to decrease throughout the observation period, as indicated by Simpson's index. Compared to fish from SW1, fish sampled at life stages above four weeks after seawater transfer, i.e. SW2 and SW3, showed more apparent difference from those in fish at FW based on the results of beta diversity. The most abundant phyla Firmicutes (mainly *Lactobacillus*) and Proteobacteria (mainly *Photobacterium*) varied among sampling time points and accounted for about 80% of the total abundance. Specifically, at FW, gut microbiota was dominated by phylum Firmicutes (e.g. genera *Lactobacillus*, *Weissella*, *Peptostreptococcus* and family *Ruminococcaceae*), followed by Proteobacteria (e.g. genera *Photobacterium*, *Deefgea* and *Pseudomonas*) and Bacteroidetes (mainly genus *Flavobacterium*). Of note, the genera *Deefgea*, *Flavobacterium* and *Pseudomonas*, as well as family *Ruminococcaceae* were detected in only a few fish, but when present, they dominated gut microbiota. Four weeks after seawater transfer, i.e. at SW1, Proteobacteria (mainly *Photobacterium*) and Firmicutes (mainly *Lactobacillus*) showed similar presence and dominated the microbiota. As fish progressed towards SW2 and SW3, Firmicutes (mainly *Lactobacillus*) and Tenericutes (mainly *Mycoplasma*) became more prominent with a corresponding decline in Proteobacteria (mainly *Photobacterium*).

Effects of functional feed ingredients

In our study, except fish at SW2, fish fed diet supplementation of these selected functional feed ingredients affected health related-biomarkers (**Paper I**) and gut microbiota (**Paper II**) of fish only marginally. At SW2, compared to those fed Ref diet, fish fed the diet with selected functional feed ingredients tended to grow slower and showed lower condition factor and plasma triglyceride levels (**Paper I**). At the same sampling time point, decreased microbial richness, microbial diversity and the lower relative abundance of LAB were also

observed in Test-fed fish (**Paper II**). At this observation period, but not at the other, it is an important growth stage from June to November. Also, the packages of functional feed ingredients differed from the packages used at the other sampling time points, in its content of a mixture of nucleotides, yeast cell walls and essential fatty acids.

The associations between microbial clades and metadata of interest

The multivariate association analysis identified 27 differentially abundant taxa (**Paper II**) to be significantly correlated with the gene expressions of gut barrier functions. Except *Flavobacterium*, the relative abundance of 26 differentially abundant taxa, including *Megasphaera*, *Photobacterium* and certain LAB, showed a negative correlation with expression levels related to barrier function genes. Moreover, the relative abundance of *Megasphaera* was positively correlated with expression levels of gut immune genes and the levels of plasma nutrients.

RTgutGC *in vitro* study

The aims of the work in **Paper III** were to strengthen knowledge on the characteristics of the RTgutGC cell line and its suitability as *in vitro* model for studies of intestinal epithelial functions, and to better understand the mechanism of action of functional feed ingredients on intestinal epithelial cells.

Overall, the biomarkers of brush border membrane enzyme activity, cell proliferation, as well as barrier and immune functions showing the characteristics of RTgutGC indicated that it is a potential for use as an efficient *in vitro* mode for investigation of effects of functional feed ingredients on intestinal epithelial functions. Specifically, for brush border membrane enzyme activities, the leucine amino peptidase (LAP) and maltase were detected in the RTgutGC cells suggesting RTgutGC develop certain intestinal digestive functions like the *in vivo* situation. The TEER level, as an indicator of cellular barrier function, increased steadily forming tight junction, and thereby strongly prevented albumin translocation from apical to basolateral chamber in our study. Meanwhile, the tight junction protein claudin 3 was clearly observed in RTgutGC via confocal fluorescence microscopy images. Regarding cell proliferation, cells fully immigrated the gap area of culture wells in 4 days. We also compared a variety of important genes related to immune, barrier function and metabolism between RTgutGC and rainbow trout *in vivo* tissues (i.e. PC, MI, DI and liver). The immune related

genes were expressed at comparable relative levels in RTgutGC cells as *in vivo* DI, whereas most genes related to barrier function and metabolism showed lower relative expression in RTgutGC than those in tissue.

LPS, i.e. a PAMP as immune stimulant, elevated gene expression levels of several pro-inflammatory cytokines, including *il1b*, *il6*, *il8* and *tnfa*, as well as tight junction gene Claudin 3 (*cldn3*), but suppressed expression of intestinal alkaline phosphatase (*ialp*). Immunostaining also indicated increased F-actin contents after LPS treatment.

Regarding the effects of functional feed ingredients on RTgutGC, the results of cell viability, based on the metabolic activity and cell membrane integrity of dose-response exposure tests, suggested that 75μg/mL nucleotides, 4 mg/mL MOS and 100μg/mL β-glucans were selected as final working concentration for further analysis. MOS significantly increased albumin translocation and the expression levels of immune-related genes and *cldn3*, but strongly suppressed ROS production, cell proliferation, as well as the gene and protein expression levels of E-cadherin. Treatment of β-glucans induced high gene expression levels of immune (*il1b* and *il8*), barrier (*cldn3* and *cdh1*) and proliferating cell nuclear antigen (*pcna*), as well as clearly increased TEER levels and F-actin content, but decreased protein expression level of E-cadherin. Compared to MOS and β-glucans, nucleotides induced expression levels of only a few genes, i.e. *il1b*, *il8* and *cdh1*.

DISCUSSION: Materials and Methods

Materials

Fish

The feeding trials presented in the work of **Paper I** and **II** were carried out in commercial sized farms. Fish in the freshwater stage were grown in two smolt production tanks, near Bodø (N67° - E14°) of Norway and observed over 15-week for prior to seawater transfer. The tanks were large closed aluminum, flow-through system (depth: 2.8 m, volume: 450 m^3) supplied with water from a nearby lake. Each tank contained about 180 000 fish. When ready for seawater transfer, the fish were transported to a sea location near Alta (N70° - E22°) of Norway. The fish from each tank were distributed into 3 sea cages, i.e. triplicate cages for each diet.

In freshwater stage, only one tank was used for each diet, as the tanks of the freshwater facility was too big, and too few, to allow use of more than one tank per diet. Our choice to help this situation with lack of replicate tanks, as tank variation was expected to be negligible, was to sample three groups of fish from each of the two tanks and consider these as proper replicates. The results of our study confirmed that the tank variation was small and that this approach should be acceptable, as the means of biomarkers of the fish in the two tanks did not differ significantly, and the variances were similar, indicating no important tank variation.

Diets

For long-term experiments under commercial conditions, fish may sense and respond to a range of environmental variables and aquaculture practices. Moreover, diet composition must change according to the nutritional requirements of the fish. As the production conditions in the north are unpredictable regarding attacks of pathogens and parasites, also the supplementation with functional feed ingredients varied throughout the observation period. They were given alone or in combination depending on the expected physiological and environmental challenges. From a scientific point of view, this complicates the discussion of the results somewhat, since environmental variables and characteristics of ingredients themselves (e.g. levels and during of administration) are important factors for

their effects (Ringø et al., 2016;Guerreiro et al., 2018;Hossain et al., 2019). However, this type of feeding trials represents an important validation step between small-scale experimental trial of limited duration and the introduction of new diets into the salmon production that resemble the current commercial practices.

Sample origin for gut microbiota profiling

For Atlantic salmon, intestinal bacterial communities vary from proximal to mid to distal intestinal regions (Gajardo et al., 2016). These differences could be attributed to the difference in nutrient concentrations between intestinal regions, since the digestive and absorptive functions removed nutrients from the chyme, as the chyme moves from the proximal to the distal region (Bakke-McKellep et al., 2000;Denstadli et al., 2004). Moreover, the level of endogenous digestive components, such as digestive enzymes secreted by the pancreas (Krogdahl et al., 2015b) and bile acid from the liver (Kortner et al., 2013;Gu et al., 2014), diminish along the intestine (also observed in **Paper I** of the current work**)** and could be of importance for microbial composition.

Some previous studies have also managed to explore independently the digesta-associated and mucosa-associated gut microbiota in Atlantic salmon, and found substantial differences between them, with higher microbial richness and diversity in the former (Gajardo et al., 2016;Gajardo et al., 2017;Gupta et al., 2019;Jaramillo-Torres et al., 2019). Digesta- and mucosa-associated gut microbiota may also respond differently to dietary modulation. Mucosa-associated gut microbiota often shows higher resilience towards changes in diet composition (Gajardo et al., 2017;Huyben et al., 2018;Rimoldi et al., 2019;Terova et al., 2019;Li et al., 2020), but contradictory results have also been reported (Jaramillo-Torres et al., 2019).

The majority of studies investigating gut microbiota of Atlantic salmon have used digesta than mucosa samples. The causes may be grouped in three. Firstly, standardized collection of mucosa samples for microbial profiling may be challenging, since collecting mucosa tissues after removing the remaining digesta strictly depends on the fish size and consistency of digesta (Gajardo et al., 2016;Gajardo et al., 2017;Gupta et al., 2019;Jaramillo-Torres et al., 2019). Secondly, based on our experience, even after multiple optimization steps for the

PCR amplification, there is still a high probability to get low quality PCR products for sequencing. Finally, although digesta-associated gut microbiota lacks the attachment to the intestinal mucosa, they are presumed to affect functions and health of the host through their metabolite production, such as short chain fatty acids (Gajardo, 2016;Petit et al., 2019). The existing knowledge on the association of these bacteria with systemic health in Atlantic salmon is still limited.

With this background, in the work of **Paper II**, we decided to investigate gut microbiota of Atlantic salmon using the distal intestinal digesta. An additional reason for focusing on the DI digesta was that lower levels of bile acids and activities of digestive enzymes, but higher levels of indigestible material, e.g. fiber, in the DI may benefit the growth of bacteria and increase the microbial abundance (Gajardo et al., 2016). Another consideration was that higher mucus production and immunological functions are observed in DI than those in other intestinal segments (Petrie and Ellis, 2006;Harstad et al., 2008;Lokka et al., 2014;Moldal et al., 2014), and therefore the samples from the DI may provide a better possibility to explore associations between gut microbiota and host gut immune functions.

Methods

Microbiota characterization by 16S ribosomal RNA gene sequencing

In the past decade, the culture-independent approaches, in particularly high-throughput sequencing of the 16S rRNA gene, have become more common for studying gut microbiota composition due to more affordable costs and rapid development of bioinformatics pipelines. These methods comprise a range of processing steps, such as sample collection, sample storage (fresh or frozen samples), DNA extraction, choice of primers targeting different hypervariable regions, library preparation, choice of sequencing platform, bioinformatics pipelines and down-stream analysis strategy. It is important to remember that bias can occur at all processing steps, which may affect the final microbiota profiling (Pollock et al., 2018). There are some initiatives or guidelines which can help decrease bias (Pollock et al., 2018;Eisenhofer et al., 2019). Also the experimental procedures may differ, due to lack of standardization, a fact which can hamper meta-analyses of salmon microbiota studies. Better standardized protocols are necessary to integrate the information and allow easy comparison across studies.

In the work presented in **Paper II**, we characterized the gut microbiota based on high-throughput sequencing of the 16S rRNA gene using the Illumina Miseq platform, which is now the most commonly used method (Perry et al., 2020). In the work of this thesis, we focused on two important, but often neglected issues using this method: bioinformatics analysis and quality control (see below in **DISCUSSION: Results**).

Operational taxonomic unit (OTU) has been the common operational definition for classifying bacterial communities by 16S rRNA gene sequencing data until 2018. This method uses the clusters of reads based on shared similarity thresholds (defined at ≥ 97% sequence similarity level) (Blaxter et al., 2005). Recently, new data processing methods have been developed requiring new terms, i.e. amplicon sequence variants (ASVs). The ASVs can avoid clustering sequences at a similarity threshold and instead uses only unique, identical 16S rRNA sequences for downstream community analyses (Callahan et al., 2017). In the present work of **Paper II**, the bioinformatics analyses were performed to generate ASVs, and downstream analyses were conducted at the genus level. Compared to the OTUs method, the ASVs approach could improve taxonomic resolution through resolving sequences that differ by as little as one base pair and avoid similarity-based clustering, as supported by one recent study (Gosmann et al., 2017). Moreover, using the ASVs-based approach, the sequences within each ASVs are identical, which means that it is easier to compare sequences between different datasets compared to using the OTUs approach (Callahan et al., 2017). However, the ASVs approach is not inherently better than OTUs. For example, regarding the alpha (i.e. measuring the diversity within a sample) and beta diversity (i.e. comparing the differences between samples), previous studies found that the ASVs approach had similar (Glassman and Martiny, 2018) or lower alpha and beta diversity compared to those using OTUs method (Capunitan, 2018). This contradicts the expectation that using the ASVs approach would increase alpha and beta diversity by improving taxonomic resolution. The reason may be that ASVs approach is too sensitive to data quality. When the data quality is very low or has sequencing errors, there will be a significant reduction in reads before downstream analysis. Each approach has advantages and disadvantages. When we put up a set of standard protocols for characterizing gut microbiota profiling, the method of bioinformatics analysis should also be considered to allow easy comparisons across studies.

Permeable membrane for RTgutGC barrier model

Two-compartment insert systems, i.e. cells cultured on a commercial polymer-based

permeable membrane insert with around 10 μm thick and 3 μm pore sizes, have been widely used for *in vitro* epithelial cell barrier models. In the work of **Paper III**, we utilized and further developed an established two-compartment barrier model system based on the RTgutGC cell line. This system has in recent years become a common culture model for studying physiological and toxicological responses in fish (Geppert et al., 2016;Langan et al., 2017;Minghetti et al., 2017;Pumputis, 2017;Langan et al., 2018;Schug et al., 2019). Given that the *in vivo* basement membrane is less than 1 μm thickness with a 3-D shape and high permeability (LeBleu et al., 2007), while the standard commercial permeable membrane inserts used are about 10 μm thick, the limitations regarding the thickness, porosity and permeability in this system may affect the relevance of some sensitive measurements (Jud et al., 2015;Drieschner et al., 2017). For example, high variation of TEER levels was found in **Paper III**. In order to better the mimicking of the structure of fish gut epithelium, an ultrathin aluminum membrane with 1 μm thickness has recently been developed for use in the two compartment system, resulting in higher permeability rates for small molecules, enabling high quality microscopy and sensitive measurement of TEER compared to those using traditional permeable membranes (Drieschner et al., 2017). Unfortunately, the released aluminum ions from aluminum membranes might interfere with metal exposure or other exposure tests. Another new membrane recommended for the RTgutGC barrier model is made by silicon nitride membranes with 500 nm thickness and 1.2 μm pore size. The permeability of this membrane has been reported to better correspond with *in vivo* basement membrane levels, and gives higher quality microscopy without negative effects of aluminum ions (Drieschner et al., 2019). These membranes will, supposedly, improve the cell barrier model and give even more reliable results. However, unlike the commercial permeable membrane inserts, these membranes are costly tools, still in a research developmental stage, and should be critically evaluated in future studies.

DISCUSSION: Results

Based on the methodological discussion above, the following topics seem to deserve further discussion beyond the discussion in **Paper I-III**.

1: Quality control for gut microbiota profiling.

2: Association between gut microbiota and host responses.

3: Possible negative effects of use of some functional feed ingredients.

4: RTgutGC as a tool for fish nutrition studies.

Quality control for gut microbiota profiling

In the experiment presented in **Paper II**, in order to critically evaluate and optimize the microbiota profiling workflow, a mock, i.e. a microbial community standard, was added for each DNA extraction batch as a positive control. The mock includes the following 8 bacteria, i.e. 12% *Listeria*, 12% *Pseudomonas*, 12% *Bacillus*, 12% *Escherichia*, 12% *Salmonella*, 12% *Lactobacillus*, 12% *Enterococcus*, 12% *Staphylococcus*, and two yeasts, i.e. 2% *Saccharomyces* and 2% *Cryptococcus*.

The analyses of relative abundance of microbiota in the mock samples (**Figure 4)** identified all eight bacteria at genus level which together accounted for about 99.9% of the total abundance, suggesting that the workflow was reliable for taxonomic profiling of the gut microbiota. The mock from 8 different DNA extraction batches showed similar microbiota profile indicating good reproducibility and no significant batch effect.

Our observations in the mock of *Lactobacillus* (33 ± 2%, mean ± S.D), *Salmonella* (19 ± 1%), *Escherichia-Shigella* (14 ± 0.3%), *Bacillus* (13 ± 0.7%), *Pseudomonas* (11 ± 0.6%), *Staphylococcus* (6 ± 1%), *Enterococcus* (1 ± 0.1%), *Listeria* (1 ± 0.1%) and other (0.4 ± 0.5%), showed different concordance between the theoretical compositions based on genomic DNA in mock. The reasons could be attributed to the bias during the microbial processing steps, since some taxa could be down or over represented due to bias introduced during DNA extraction and PCR amplification (Brooks et al., 2015;Earl et al., 2018;McLaren et al., 2019). There is a need to develop the methodologies to overcome this. For example, sequencing of the whole 16S rRNA gene, rather than a small sequence region would be an improvement (Klemetsen et al., 2019). Whether a study has employed, the Mock samples

should be taken into account when comparing studies with different methods.

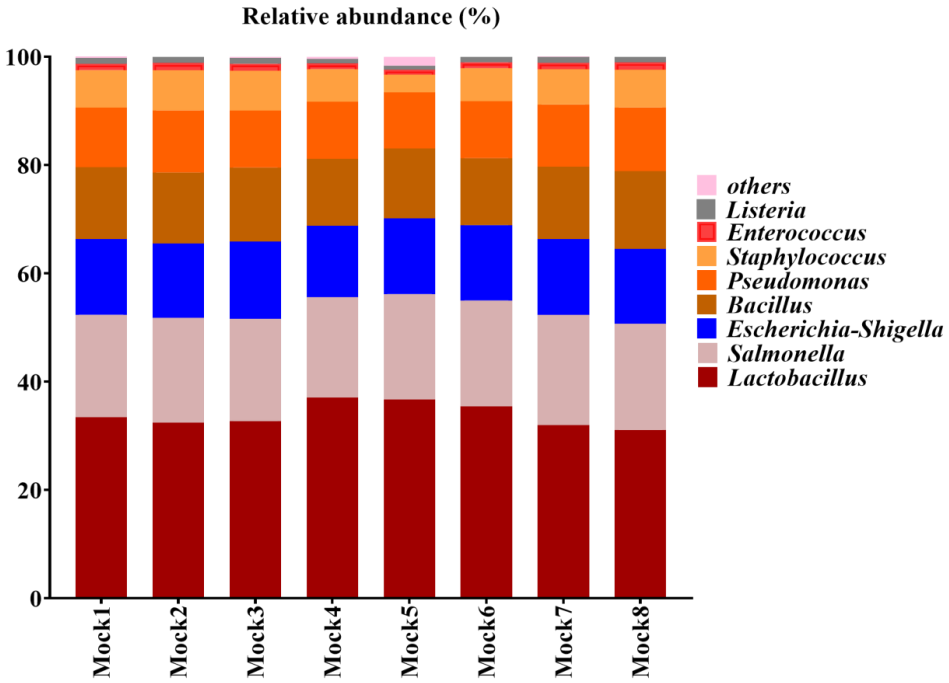

Figure 4: *The relative abundance of microbiota in mock samples from 8 DNA extraction batches.*

Contamination is a common challenge in gut microbiota studies using the 16S rRNA gene sequencing. The contamination problems can be reduced following appropriate laboratory practices, but do not eliminate it, since they are also easily caused by the contaminated reagents, such as PCR master mixes and water (Salter et al., 2014;Eisenhofer et al., 2019). In the work of **Paper II,** the contaminants were identified based on their presence in negative controls and their inverse relationship with bacterial DNA levels according to the description of Davis et al. (2018). The removed sequences were classified as *Acinetobacter*, *Aeromonas, Cutibacterium, Flavobacterium, Leptothrix*, *Pseudomonas,* an unclassified bacterium of order *Chitinophagales* and *Betaproteobacteriales*, as well as *Streptophyta*. Among these taxa, *Streptophyta* is usually assumed as chloroplast sequences to reflect non-bacterial-associated taxa (Parris et al., 2016). *Pseudomonas* and *Cutibacterium* have been reported as contaminants in salmon microbiota studies previously (Krogdahl et al., 2020;Li et al., 2020), while *Acinetobacter*, *Flavobacterium* and *Leptothrix* have also been commonly found in

negative controls (Eisenhofer et al., 2019). Notably, some removed taxa, such as *Pseudomonas* and *Flavobacterium*, have been reported to be core bacteria of gut microbiota in Atlantic salmon whether using culture-based (Cantas et al., 2011;Hatje et al., 2014) or culture-independent methods (Gajardo et al., 2016;Gajardo et al., 2017;Rudi et al., 2018;Lokesh et al., 2019). One potential explanation for this discrepancy is that it is still hard to distinguish contaminating species from true community members based on taxonomic labels as the amplicon sequencing taxonomic resolution is very low. On the other hand, the method of Davis et al. (2018) still has one limitation to remove the cross-contamination. Contamination issues should always be addressed in microbiome analyses, and strategies for removal in microbiota studies should be employed, as it may influence the outcome of microbial community survey.

Association between gut microbiota and host responses

When evaluating gut microbiota, the question arises: do the observed changes in microbiota composition have any implications for host responses? In mammals, intestinal bacterial communities have been found to play key roles in a range of host physiological processes, such as mucosal immune responses (Kamada and Nunez, 2013;Sommer and Backhed, 2013), lipid metabolism (Petersen et al., 2019), the central nervous system via brain-gut microbiota axis (Lyte and Cryan, 2014), function and maturation of mucus layer (Petersson et al., 2011;Schroeder, 2019) and energy homeostasis (Rosenbaum et al., 2015). Although the knowledge of gut microbiota in fish lags that of mammals, it is clear that alterations in intestinal bacterial composition may influence host physiological responses and induce disease development, such as nutrient digestion (Ray et al., 2012;Semova et al., 2012;Falcinelli et al., 2015), development and maturation of gut-associated lymphoid tissue (Austin, 2006;Gomez and Balcazar, 2008;Merrifield and Ringø, 2014), energy homeostasis (Butt and Volkoff, 2019), gut immunity (Lopez Nadal et al., 2020) and dysbiosis (Infante-Villamil et al., 2020).

In the work of **Paper II**, we identified differentially abundant taxa to be significantly associated with gut immune and barrier gene expressions, and plasma nutrients. Our finding is an important step to reveal the potential implications of variation in gut microbiota and could supply valuable information for further manipulation of these observed taxa to test hypothesis regarding their action. Very recent studies, in Atlantic salmon, have shown

significant association between gut microbiota composition and growth performance (Bozzi et al., 2020), flesh pigmentation (Nguyen et al., 2020a;Nguyen et al., 2020b), lipid metabolism (Dvergedal et al., 2020), immune responses in head kidney (Parra et al., 2020), as well as immune and barrier functions in the gut (Li et al., 2020).

The genus *Photobacterium* is often reported to belong to the core bacteria of the Atlantic salmon gut (Gajardo et al., 2016;Gajardo et al., 2017;Catalan et al., 2018;Rudi et al., 2018;Fogarty et al., 2019;Jaramillo-Torres et al., 2019;Lokesh et al., 2019), and is commonly found in the seawater where Atlantic salmon sampled environment (Rud et al., 2017). Its increasing abundance after seawater transfer may be attributed to the drinking behavior of post-smolt salmon to prevent dehydration, as supported by the work in **Paper II**. Multivariate association analysis identified a negative relationship between the expression levels of barrier function genes and relative abundance of genus *Photobacterium* (**Paper II**). In this context, it is interesting to note that previous studies have demonstrated that certain *Photobacterium* species may cause disease outbreaks in salmon right after seawater transfer, and that this may be linked to malfunction of the gut barrier (Osorio et al., 2000;Zhao et al., 2009). Further studies are therefore recommended to investigate the potential relationship between *Photobacterium* species, gut barrier functions, and disease resistance in salmon.

Our findings in **Paper II** clearly demonstrated that the relative abundance of *Megasphaera,* a genus of phylum Firmicutes, was positively correlated with gut immune gene expressions. This finding is partly in line with the work of Parra et al. (2020) showing filifolinone-induced immune responses via increasing the abundance of phylum Firmicutes. One mucosa-enriched taxa *Brevinema andersonii* in Atlantic salmon has also been found to be correlated with expression levels of gut immune genes (Li et al., 2020). Published studies revealing effects of variation in gut microbiota on immune responses in Atlantic salmon are still limited, but gut microbiota in zebrafish has been found to be related to 212 function genes, including immune functions (Rawls et al., 2004) and to stimulate immune system (Rolig et al., 2015). It is assumed that these bacteria shape the host's immune functions and regulate host metabolic functions.

Despite that our findings fill certain knowledge gaps regarding the association between gut microbiota and host functions, it is urgent to reveal the mechanisms behind these associations

as many knowledge gaps still dominate the current status. The general lack of information has stimulated us to recommend using more holistic technologies and methods to supply improved resolutions to reveal their interplay between systemic functionality. The novel technologies, such as multi-omics approaches (e.g. genomics, transcriptomics, proteomics and metabolomics), immunohistochemistry, fluorescence-activated cell sorting and imaging, have been used in fish, which could provide comprehensive insights into the suite of functional contributions to the host (Legrand et al., 2020). The existing manipulative methods, such as bacteria isolation, fecal transplants and germ-free salmon models, are required to determine whether these observed bacteria are responsible for these functional roles, rather than associated with host responses.

Possible negative effects of use of some functional feed ingredients.

The lack of predicted effects of functional feed ingredients in the present experiments of **Paper I and II** raises questions regarding the representativeness of the majority of relevant scientific studies (reviewed by (Merrifield and Ringø, 2014;Rignø, 2014;Song et al., 2014;Akhter et al., 2015;Dawood et al., 2018;Guerreiro et al., 2018;Hossain et al., 2019)). Our findings indicated that certain functional feed ingredients when supplemented to Atlantic salmon feed do not always exert beneficial actions, and may represent a cost regarding energy utilization and growth. It is, however, known that, besides characteristics of functional feed ingredients themselves, dosage and duration of oral administration are two key determinants in such effects, as discussed below.

Dosage and duration of administration

It is widely accepted that high dosage of some functional feed ingredients, beyond what is indicated as optimum dosage, may reduce performance and affect physiological functions in fish. This has been shown in studies of red sea bream, fed high levels of nucleotides, showing clear negative effects on growth performance and stress resistance (Hossain et al., 2016a;Hossain et al., 2016b;Hossain et al., 2016c). The possible explanation is the suppression of digestive enzyme and immune functions. Increasing nucleotides levels have been observed to cause reduction in digestive enzyme activities, as shown for alkaline protease, lipase and amylase (Safari et al., 2015), as well as reduction in immune responses, such as in lysozyme and bactericidal activities (Burrells et al., 2001a;Welker et al., 2011;Hossain et al., 2016a). In the work presented in **Paper I** and **II**, the levels of functional

feed ingredients cannot be disclosed due to commercial interests and production of intellectual rights. However, they are within the ranges suggested by the producers. Therefore, excessive level is unlikely to be the explanation for the seemingly negative effects observed in the present study.

Whether or not supplementation with some functional feed ingredients have beneficial effects may depend on the duration of the exposure (Sakai, 1999). For instance, 8-week administration of nucleotide to hybrid striped bass increased antibody responses significantly, as well as enhanced disease resistance against *S. iniae*, while 16-week administration did not show such enhancement of immunity and disease resistance (Li et al., 2004). Compared to short-term administration, long-term administration causing detrimental effects were also observed for β-glucan supplementation in previous studies of catfish (Yoshida et al., 1995), turbot (Ogier de Baulny et al., 1996) and sea bass (Bagni et al., 2005), as well as for nucleotide-supplemented diets fed to red drum (Li et al., 2007). Our results presented in **Paper I** and **II** showed that at SW2 Atlantic salmon fed diet supplemented with a mixture of nucleotides, yeast cell walls and essential fatty acids, tended to grow slower and showed significantly lower CF and plasma triglyceride levels. Also, the microbial richness and diversity, and the relative abundance of LAB were significantly reduced at this time point. These results may suggest that long term exposure, in this case a 10-week administration, may cause negative effects on fish health, and have economic consequences for the farmer. Similarly, more than 20-week administration of a mixture of nucleotides, a prebiotic and essential fatty acids in fish at SW3 showed higher proportion of mild steatosis and a tendency to slower growth (**Paper I**). There is a need to define the optimal use of functional feed ingredients, which probably will differ between ingredients and conditions for the fish. It is obvious that available scientific literature has large knowledge gaps regarding effects of duration of administration for functional feed ingredients, as stated in recent reviews (Dawood et al., 2018;Guerreiro et al., 2018;Hossain et al., 2019).

Are the elicited immune responses or changes of microbiota always beneficial?

The claims regarding mechanism underlying health benefits of functional feed ingredients in fish are supposedly directly or indirectly regulated via two ways: one regards stimulation of the immune system, the other regards the growth of bacteria considered beneficial for the animal. For both categories improvement of disease resistance is a goal (Song et al.,

2014;Hossain et al., 2019).

For immune responses, lack of effects of functional feed ingredients on immune related genes were observed in the *in vivo* trial (**Paper I**), while selected functional feed ingredients mostly induced immune gene expressions in the *in vitro* treatments (**Paper III**). The question arises whether or not functional feed ingredients induced immune responses which will result in increasing immunity to alleviate stress and ward off pathogens? Unfortunately, most of the available data in the literature focus on the host immune responses (mainly cytokine parameters) and are interpreted to modulate the immune system, whereas far fewer studies shed light regarding effects in challenge trials, and only a few publications report both (as reviewed (Song et al., 2014;Akhter et al., 2015;Guerreiro et al., 2018;Hossain et al., 2019). Some functional feed ingredients may induce either pro- or anti-inflammatory cytokine responses, as shown in our work presented in **Paper III**, making implications for disease resistance difficult to predict without challenge test.

With this background, another question arises whether some immune responses induced by functional feed ingredients may have negative effects on fish health? Regarding humans, it is suggested that beneficial effects of prebiotics in terms of enhancing immune system are regulated via increasing expression of anti-inflammatory cytokines genes and decreasing expression of pro-inflammatory cytokines genes (Shokryazdan et al., 2017). Immune responses, such as regulation of pro-inflammatory cytokines, may therefore indicate detrimental effects in the host, since the up-regulation of expressions of genes of pro-inflammatory cytokines is often a result of infection or inflammation responses (Ibrahim and El-Sayed, 2016) and enteritis in Atlantic salmon (Krogdahl et al., 2015a). As such, in the absence of immunological challenge trials or other health related indictors, the molecular immune biomarkers alone may not predict real effects on fish health.

Regarding gut microbiota, LAB have been identified as a major player in the Atlantic salmon gut and are presumed to have beneficial effects on the host through modulation of immune responses, improvement of digestive processes and protection against pathogenic bacteria, at least under certain conditions (Ringø and Gatesoupe, 1998;Balcázar et al., 2007;Ringø et al., 2010). In our study, diets with functional feed ingredients caused a decrease in the relative abundance of LAB (mainly genus *Lactobacillus*) in fish at SW2 (**Paper II**), in contrast to

what was expected. Whether this effect was related to the indication of increased metabolic cost and growth retardation (**Paper I**), cannot be decided. The presumption that LAB are beneficial, is challenged as there are indications that increased population of LAB may not always be beneficial in our study. This is in line with the previous concerns regarding the efficacy and safety of feeding some probiotics, such as some *Lactobacillus* strains to mammals (reviewed by (Martinez et al., 2015;Lerner et al., 2019)). Their concerns are supported by previous studies that *Lactobacillus plantarum*, which is a potent strain of probiotics, could disrupt healthy intestinal tissues in humans (Tsilingiri et al., 2012) and worsen colitis in mice (Mileti et al., 2009). The increasing susceptibility to infectious disease has also been observed in tilapia following the suspension of administration of some probiotic *Lactobacillus* strains (Liu et al., 2016). Regarding Atlantic salmon, previous studies have shown that salmon fed soybean meal, replacing fish meal, which developed the classical soy-induced enteritis in the distal intestine, also showed high levels of LAB in the digesta of the distal intestine (Reveco et al., 2014;Gajardo, 2016). From these studies LAB does not appear to protect against immune challenging conditions (Reveco et al., 2014;Schmidt et al., 2016;Gajardo et al., 2017;Krogdahl et al., 2020). Therefore, present knowledge is not sufficient to conclude the population of LAB leads to beneficial or detrimental effects on salmon gut health. It may for example depend on the challenge and level of LAB.

It is noteworthy that, compared to the great number of previous studies that claim to show positive effects of functional feed ingredients, very few studies published to date have shown non-beneficial effects of functional fish feeds. For aquaculture production, lack of intended, beneficial effect of inclusion of functional feed ingredients, as we demonstrate in **Paper I** and **II**, has merit and deserves attention and support to increase the basis for taking decisions regarding how and when functional feed ingredients should be used.

RTgutGC as a tool for fish nutrition studies

Intestinal epithelial cell lines are useful tools for *in vitro* study of intestinal epithelial responses to various components, including nutrients and dietary additives. Regarding fish, RTgutGC, the only fish cell line available for similar studies, still under development and characterization, has given the opportunity to explore mechanisms underlying effects of fish feed additives.

Our observations presented in **Paper III** confirmed the possibilities of RTgutGC for exploring the effects of bioactive compounds and found that treatment with functional feed ingredients, i.e. MOS, nucleotides and β-glucan, significantly increased gene expression levels of immune functions. This seems to be in line with earlier findings that β-glucan treatment increased expression of il1β at the both transcript and protein levels (Schmitt et al., 2015). The observation of increasing TEER levels after beta-glucan treatment suggests the increase in barrier function in our study (**Paper III**). Similar finding has been found in phytochemical naringenin treatment (Pumputis et al., 2020). Contrarily, MOS treatment seems to weaken barrier functions, as supported by the increasing fluorescently-labeled albumin permeation from apical to basolateral chamber (**Paper III**). Moreover, like MOS treatment in the **Paper III**, phytochemical naringenin treatment impeded cell migration (Pumputis et al., 2020). Collectively, these findings indicate that the RTgutGC cell line responds to these components showing various physiological functionalities and features, which can accumulate valuable information helping predict responses *in vivo*.

It is also suggested that RTgutGC cell line may lose some of inherent functionalities and features due to the decreased number of stimuli under continuous cultivation. As such, a more comprehensive characterization of RTgutGC cell line should be completed based on their purpose of use, as we conducted in **Paper III**. Additionally, the limitations of establishing intestinal barrier model using the commercial polymer-based permeable membrane have stimulated the researchers to explore improved membranes or culture models, such as fish-gut-on-chip model based on the co-culture of RTgutGC and RTgutF (intestinal fibroblasts) (Drieschner et al., 2019). These improved models should highly mimic fish gut complexity and narrow the gap between *in vitro* and *in vivo*, and thereby support more detailed and accurate features and functions underlying effects of aquatic feed ingredients.

MAIN CONCLUSIONS

Sampling time points

- Gut functions, health biomarkers and gut microbiota composition varied greatly between sampling time points.
- Seawater transfer caused great temporary reductions in plasma cholesterol and triglycerides levels, as well as expression of genes involved in intestinal immune and barrier functions.
- Three major genera *Lactobacillus*, *Photobacterium* and *Mycoplasma* varied between sampling time points, while *Lactobacillus* and *Mycoplasma* increased its relative abundance with time.

Functional feed ingredients

- Inclusion of functional feed ingredients, alone or in a mixture of nucleotides, yeast cell walls, a prebiotic and essential fatty acids, affected the fish only marginally.
- At SW2, when fish fed diet with a mixture of nucleotides, yeast cell walls and essential fatty acids, lower performance and reduced microbial richness and diversity, and reduced relative abundance of LAB were observed.
- Supplementation with some functional feed ingredients may represent a metabolic cost and may not always be beneficial for fish.

Multivariate association

- Different taxa, in particularly genus *Megasphaera*, were significantly associated with gut immune and barrier gene expressions, as well as plasma nutrient levels.

RTgutGC *in vitro* study

- The RTgutGC cell line possesses many features similar to that intestinal epithelial cells *in vivo*, indicating its usefulness for use as an efficient *in vitro* model for the evaluation of effects of functional feed ingredients on intestinal epithelial functions.
- MOS appeared as a potent modulator of immune and barrier functions in

RTgutGC.

- Treatment with beta-glucans regulated RTgutGC immune functions, increased barrier functions.
- Nucleotides induced expression levels of only a few immune and barrier genes.

FUTURE PERSPECTIVES

This thesis provides new information of growth performance, gut functions and health, as well as gut microbiota in farmed Atlantic salmon from freshwater to seawater production period in the Arctic region and effects of functional feed ingredients. Moreover, the present thesis suggests that the RTgutGC cell line can be a potential efficient *in vitro* model to gain new knowledge on how functional feed ingredients can affect intestinal epithelial functions in fish. Based on the conducted experiments and the results obtained, the following questions are among those which should be addressed in the future studies:

- What are the causes and consequences of the suppression of gut immune functions of farmed Atlantic salmon right after seawater transfer?
- How to improve host health during freshwater to seawater transition?
- What are nutritional and health consequences of variation in LAB and other microbes in the gut microbiota?
- How can we modulate gut microbiota to improve host health?
- Why does application of functional feed ingredients not always have beneficial effects?
- How can the correlation between *in vitro* and *in vivo* studies be increased?

REFERENCES

Akhter, N., Wu, B., Memon, A.M., and Mohsin, M. (2015). Probiotics and prebiotics associated with aquaculture: A review. *Fish Shellfish Immunol* 45, 733-741.

Almås, K.A., and Ratvik, I. (2017). OC2017 A-092-Sjøkart mot 2050-Tiltak for utvikling av biologisk baserte marine næringer mot 2050.

Amasheh, M., Andres, S., Amasheh, S., Fromm, M., and Schulzke, J.D. (2009). Barrier effects of nutritional factors. *Ann N Y Acad Sci* 1165, 267-273.

Austin, B. (2006). The bacterial microflora of fish, revised. *ScientificWorldJournal* 6, 931-945.

Bagni, M., Romano, N., Finoia, M., Abelli, L., Scapigliati, G., Tiscar, P.G., Sarti, M., and Marino, G. (2005). Short-and long-term effects of a dietary yeast β-glucan (Macrogard) and alginic acid (Ergosan) preparation on immune response in sea bass (*Dicentrarchus labrax*). *Fish Shellfish Immunol* 18, 311-325.

Bakke-Mckellep, A.M., Froystad, M.K., Lilleeng, E., Dapra, F., Refstie, S., Krogdahl, A., and Landsverk, T. (2007). Response to soy: T-cell-like reactivity in the intestine of Atlantic salmon, *Salmo salar* L. *J Fish Dis* 30, 13-25.

Bakke-Mckellep, A.M., Nordrum, S., Krogdahl, Å., and Buddington, R. (2000). Absorption of glucose, amino acids, and dipeptides by the intestines of Atlantic salmon (*Salmo salar* L.). *Fish Physiol* 22, 33-44.

Bakke, A.M., Glover, C., and Krogdahl, Å. (2010). "Feeding, digestion and absorption of nutrients," in *Fish physiology*. Elsevier), 57-110.

Balcázar, J.L., De Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Gironés, O., Muzquiz, J.L.J.F.I., and Microbiology, M. (2007). Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). *FEMS Microbiol Ecol* 51, 185-193.

Beck, B.H., and Peatman, E. (2015). *Mucosal health in aquaculture.* Amsterdam ; New York: Elsevier/AP, Academic Press is an imprint of Elsevier.

Bengmark, S. (2013). Gut microbiota, immune development and function. *Pharmacol Res* 69, 87-113.

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

Shellfish Immunol 107, 172-186.

Blaxter, M., Mann, J., Chapman, T., Thomas, F., Whitton, C., Floyd, R., and Abebe, E.J.P.T.O.T.R.S.B.B.S. (2005). Defining operational taxonomic units using DNA barcode data. *Philos Trans R Soc Lond B Biol Sci* 360, 1935-1943.

Bozzi, D., Rasmussen, J.A., Carøe, C., Sveier, H., Nordøy, K., Gilbert, M.T.P., and Limborg, M.T. (2020). Salmon Gut Microbiota Correlates With Disease Infection Status: Potential for Monitoring Health in Farmed Animals.

Brooks, J.P., Edwards, D.J., Harwich, M.D., Rivera, M.C., Fettweis, J.M., Serrano, M.G., Reris, R.A., Sheth, N.U., Huang, B., and Girerd, P. (2015). The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies. *BMC Microbiol* 15, 66.

Buchinger, T.J., Li, W., and Johnson, N.S. (2014). Bile salts as semiochemicals in fish. *Chemical senses* 39, 647-654.

Burrells, C., Williams, P., and Forno, P.J.A. (2001a). Dietary nucleotides: a novel supplement in fish feeds: 1. Effects on resistance to disease in salmonids. *Aquacu* 199, 159-169.

Burrells, C., Williams, P., Southgate, P., and Wadsworth, S. (Year). "The effects of a nucleotide-enriched diet on experimental infestation with sea lice (Lepeophtheirus salmonis) and on re-infestation rates following anti-louse bath treatment with cyperthin", in: *SCI Conference, Aberdeen*).

Burrells, C., Williams, P., Southgate, P., and Wadsworth, S.J.A. (2001c). Dietary nucleotides: a novel supplement in fish feeds: 2. Effects on vaccination, salt water transfer, growth rates and physiology of Atlantic salmon (*Salmo salar* L.). *Aquacu* 199, 171-184.

Butt, R.L., and Volkoff, H. (2019). Gut Microbiota and Energy Homeostasis in Fish. *Front Endocrinol (Lausanne)* 10, 9.

Callahan, B.J., Mcmurdie, P.J., and Holmes, S.P. (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J* 11, 2639-2643.

Cantas, L., Fraser, T.W.K., Fjelldal, P.G., Mayer, I., and Sorum, H. (2011). The culturable intestinal microbiota of triploid and diploid juvenile Atlantic salmon (*Salmo salar*) - a comparison of composition and drug resistance. *BMC Vet Res* 7, 71.

Capunitan, D. (2018). Comparison of OTUs and ASVs in 73 Bird Species from Equatorial Guinea.

Carbone, D., and Faggio, C. (2016). Importance of prebiotics in aquaculture as

immunostimulants. Effects on immune system of *Sparus aurata* and *Dicentrarchus labrax*. *Fish Shellfish Immunol* 54, 172-178.

Carolina, A. (2018). Modulation of selected inflammatory responses and non-specific defenses in Atlantic salmon induced by beta-1, 3/1, 6-glucans (Macrogard®). Norwegian University of Life Sciences, Ås.

Catalan, N., Villasante, A., Wacyk, J., Ramirez, C., and Romero, J. (2018). Fermented Soybean Meal Increases Lactic Acid Bacteria in Gut Microbiota of Atlantic Salmon (*Salmo salar*). *Probiotics Antimicrob. Proteins* 10, 566-576.

Davis, N.M., Proctor, D.M., Holmes, S.P., Relman, D.A., and Callahan, B.J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* 6, 226.

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

Dehler, C.E., Secombes, C.J., and Martin, S.A. (2017). Environmental and physiological factors shape the gut microbiota of Atlantic salmon parr (*Salmo salar* L.). *Aquacu* 467, 149-157.

Denstadli, V., Vegusdal, A., Krogdahl, Å., Bakke-Mckellep, A.M., Berge, G.M., Holm, H., Hillestad, M., and Ruyter, B. (2004). Lipid absorption in different segments of the gastrointestinal tract of Atlantic salmon (*Salmo salar* L.). *Aquacu*240, 385-398.

Dimitroglou, A., Merrifield, D.L., Moate, R., Davies, S.J., Spring, P., Sweetman, J., and Bradley, G. (2009). Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Anim. Sci. J* 87, 3226-3234.

Dimitroglou, A., Reynolds, P., Ravnoy, B., Johnsen, F., Sweetman, J., Johansen, J., and Davies, S.J.J.a.R.D.S. (2011). The effect of mannan oligosaccharide supplementation on Atlantic salmon smolts (*Salmo salar* L.) fed diets with high levels of plant proteins. *J Aquacult Res Dev S* 1, 011.

Dongarra, M.L., Rizzello, V., Muccio, L., Fries, W., Cascio, A., Bonaccorsi, I., and Ferlazzo, G. (2013). Mucosal immunology and probiotics. *Curr Allergy Asthma Rep* 13, 19-26.

Drieschner, C., Konemann, S., Renaud, P., and Schirmer, K. (2019). Fish-gut-on-chip: development of a microfluidic bioreactor to study the role of the fish intestine in vitro. *Lab Chip* 19, 3268-3276.

Drieschner, C., Minghetti, M., Wu, S.M., Renaud, P., and Schirmer, K. (2017). Ultrathin

Alumina Membranes as Scaffold for Epithelial Cell Culture from the Intestine of Rainbow Trout. *Acs Applied Materials & Interfaces* 9, 9496-9505.

Dvergedal, H., Sandve, S.R., Klemetsdal, G., Rudi, K., and Angell, I.L. (2020). Association of gut microbiota with metabolism in juvenile Atlantic Salmon. *BioRxiv*.

Earl, J.P., Adappa, N.D., Krol, J., Bhat, A.S., Balashov, S., Ehrlich, R.L., Palmer, J.N., Workman, A.D., Blasetti, M., Sen, B., Hammond, J., Cohen, N.A., Ehrlich, G.D., and Mell, J.C. (2018). Species-level bacterial community profiling of the healthy sinonasal microbiome using Pacific Biosciences sequencing of full-length 16S rRNA genes. *Microbiome* 6, 190.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., and Ross, R.P. (2018). The Gut Microbiota of Marine Fish. *Front Microbiol* 9, 873.

Eisenhofer, R., Minich, J.J., Marotz, C., Cooper, A., Knight, R., and Weyrich, L.S. (2019). Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. *Trends Microbiol* 27, 105-117.

Ellis, A.E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Dev Comp Immunol* 25, 827-839.

Falcinelli, S., Picchietti, S., Rodiles, A., Cossignani, L., Merrifield, D.L., Taddei, A.R., Maradonna, F., Olivotto, I., Gioacchini, G., and Carnevali, O. (2015). *Lactobacillus rhamnosus* lowers zebrafish lipid content by changing gut microbiota and host transcription of genes involved in lipid metabolism. *Sci Rep* 5, 9336.

Fao (2020). The State of World Fisheries and Aquaculture 2020-sustainability in action. *Food Agriculture Organization of the United Nations*.

Fast, M.D., Johnson, S.C., and Jones, S.R. (2007). Differential expression of the pro-inflammatory cytokines IL-1beta-1, TNFalpha-1 and IL-8 in vaccinated pink (*Oncorhynchus gorbuscha*) and chum (*Oncorhynchus keta*) salmon juveniles. *Fish Shellfish Immunol* 22, 403-407.

Fogarty, C., Burgess, C.M., Cotter, P.D., Cabrera-Rubio, R., Whyte, P., Smyth, C., and Bolton, D. (2019). Diversity and composition of the gut microbiota of Atlantic salmon (*Salmo salar*) farmed in Irish waters. *J Appl Microbiol* 127.3, 648-657

Gajardo, K. (2016). Nutrition and intestinal health in Atlantic salmon (*Salmo salar*): involvement of antinutrients and microbiota. Norwegian University of Life Sciences, Oslo.

Gajardo, K., Jaramillo-Torres, A., Kortner, T.M., Merrifield, D.L., Tinsley, J., Bakke, A.M.,

and Krogdahl, A. (2017). Alternative Protein Sources in the Diet Modulate Microbiota and Functionality in the Distal Intestine of Atlantic Salmon (*Salmo salar*). *Appl Environ Microbiol* 83.

Gajardo, K., Rodiles, A., Kortner, T.M., Krogdahl, A., Bakke, A.M., Merrifield, D.L., and Sorum, H. (2016). A high-resolution map of the gut microbiota in Atlantic salmon (*Salmo salar*): A basis for comparative gut microbial research. *Sci Rep* 6, 30893.

Geppert, M., Sigg, L., and Schirmer, K. (2016). A novel two-compartment barrier model for investigating nanoparticle transport in fish intestinal epithelial cells. *Environmental Science: Nano* 3, 388-395.

Gibson, G.R., Scott, K.P., Rastall, R.A., Tuohy, K.M., Hotchkiss, A., Dubert-Ferrandon, A., Gareau, M., Murphy, E.F., Saulnier, D., and Loh, G. (2010). Dietary prebiotics: current status and new definition. *Food Sci Technol Bull Funct Foods* 7, 1-19.

Gil, A. (2002). Modulation of the immune response mediated by dietary nucleotides. *European Journal of Clinical Nutrition* 56, 1-4.

Glassman, S.I., and Martiny, J.B.J.M. (2018). Broadscale ecological patterns are robust to use of exact sequence variants versus operational taxonomic units. 3.

Gomez, D., Sunyer, J.O., and Salinas, I. (2013). The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. *Fish Shellfish Immunol* 35, 1729-1739.

Gomez, G.D., and Balcazar, J.L. (2008). A review on the interactions between gut microbiota and innate immunity of fish. *FEMS Immunol Med Microbiol* 52, 145-154.

González, J., and Troncoso, J. (Year). "Reducing Caligus rogercresseyi infestation in *Salmo salar* fed a diet supplemented with microbial based nucleotides an d immunostimulants", in: *Abstract from the World Aquaculture Conference, Veracruz, Mexico*), 25-29.

Gosmann, C., Anahtar, M.N., Handley, S.A., Farcasanu, M., Abu-Ali, G., Bowman, B.A., Padavattan, N., Desai, C., Droit, L., Moodley, A., Dong, M., Chen, Y., Ismail, N., Ndung'u, T., Ghebremichael, M.S., Wesemann, D.R., Mitchell, C., Dong, K.L., Huttenhower, C., Walker, B.D., Virgin, H.W., and Kwon, D.S. (2017). *Lactobacillus*-Deficient Cervicovaginal Bacterial Communities Are Associated with Increased HIV Acquisition in Young South African Women. *Immunity* 46, 29-37.

Green, T.J., Smullen, R., and Barnes, A.C.J.V.M. (2013). Dietary soybean protein concentrate-induced intestinal disorder in marine farmed Atlantic salmon, *Salmo*

salar is associated with alterations in gut microbiota. *Vet. Microbiol* 166, 286-292.

Grisdale-Helland, B., Helland, S.J., and Gatlin, D.M. (2008). The effects of dietary supplementation with mannanoligosaccharide, fructooligosaccharide or galactooligosaccharide on the growth and feed utilization of Atlantic salmon (*Salmo salar*). *Aquac* 283, 163-167.

Gu, M., Kortner, T.M., Penn, M., Hansen, A.K., and Krogdahl, A. (2014). Effects of dietary plant meal and soya-saponin supplementation on intestinal and hepatic lipid droplet accumulation, lipoprotein and sterol metabolism in Atlantic salmon (*Salmo salar* L.) - CORRIGENDUM. *Br J Nutr* 111, 2046.

Guerreiro, I., Oliva-Teles, A., and Enes, P. (2018). Prebiotics as functional ingredients: focus on Mediterranean fish aquaculture. *Rev Aquac* 10, 800-832.

Gupta, S., Lokesh, J., Abdelhafiz, Y., Siriyappagouder, P., Pierre, R., Sorensen, M., Fernandes, J.M.O., and Kiron, V. (2019). Macroalga-Derived Alginate Oligosaccharide Alters Intestinal Bacteria of Atlantic Salmon. *Front. Microbiol* 10.

Harstad, H., Lukacs, M.F., Bakke, H.G., and Grimholt, U. (2008). Multiple expressed MHC class II loci in salmonids; details of one non-classical region in Atlantic salmon (*Salmo salar*). *BMC Genomics* 9, 193.

Hatje, E., Neuman, C., Stevenson, H., Bowman, J.P., and Katouli, M. (2014). Population dynamics of *Vibrio* and *Pseudomonas* species isolated from farmed Tasmanian Atlantic salmon (*Salmo salar* L.): a seasonal study. *Microb Ecol* 68, 679-687.

He, Y., Chu, S.H., and Walker, W.A. (1993). Nucleotide supplements alter proliferation and differentiation of cultured human (Caco-2) and rat (IEC-6) intestinal epithelial cells. *J Nutr* 123, 1017-1027.

Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., and Sony, N.M. (2016a). Dietary nucleotide administration influences growth, immune responses and oxidative stress resistance of juvenile red sea bream (*Pagrus major*). *Aquac* 455, 41-49.

Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., and Sony, N.M. (2016b). Effects of dietary administration of guanosine monophosphate on the growth, digestibility, innate immune responses and stress resistance of juvenile red sea bream, *Pagrus major*. *Fish Shellfish Immunol* 57, 96-106.

Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M., Dawood, M.A., Kader, M.A., Bulbul, M., and Fujieda, T. (2016c). Efficacy of nucleotide related products on growth, blood chemistry, oxidative stress and growth factor gene expression of

juvenile red sea bream, *Pagrus major*. *Aquac* 464, 8-16.

Hossain, M.S., Koshio, S., and Kestemont, P. (2019). Recent advances of nucleotide nutrition research in aquaculture: a review. *Rev Aquac* 12.2, 1028-1053

Hovda, M.B., Fontanillas, R., Mcgurk, C., Obach, A., and Rosnes, J.T. (2012). Seasonal variations in the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.). *Aquac. Res.* 43, 154-159.

Hu, H., Kortner, T.M., Gajardo, K., Chikwati, E., Tinsley, J., and Krogdahl, A. (2016). Intestinal Fluid Permeability in Atlantic Salmon (*Salmo salar* L.) Is Affected by Dietary Protein Source. *PLoS One* 11, e0167515.

Huyben, D., Sun, L., Moccia, R., Kiessling, A., Dicksved, J., and Lundh, T.J.J.O.a.M. (2018). Dietary live yeast and increased water temperature influence the gut microbiota of rainbow trout. *J. Appl. Microbiol* 124, 1377-1392.

Ibrahim, K., and El-Sayed, E. (2016). Potential role of nutrients on immunity. *International Food Research Journal* 23.

Infante-Villamil, S., Huerlimann, R., and Jerry, D.R. (2020). Microbiome diversity and dysbiosis in aquaculture. *Rev Aquac*.

Jaramillo-Torres, A., Rawling, M., Rodiles, A., Mikalsen, H.E., Johansen, L.H., Tinsley, J., Forberg, T., Aasum, E., Castex, M., and Merrifield, D.L.J.F.I.M. (2019). Influence of dietary supplementation of probiotic Pediococcus acidilactici MA18/5M during the transition from freshwater to seawater on intestinal health and microbiota of Atlantic salmon (*Salmo salar* L.). *Front. Microbiol* 10, 2243.

Johansson, L.-H., Timmerhaus, G., Afanasyev, S., Jørgensen, S.M., and Krasnov, A. (2016). Smoltification and seawater transfer of Atlantic salmon (*Salmo salar* L.) is associated with systemic repression of the immune transcriptome. *Fish shellfish immunol* 58, 33-41.

Jud, C., Ahmed, S., Müller, L., Kinnear, C., Vanhecke, D., Umehara, Y., Frey, S., Liley, M., Angeloni, S., and Petri-Fink, A. (2015). Ultrathin ceramic membranes as scaffolds for functional cell coculture models on a biomimetic scale. *BioResearch open access* 4, 457-468.

Kamada, N., and Nunez, G. (2013). Role of the gut microbiota in the development and function of lymphoid cells. *J Immunol* 190, 1389-1395.

Karlsen, C., Ottem, K.F., Brevik, O.J., Davey, M., Sorum, H., and Winther-Larsen, H.C. (2017). The environmental and host-associated bacterial microbiota of Arctic

seawater-farmed Atlantic salmon with ulcerative disorders. *J. Fish Dis.* 40, 1645-1663.

Karlsen, C., Ytteborg, E., Timmerhaus, G., Host, V., Handeland, S., Jorgensen, S.M., and Krasnov, A. (2018). Atlantic salmon skin barrier functions gradually enhance after seawater transfer. *Sci Rep* 8, 9510.

Kawano, A. (2009). Developing and characterizing a salmonid intestinal epithelial cell line for use in studies of inflammation in the fish gastrointestinal tract. *Master's thesis, University of Waterloo*.

Kawano, A., Haiduk, C., Schirmer, K., Hanner, R., Lee, L.E.J., Dixon, B., and Bols, N.C. (2011). Development of a rainbow trout intestinal epithelial cell line and its response to lipopolysaccharide. *Aquac Nut* 17, e241-e252.

Kiron, V. (2012). Fish immune system and its nutritional modulation for preventive health care. *Anim. Feed Sci. Technol* 173, 111-133.

Kiron, V., Kulkarni, A., Dahle, D., Vasanth, G., Lokesh, J., and Elvebo, O. (2016). Recognition of purified beta 1,3/1,6 glucan and molecular signalling in the intestine of Atlantic salmon. *Dev Comp Immunol* 56, 57-66.

Klemetsen, T., Willassen, N.P., and Karlsen, C.R. (2019). Full-length 16S rRNA gene classification of Atlantic salmon bacteria and effects of using different 16S variable regions on community structure analysis. *Microbiologyopen* 8, e898.

Kononova, S.V., Zinchenko, D.V., Muranova, T.A., Belova, N.A., and Miroshnikov, A.I. (2019). Intestinal microbiota of salmonids and its changes upon introduction of soy proteins to fish feed. *Aquac. Int.* 27, 475-496.

Kortner, T.M., Bjorkhem, I., Krasnov, A., Timmerhaus, G., and Krogdahl, A. (2014). Dietary cholesterol supplementation to a plant-based diet suppresses the complete pathway of cholesterol synthesis and induces bile acid production in Atlantic salmon (*Salmo salar* L.). *Br J Nutr* 111, 2089-2103.

Kortner, T.M., Gu, J., Krogdahl, A., and Bakke, A.M. (2013). Transcriptional regulation of cholesterol and bile acid metabolism after dietary soyabean meal treatment in Atlantic salmon (*Salmo salar* L.). *Br J Nutr* 109, 593-604.

Kosinska, A., and Andlauer, W. (2013). Modulation of tight junction integrity by food components. *Food Res. Int* 54, 951-960.

Krogdahl, A., Gajardo, K., Kortner, T.M., Penn, M., Gu, M., Berge, G.M., and Bakke, A.M. (2015a). Soya Saponins Induce Enteritis in Atlantic Salmon (*Salmo salar* L.). *J Agric*

Food Chem 63, 3887-3902.

Krogdahl, A., and Sundby, A. (1999). Characteristics of pancreatic function in fish. *Biology of the Pancreas in Growing Animals* 28, 437-458.

Krogdahl, Å., Kortner, T.M., Jaramillo-Torres, A., Gamil, A.a.A., Chikwati, E., Li, Y., Schmidt, M., Herman, E., Hymowitz, T., Teimouri, S., and Storebakken, T. (2020). Removal of three proteinaceous antinutrients from soybean does not mitigate soybean-induced enteritis in Atlantic salmon (*Salmo salar*, L). *Aquac* 514.

Krogdahl, Å., Sundby, A., and Holm, H. (2015b). Characteristics of digestive processes in Atlantic salmon (*Salmo salar*). Enzyme pH optima, chyme pH, and enzyme activities. *Aquacu* 449, 27-36.

Langan, L.M., Harper, G.M., Owen, S.F., Purcell, W.M., Jackson, S.K., and Jha, A.N. (2017). Application of the rainbow trout derived intestinal cell line (RTgutGC) for ecotoxicological studies: molecular and cellular responses following exposure to copper. *Ecotoxicology* 26, 1117-1133.

Langan, L.M., Owen, S.F., Trznadel, M., Dodd, N.J.F., Jackson, S.K., Purcell, W.M., and Jha, A.N. (2018). Spheroid Size Does not Impact Metabolism of the beta-blocker Propranolol in 3D Intestinal Fish Model. *Front. Pharmacol* 9.

Lazado, C.C., and Caipang, C.M. (2014). Mucosal immunity and probiotics in fish. *Fish Shellfish Immunol* 39, 78-89.

Lebleu, V.S., Macdonald, B., and Kalluri, R. (2007). Structure and function of basement membranes. *Exp Biol Med (Maywood)* 232, 1121-1129.

Legrand, T.P., Wynne, J.W., Weyrich, L.S., and Oxley, A.P. (2020). A microbial sea of possibilities: current knowledge and prospects for an improved understanding of the fish microbiome. *Rev Aquac* 12, 1101-1134.

Lerner, A., Shoenfeld, Y., and Matthias, T. (2019). Probiotics: if it does not help it does not do any harm. Really? *Microorganisms* 7, 104.

Li, P. (2004). Dietary brewers yeast and the prebiotic Grobiotic™AE influence growth performance, immune responses and resistance of hybrid striped bass (*Morone chrysops×M. saxatilis*) to Streptococcus iniae infection. *Aquac* 231, 445-456.

Li, P., Gatlin Iii, D.M., and Neill, W.H. (2007). Dietary supplementation of a purified nucleotide mixture transiently enhanced growth and feed utilization of juvenile red drum, *Sciaenops ocellatus*. *JWAS* 38, 281-286.

Li, P., Lewis, D.H., and Gatlin, D.M., 3rd (2004). Dietary oligonucleotides from yeast RNA

influence immune responses and resistance of hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) to Streptococcus iniae infection. *Fish Shellfish Immunol* 16, 561-569.

Li, Y., Bruni, L., Jaramillo-Torres, A., Gajardo, K., Kortner, T.M., and Krogdahl, Å. (2020). Differential Response of Digesta-and Mucosa-Associated Intestinal Microbiota to Dietary Black Soldier Fly (*Hermetia illucens*) Larvae Meal in Seawater Phase Atlantic Salmon (*Salmo salar*). *BioRxiv*.

Li, Y., Li, Y., Cao, X., Jin, X., and Jin, T. (2017). Pattern recognition receptors in zebrafish provide functional and evolutionary insight into innate immune signaling pathways. *Cell Mol Immunol* 14, 80-89.

Lilleeng, E., Froystad, M.K., Ostby, G.C., Valen, E.C., and Krogdahl, A. (2007). Effects of diets containing soybean meal on trypsin mRNA expression and activity in Atlantic salmon (*Salmo salar* L). *Comp Biochem Physiol A Mol Integr Physiol* 147, 25-36.

Lilleeng, E., Penn, M.H., Haugland, O., Xu, C., Bakke, A.M., Krogdahl, A., Landsverk, T., and Froystad-Saugen, M.K. (2009). Decreased expression of TGF-beta, GILT and T-cell markers in the early stages of soybean enteropathy in Atlantic salmon (*Salmo salar* L.). *Fish Shellfish Immunol* 27, 65-72.

Liu, Z., Liu, W., Ran, C., Hu, J., and Zhou, Z. (2016). Abrupt suspension of probiotics administration may increase host pathogen susceptibility by inducing gut dysbiosis. *Sci Rep* 6, 23214.

Lokesh, J., and Kiron, V. (2016). Transition from freshwater to seawater reshapes the skin-associated microbiota of Atlantic salmon. *Sci Rep* 6, 19707.

Lokesh, J., Kiron, V., Sipkema, D., Fernandes, J.M.O., and Moum, T. (2019). Succession of embryonic and the intestinal bacterial communities of Atlantic salmon (*Salmo salar*) reveals stage-specific microbial signatures. *Microbiologyopen* 8, e00672.

Lokka, G., Austbo, L., Falk, K., Bromage, E., Fjelldal, P.G., Hansen, T., Hordvik, I., and Koppang, E.O. (2014). Immune parameters in the intestine of wild and reared unvaccinated and vaccinated Atlantic salmon (*Salmo salar* L.). *Dev Comp Immunol* 47, 6-16.

Lopez Nadal, A., Ikeda-Ohtsubo, W., Sipkema, D., Peggs, D., Mcgurk, C., Forlenza, M., Wiegertjes, G.F., and Brugman, S. (2020). Feed, Microbiota, and Gut Immunity: Using the Zebrafish Model to Understand Fish Health. *Front Immunol* 11, 114.

Lyte, M., and Cryan, J.F. (2014). *Microbial endocrinology: the microbiota-gut-brain axis in*

health and disease. Springer.

Madsen, S.S., Olesen, J.H., Bedal, K., Engelund, M.B., Velasco-Santamaria, Y.M., and Tipsmark, C.K. (2011). Functional characterization of water transport and cellular localization of three aquaporin paralogs in the salmonid intestine. *Front Physiol* 2, 56.

Maren Amasheh, S.S., Salah Amasheh, Joachim Mankertz, Martin Zeitz,, and Michael Fromm, A.J.R.D.S. (2008). Quercetin enhances epithelial barrier function and increases claudin-4 expression in Caco-2 cells. *J. Nutr* 138.6, 1067-1073.

Marjara, I.S., Chikwati, E.M., Valen, E.C., Krogdahl, A., and Bakke, A.M. (2012). Transcriptional regulation of IL-17A and other inflammatory markers during the development of soybean meal-induced enteropathy in the distal intestine of Atlantic salmon (*Salmo salar* L.). *Cytokine* 60, 186-196.

Martin, S.A., Dehler, C.E., and Krol, E. (2016). Transcriptomic responses in the fish intestine. *Dev Comp Immunol* 64, 103-117.

Martinez, R.C., Bedani, R., and Saad, S.M. (2015). Scientific evidence for health effects attributed to the consumption of probiotics and prebiotics: an update for current perspectives and future challenges. *Br J Nutr* 114, 1993-2015.

Mclaren, M.R., Willis, A.D., and Callahan, B.J. (2019). Consistent and correctable bias in metagenomic sequencing experiments. *Elife* 8.

Merrifield, D.L., and Ringø, E. (2014). *Aquaculture nutrition: gut health, probiotics and prebiotics.* John Wiley & Sons.

Micallef, G., Cash, P., Fernandes, J.M., Rajan, B., Tinsley, J.W., Bickerdike, R., Martin, S.A., and Bowman, A.S. (2017). Dietary Yeast Cell Wall Extract Alters the Proteome of the Skin Mucous Barrier in Atlantic Salmon (*Salmo salar*): Increased Abundance and Expression of a Calreticulin-Like Protein. *PLoS One* 12, e0169075.

Mileti, E., Matteoli, G., Iliev, I.D., and Rescigno, M. (2009). Comparison of the immunomodulatory properties of three probiotic strains of *Lactobacilli* using complex culture systems: prediction for *in vivo* efficacy. *PLoS One* 4, e7056.

Minghetti, M., Drieschner, C., Bramaz, N., Schug, H., and Schirmer, K. (2017). A fish intestinal epithelial barrier model established from the rainbow trout (*Oncorhynchus mykiss*) cell line, RTgutGC. *Cell Biol Toxicol* 33, 539-555.

Moldal, T., Løkka, G., Wiik-Nielsen, J., Austbø, L., Torstensen, B.E., Rosenlund, G., Dale, O.B., Kaldhusdal, M., and Koppang, E.O. (2014). Substitution of dietary fish oil with

plant oils is associated with shortened mid intestinal folds in Atlantic salmon (*Salmo salar*). *BMC Vet. Res* 10, 60.

Munir, M.B., Hashim, R., Chai, Y.H., Marsh, T.L., and Nor, S.a.M. (2016). Dietary prebiotics and probiotics influence growth performance, nutrient digestibility and the expression of immune regulatory genes in snakehead (*Channa striata*) fingerlings. *Aquac* 460, 59-68.

Nguyen, C.D.H., Amoroso, G., Ventura, T., and Elizur, A. (2020a). Assessing the Pyloric Caeca and Distal Gut Microbiota Correlation with Flesh Color in Atlantic Salmon (*Salmo salar* L., 1758). *Microorganisms* 8.

Nguyen, C.D.H., Amoroso, G., Ventura, T., Minich, J.J., and Elizur, A. (2020b). Atlantic Salmon (*Salmo salar* L., 1758) Gut Microbiota Profile Correlates with Flesh Pigmentation: Cause or Effect? *Mar Biotechnol (NY)*.

Nylund, A., Hansen, H., Brevik, O.J., Hustoft, H., Markussen, T., Plarre, H., and Karlsbakk, E. (2018). Infection dynamics and tissue tropism of Parvicapsula pseudobranchicola (*Myxozoa: Myxosporea*) in farmed Atlantic salmon (*Salmo salar*). *Parasit Vectors* 11, 17.

Nylund, A., Karlsbakk, E., Sæther, P., Koren, C., Larsen, T., Nielsen, B., Brøderud, A., Høstlund, C., Fjellsøy, K., and Lervik, K. (2005). Parvicapsula pseudobranchicola (*Myxosporea*) in farmed Atlantic salmon *Salmo salar*: tissue distribution, diagnosis and phylogeny. *Diseases of aquatic organisms* 63, 197-204.

Ogier De Baulny, M., Quentel, C., Fournier, V., Lamour, F., and Le Gouvello, R. (1996). Effect of long-term oral administration of beta-glucan as an immunostimulant or an adjuvant on some non-specific parameters of the immune response of turbot Scophthalmus maximus. *Dis. Aquat. Org* 26, 139-147.

Osorio, C.R., Toranzo, A.E., Romalde, J.L., and Barja, J.L. (2000). Multiplex PCR assay for ureC and 16S rRNA genes clearly discriminates between both subspecies of Photobacterium damselae. *Dis. Aquat. Org* 40, 177-183.

Oulad, S., Abedian, A.M., Khodabandeh, S.J.C.B., and Physiology, P.A. (2009). Effect of dietary nucleotide on the osmoregulatory function of pyloric caeca in Caspian Sea salmon, *Salmo trutta caspius*. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol* 2, S81.

Parra, M., Espinoza, D., Valdes, N., Vargas, R., Gonzalez, A., Modak, B., and Tello, M. (2020). Microbiota Modulates the Immunomodulatory Effects of Filifolinone on

Atlantic Salmon. *Microorganisms* 8.

Parris, D.J., Brooker, R.M., Morgan, M.A., Dixson, D.L., and Stewart, F.J. (2016). Whole gut microbiome composition of damselfish and cardinalfish before and after reef settlement. *Peerj* 4.

Perry, W.B., Lindsay, E., Payne, C.J., Brodie, C., and Kazlauskaite, R. (2020). The role of the gut microbiome in sustainable teleost aquaculture. *Proc Biol Sci* 287, 20200184.

Petersen, C., Bell, R., Klag, K.A., Lee, S.H., Soto, R., Ghazaryan, A., Buhrke, K., Ekiz, H.A., Ost, K.S., Boudina, S., O'connell, R.M., Cox, J.E., Villanueva, C.J., Stephens, W.Z., and Round, J.L. (2019). T cell-mediated regulation of the microbiota protects against obesity. *Science* 365.

Petersson, J., Schreiber, O., Hansson, G.C., Gendler, S.J., Velcich, A., Lundberg, J.O., Roos, S., Holm, L., and Phillipson, M. (2011). Importance and regulation of the colonic mucus barrier in a mouse model of colitis. *Am J Physiol Gastrointest Liver Physiol* 300, G327-333.

Petit, J., De Bruijn, I., Brugman, S., Forlenza, M., Wiegertjes, G.F.J.a.F., and Immunology, S. (2019). B-glucan immuno-modulation in common carp intestine: A role for microbiota and its metabolites. *Fish shellfish immunol* 91, 440.

Petrie, A.G., and Ellis, A.E. (2006). Evidence of particulate uptake by the gut of Atlantic salmon (*Salmo salar* L.). *Fish shellfish immunol* 20, 660-664.

Pollock, J., Glendinning, L., Wisedchanwet, T., and Watson, M. (2018). The Madness of Microbiome: Attempting To Find Consensus "Best Practice" for 16S Microbiome Studies. *Appl Environ Microbiol* 84.

Pontigo, J.P., Aguero, M.J., Sanchez, P., Oyarzun, R., Vargas-Lagos, C., Mancilla, J., Kossmann, H., Morera, F.J., Yanez, A.J., and Vargas-Chacoff, L. (2016). Identification and expressional analysis of NLRC5 inflammasome gene in smolting Atlantic salmon (*Salmo salar*). *Fish Shellfish Immunol* 58, 259-265.

Pumputis, P.G. (2017). *Development of RTgutGC as a Tool for Fish Feed Development.* University of Waterloo.

Pumputis, P.G., Dayeh, V.R., Lee, L.E., Hamilton, M.E., Liu, Z., Viththiyapaskaran, S., and Bols, N.C. (2020). Beneficial and detrimental effects of the phytochemical naringenin on rainbow trout intestinal epithelial cells. *J. Funct. Foods* 64, 103653.

Rawls, J.F., Samuel, B.S., and Gordon, J.I. (2004). Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proc Natl Acad Sci U S A*

101, 4596-4601.

Ray, A., Ghosh, K., and Ringø, E.J.a.N. (2012). Enzyme-producing bacteria isolated from fish gut: a review. 18, 465-492.

Refstie, S., Baeverfjord, G., Seim, R.R., and Elvebø, O. (2010). Effects of dietary yeast cell wall β-glucans and MOS on performance, gut health, and salmon lice resistance in Atlantic salmon (*Salmo salar*) fed sunflower and soybean meal. *Aquac* 305, 109-116.

Resta-Lenert, S.C., and Barrett, K.E. (2009). Modulation of intestinal barrier properties by probiotics: role in reversing colitis. *Ann N Y Acad Sci* 1165, 175-182.

Reveco, F.E., Øverland, M., Romarheim, O.H., and Mydland, L.T. (2014). Intestinal bacterial community structure differs between healthy and inflamed intestines in Atlantic salmon (*Salmo salar* L.). *Aquac* 420-421, 262-269.

Rignø, D.A. (2014). Aquaculture Nutrition. Gut health probiotics and prebiotics. *Book*.

Rimoldi, S., Gini, E., Iannini, F., Gasco, L., and Terova, G. (2019). The Effects of Dietary Insect Meal from *Hermetia illucens* Prepupae on Autochthonous Gut Microbiota of Rainbow Trout (*Oncorhynchus mykiss*). *Animals* 9.

Ringo, E., Sperstad, S., Myklebust, R., Mayhew, T.M., and Olsen, R.E. (2006). The effect of dietary inulin on aerobic bacteria associated with hindgut of Arctic charr (*Salvelinus alpinus* L.). *Aquac Res* 37, 891-897.

Ringø, E., Erik Olsen, R., Gonzalez Vecino, J.L., and Wadsworth, S. (2012). Use of Immunostimulants and Nucleotides in Aquaculture: A Review. *Journal of Marine Science: Research & Development* 02.

Ringø, E., and Gatesoupe, F.J. (1998). Lactic acid bacteria in fish: a review. *Aquacu* 160, 177-203.

Ringø, E., Lovmo, L., Kristiansen, M., Bakken, Y., Salinas, I., Myklebust, R., Olsen, R.E., and Mayhew, T.M. (2010). Lactic acid bacteria vs. pathogens in the gastrointestinal tract of fish: a review. *Aquacu Res* 41, 451-467.

Ringø, E., Zhou, Z., Vecino, J.L.G., Wadsworth, S., Romero, J., Krogdahl, Å., Olsen, R.E., Dimitroglou, A., Foey, A., Davies, S., Owen, M., Lauzon, H.L., Martinsen, L.L., De Schryver, P., Bossier, P., Sperstad, S., and Merrifield, D.L. (2016). Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? *Aquac Nut* 22, 219-282.

Rolig, A.S., Parthasarathy, R., Burns, A.R., Bohannan, B.J., and Guillemin, K. (2015). Individual Members of the Microbiota Disproportionately Modulate Host Innate

Immune Responses. *Cell Host Microbe* 18, 613-620.

Rombout, J.H., Abelli, L., Picchietti, S., Scapigliati, G., and Kiron, V. (2011). Teleost intestinal immunology. *Fish Shellfish Immunol* 31, 616-626.

Rosenbaum, M., Knight, R., and Leibel, R.L. (2015). The gut microbiota in human energy homeostasis and obesity. *Trends Endocrinol Metab* 26, 493-501.

Rud, I., Kolarevic, J., Holan, A.B., Berget, I., Calabrese, S., and Terjesen, B.F. (2017). Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production. *Aquac Eng* 78, 50-62.

Rudi, K., Angell, I.L., Pope, P.B., Vik, J.O., Sandve, S.R., and Snipen, L.G. (2018). Stable Core Gut Microbiota across the Freshwater-to-Saltwater Transition for Farmed Atlantic Salmon. *Aquac Eng* 84.

Safari, O., Shahsavani, D., Paolucci, M., and Mehraban Sang Atash, M. (2015). The effects of dietary nucleotide content on the growth performance, digestibility and immune responses of juvenile narrow clawed crayfish, A stacus leptodactylus leptodactylus Eschscholtz, 1823. *Aquac res* 46, 2685-2697.

Sakai, M.J.A. (1999). Current research status of fish immunostimulants. *Aquac* 172, 63-92.

Salinas, I. (2015). The Mucosal Immune System of Teleost Fish. *Biology (Basel)* 4, 525-539.

Salter, S.J., Cox, M.J., Turek, E.M., Calus, S.T., Cookson, W.O., Moffatt, M.F., Turner, P., Parkhill, J., Loman, N.J., and Walker, A.W. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol* 12, 87.

Schmidt, V., Amaral-Zettler, L., Davidson, J., Summerfelt, S., and Good, C. (2016). Influence of Fishmeal-Free Diets on Microbial Communities in Atlantic Salmon (*Salmo salar*) Recirculation Aquaculture Systems. *Appl Environ Microbiol* 82, 4470-4481.

Schmitt, P., Wacyk, J., Morales-Lange, B., Rojas, V., Guzman, F., Dixon, B., and Mercado, L. (2015). Immunomodulatory effect of cathelicidins in response to a beta-glucan in intestinal epithelial cells from rainbow trout. *Dev Comp Immunol* 51, 160-169.

Schroeder, B.O. (2019). Fight them or feed them: how the intestinal mucus layer manages the gut microbiota. *Gastroenterology Report* 7, 3-12.

Schug, H., Yue, Y., Krese, R., Fischer, S., Kortner, T.M., and Schirmer, K. (2019). Time- and concentration-dependent expression of immune and barrier genes in the RTgutGC

fish intestinal model following immune stimulation. *Fish Shellfish Immunol* 88, 308-317.

Secombes, C.J. (2016). What's new in fish cytokine research? *Fish Shellfish Immunol* 53, 1-3.

Secombes, C.J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K.J., Cunningham, C., and Zou, J. (2001). Cytokines and innate immunity of fish. *Dev Comp Immunol* 25, 713-723.

Semova, I., Carten, J.D., Stombaugh, J., Mackey, L.C., Knight, R., Farber, S.A., and Rawls, J.F. (2012). Microbiota Regulate Intestinal Absorption and Metabolism of Fatty Acids in the Zebrafish. *Cell Host & Microbe* 12, 277-288.

Shokryazdan, P., Faseleh Jahromi, M., Navidshad, B., and Liang, J.B. (2017). Effects of prebiotics on immune system and cytokine expression. *Med Microbiol Immunol* 206, 1-9.

Sissener, N.H., Sanden, M., Bakke, A.M., Krogdahl, Å., and Hemre, G.I. (2009). A long term trial with Atlantic salmon (*Salmo salar* L.) fed genetically modified soy; focusing general health and performance before, during and after the parr–smolt transformation. *Aquac* 294, 108-117.

Sommer, F., and Backhed, F. (2013). The gut microbiota--masters of host development and physiology. *Nat Rev Microbiol* 11, 227-238.

Song, S.K., Beck, B.R., Kim, D., Park, J., Kim, J., Kim, H.D., and Ringo, E. (2014). Prebiotics as immunostimulants in aquaculture: a review. *Fish Shellfish Immunol* 40, 40-48.

Staykov, Y., Spring, P., Denev, S., and Sweetman, J. (2007). Effect of a mannan oligosaccharide on the growth performance and immune status of rainbow trout (*Oncorhynchus mykiss*). *Aquac. Int* 15, 153-161.

Sundell, K.S., and Sundh, H. (2012). Intestinal fluid absorption in anadromous salmonids: importance of tight junctions and aquaporins. *Front Physiol* 3, 388.

Sundh, H., Nilsen, T.O., Lindstrom, J., Hasselberg-Frank, L., Stefansson, S.O., Mccormick, S.D., and Sundell, K. (2014). Development of intestinal ion-transporting mechanisms during smoltification and seawater acclimation in Atlantic salmon *Salmo salar*. *J Fish Biol* 85, 1227-1252.

Sveen, L.R., Grammes, F.T., Ytteborg, E., Takle, H., and Jorgensen, S.M. (2017). Genome-wide analysis of Atlantic salmon (*Salmo salar*) mucin genes and their role as

biomarkers. *PLoS One* 12, e0189103.

Sweetman, J., Dimitroglou, A., Davies, S., and Torrecillas, S.J.I.A. (2008). Nutrient uptake: gut morphology a key to efficient nutrition. *International AquaFeed* 11, 27-30.

Tacchi, L., Bickerdike, R., Douglas, A., Secombes, C.J., and Martin, S.A. (2011). Transcriptomic responses to functional feeds in Atlantic salmon (*Salmo salar*). *Fish Shellfish Immunol* 31, 704-715.

Tahmasebi-Kohyani, A., Keyvanshokooh, S., Nematollahi, A., Mahmoudi, N., and Pasha-Zanoosi, H. (2011). Dietary administration of nucleotides to enhance growth, humoral immune responses, and disease resistance of the rainbow trout (*Oncorhynchus mykiss*) fingerlings. *Fish Shellfish Immunol* 30, 189-193.

Tao, S., Zhu, L., Lee, P., Lee, W.M., Knox, K., Chen, J., Di, Y.P., and Chen, Y. (2012). Negative control of TLR3 signaling by TICAM1 down-regulation. *Am J Respir Cell Mol Biol* 46, 660-667.

Terova, G., Rimoldi, S., Ascione, C., Gini, E., Ceccotti, C., and Gasco, L. (2019). Rainbow trout (*Oncorhynchus mykiss*) gut microbiota is modulated by insect meal from Hermetia illucens prepupae in the diet. *Rev. Fish Biol. Fish* 29, 465-486.

Tipsmark, C.K., and Madsen, S.S. (2012). Tricellulin, occludin and claudin-3 expression in salmon intestine and kidney during salinity adaptation. *Comp Biochem Physiol A Mol Integr Physiol* 162, 378-385.

Tipsmark, C.K., Sorensen, K.J., Hulgard, K., and Madsen, S.S. (2010a). Claudin-15 and -25b expression in the intestinal tract of Atlantic salmon in response to seawater acclimation, smoltification and hormone treatment. *Comp Biochem Physiol A Mol Integr Physiol* 155, 361-370.

Tipsmark, C.K., Sorensen, K.J., and Madsen, S.S. (2010b). Aquaporin expression dynamics in osmoregulatory tissues of Atlantic salmon during smoltification and seawater acclimation. *J Exp Biol* 213, 368-379.

Torrecillas, S., Makol, A., Betancor, M.B., Montero, D., Caballero, M.J., Sweetman, J., and Izquierdo, M. (2013). Enhanced intestinal epithelial barrier health status on European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides. *Fish Shellfish Immunol* 34, 1485-1495.

Torrecillas, S., Montero, D., and Izquierdo, M. (2014). Improved health and growth of fish fed mannan oligosaccharides: potential mode of action. *Fish Shellfish Immunol* 36, 525-544.

Tsilingiri, K., Barbosa, T., Penna, G., Caprioli, F., Sonzogni, A., Viale, G., and Rescigno, M. (2012). Probiotic and postbiotic activity in health and disease: comparison on a novel polarised ex-vivo organ culture model. *Gut* 61, 1007-1015.

Van Der Marel, M., Adamek, M., Gonzalez, S.F., Frost, P., Rombout, J.H., Wiegertjes, G.F., Savelkoul, H.F., Steinhagen, D.J.F., and Immunology, S. (2012). Molecular cloning and expression of two β-defensin and two mucin genes in common carp (*Cyprinus carpio* L.) and their up-regulation after β-glucan feeding. *Fish Shellfish Immunol* 32, 494-501.

Volman, J.J., Helsper, J.P., Wei, S., Baars, J.J., Van Griensven, L.J., Sonnenberg, A.S., Mensink, R.P., and Plat, J. (2010). Effects of mushroom-derived beta-glucan-rich polysaccharide extracts on nitric oxide production by bone marrow-derived macrophages and nuclear factor-kappaB transactivation in Caco-2 reporter cells: can effects be explained by structure? *Mol Nutr Food Res* 54, 268-276.

Wang, A.R., Ran, C., Ringø, E., and Zhou, Z.G. (2017). Progress in fish gastrointestinal microbiota research. *Rev Aquac* 10.3, 626-640.

Wei, Z., Yi, L., Xu, W., Zhou, H., Zhang, Y., Zhang, W., and Mai, K. (2015). Effects of dietary nucleotides on growth, non-specific immune response and disease resistance of sea cucumber *Apostichopus japonicas*. *Fish Shellfish Immunol* 47, 1-6.

Welker, T.L., Lim, C., Yildirim-Aksoy, M., and Klesius, P.H. (2011). Effects of dietary supplementation of a purified nucleotide mixture on immune function and disease and stress resistance in channel catfish, Ictalurus punctatus. *Aquac Res* 42, 1878-1889.

Xu, L., Ran, C., He, S., Zhang, J., Hu, J., Yang, Y., Du, Z., Yang, Y., and Zhou, Z. (2015). Effects of dietary yeast nucleotides on growth, non-specific immunity, intestine growth and intestinal microbiota of juvenile hybrid tilapia *Oreochromis niloticus* female symbol x *Oreochromis aureus* male symbol. *Anim Nutr* 1, 244-251.

Yoshida, T., Kruger, R., and Inglis, V. (1995). Augmentation of non-specific protection in African catfish, *Clarias gariepinus* (Burchell), by the long-term oral administration of immunostimulants. *J. Fish Dis* 18, 195-198.

Zarkasi, K.Z., Abell, G.C., Taylor, R.S., Neuman, C., Hatje, E., Tamplin, M.L., Katouli, M., and Bowman, J.P. (2014). Pyrosequencing-based characterization of gastrointestinal bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system. *J Appl Microbiol* 117, 18-27.

Zarkasi, K.Z., Taylor, R.S., Abell, G.C., Tamplin, M.L., Glencross, B.D., and Bowman, J.P.

(2016). Atlantic Salmon (*Salmo salar* L.) Gastrointestinal Microbial Community Dynamics in Relation to Digesta Properties and Diet. *Microb Ecol* 71, 589-603.

Zhao, D.H., Sun, J.J., Liu, L., Zhao, H.H., Wang, H.F., Liang, L.Q., Liu, L.B., and Li, G.F.J.J.O.T.W.a.S. (2009). Characterization of two phenotypes of *Photobacterium damselae* subsp. *damselae* isolated from diseased juvenile *Trachinotus ovatus* reared in cage mariculture. *JWAS* 40, 281-289.

Zhao, X., Findly, R.C., and Dickerson, H.W. (2008). Cutaneous antibody-secreting cells and B cells in a teleost fish. *Dev Comp Immunol* 32, 500-508.

Zhou, Z.X., Zhang, B.C., and Sun, L. (2014). Poly(I:C) induces antiviral immune responses in Japanese flounder (*Paralichthys olivaceus*) that require TLR3 and MDA5 and is negatively regulated by Myd88. *PLoS One* 9, e112918.

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Gut immune functions and health in Atlantic salmon (*Salmo salar*) from late freshwater stage until one year in seawater and effects of functional ingredients: A case study from a commercial sized research site in the Arctic region

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ABSTRACT

The present study was conducted to strengthen the knowledge on gut immune functions and health in Atlantic salmon under large scale, commercial conditions in the Arctic region of Norway. Two groups of fish were monitored, one fed a series of diets without functional ingredients (Ref) and the other diets with functional ingredients (Test). The nutritional composition of the two diet series varied in parallel according to the nutrient requirements of the fish during the observation time. The content of functional ingredients in the Test diets, i.e. nucleotides, yeast cell walls, a prebiotic and essential fatty acids, varied in accordance with a strategy developed by the feed company. The fish were observed at four sampling time points, the first (FW) in May 2016 two weeks before seawater transfer, the other three throughout the following seawater period until the fish reached a size of about 2 kg, i.e. in June, four weeks after seawater transfer (SW1); in November (SW2), and in April the following year (SW3). Gut health was assessed based on histopathological indicators of lipid malabsorption and gut inflammation, expression of gut immune, barrier and other health related genes, plasma biomarkers, somatic indices of intestinal sections, as well as biomarkers of digestive functions.

Seawater transfer of the fish (SW1 compared to FW) caused a marked lowering of expression of genes related to immune and barrier functions in the distal intestine, i.e. cytokines (*il1β*, *il10*, *tgfβ*, *ifnγ*), T-cell markers (*cd3γδ*), *myd88* and tight junction proteins (*zo-1*, *claudin-15*, *claudin-25b*), indicating suppressed immune and barrier functions. At SW2 and SW3, most of the immune biomarkers showed values similar to those observed at FW. The development of plasma cholesterol and triglyceride levels showed similar picture, with markedly lower levels after seawater transfer. Lipid malabsorption was observed in particular in fish from SW1 and SW2, as indicated by hyper-vacuolation of the pyloric caeca enterocytes with concurrently increased expression levels of *plin2*. Regarding effects of functional ingredients, significantly lower condition factor and plasma triglyceride level were observed for Test-fed fish at SW2, indicating a metabolic cost of use of a mixture of nucleotides, yeast cell walls and essential fatty acids. No clear effects of functional ingredients on expression of gut immune genes and other health indexes were observed through the observation period. The great, temporary lowering of expression of gut immune and barrier genes at SW1 is suggested to be an important factor underlying the increased vulnerability of the fish at this time point. Our findings regarding supplementation with functional ingredients raise questions whether some of these ingredients overall are beneficial or might come with a metabolic cost. Our results highlight the need for a better understanding of the cause and consequences of the suppression of gut immune functions of farmed Atlantic salmon just after seawater transfer, and the use of functional ingredients under commercial conditions.

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1. Introduction

Norwegian Atlantic salmon (*Salmo salar*) production has the potential to reach a level 4–5 times higher than today by 2050 [1,2]. Conditions for fulfilling such projected growth in production are considered to include expansion of aquaculture production in the Arctic region [3,4]. However, the environmental conditions are extreme in the north with very low average temperature and extreme variation in photoperiod. Use of diets formulated for fish in the south, may not be optimal for fish in the north. Adjustments to local conditions may be required to obtain optimal function and health. Literature searches reveal only limited information about nutrient requirement of fish produced under Arctic conditions. Therefore, as feeds used in the northern regions are quite similar to those in the south, it is not unlikely that the diets may be deficient and imbalanced for some nutrients.

It is well documented that suboptimal feed and feeding strategies may affect immune functions and health in fish [5–7]. In Atlantic salmon, the consequences may be most severe during the physiological transitions taking place in the fish in the period of transfer from freshwater to seawater [8–11]. Available information from studies conducted in the south of Norway show clear alterations in immune functions in Atlantic salmon upon seawater transfer with decreases in immune related plasma proteins and levels of lysozyme, IgM and leucocyte [12–14]. A study of expression of immune genes during smoltification and seawater transfer in tissue from pyloric region, employing microarrays, showed alteration in about 300 immune genes including cytokines and T cells marker genes [15]. Also in the distal intestine of Atlantic salmon, which is considered to play the most important role in immune-related gut functions [16] altered expression of immune-related genes has been observed [17].

In the present situation, with a weak knowledge basis for optimizing the nutrient content of the diet, preventative use of so-called functional ingredients is common. Such compounds are claimed to prevent or milden disease outbreaks and improve function and health during periods expected to be particularly challenging, for example the period just before and after seawater transfer [7,18]. The most commonly used functional ingredients for fish include certain nutrients (e.g. essential fatty acids and nucleotides), a range of polysaccharides (e.g. prebiotics) and microbial extracts (e.g. compounds from yeast cell walls) [7,18]. Such functional ingredients are claimed to have beneficial effects locally within the intestine with possible direct or indirect modulatory effects on gut immune responses [18,19], gut barrier functions [20] and gut microbiota [21]. Functional ingredients have different main functions. They are therefore used alone or in combination throughout the production cycle depending on the expected physiological and environmental challenges, to improve the robustness of the fish. Additionally, in the commercial production cycle, Atlantic salmon sense and respond to various changeable environmental factors (e.g. daylight, temperature, salinity and dissolved oxygen), aquaculture practices (e.g. netting and transferring of fish), as well as adjustments of macronutrient composition to meet fish requirement according to growth rate and body composition. Hence, long-term, lager scale feeding trials, conducted under commercial conditions, would represent an important validation step between small-scale feeding experiments of limited duration and the introduction of new diets into the commercial production [22].

The present study was therefore conducted to gain knowledge on fluctuations in gut immune functions and health of Atlantic salmon from late freshwater stage until one year in seawater farmed under large scale, commercial conditions in Arctic regions, and whether use of functional ingredients would benefit gut functions and health.

2. Materials and methods

2.1. Fish husbandry

This experiment was conducted following the Norwegian laws and regulations of the experimentation with live animals according to Norwegian Animal Research Authority. Atlantic salmon fry, from the same batch of eggs hatched in the spring of 2015, were raised at two smolt production tanks in the Bodø area of Norway (N67° – E14°), each tank containing about 180 000 fish. When ready for seawater transfer, the fish were transported to the sea location in Alta area (N70° – E22°) of Finnmark County by boat where each tank was split into triplicated net pens, each containing about 55 000 fish.

For water parameters, a vertically devise connected to an automatic winch (HF5000, Belitronics, Lunde, Sweden) was used to monitor water salinity, temperature and oxygen levels at 3 m depth throughout the experimental period. The temperature followed natural fluctuations in the water and averaged, 6.8 ± 2.6 °C (mean ± SD) for the entire period. Oxygen and salinity levels averaged 11.8 ± 1.3 mg/L and 29.8 ± 3.8‰ throughout the experimental period, respectively (Fig. S1).

2.2. Diets and feeding routines

Two groups of fish were monitored, one fed a series of diets without functional ingredients (Ref) and the other with functional ingredients (Test). The nutritional composition of the two diet series varied in parallel according to the development in nutrient requirements. As the production conditions in the north are unpredictable regarding attacks of pathogens and parasites, feeding with functional ingredients were chosen. The functional ingredients, i.e. nucleotides, yeast cell walls, a prebiotic and essential fatty acids, were supplemented to the Test diets either as single ingredient or as a mixture according to a strategy developed by the feed producer. Table 1 shows the composition of the diets fed during a period of several weeks before each of the four sampling time points. In freshwater, fish were fed continuously to satiation using automatic feeders. Also, during the first weeks in seawater, fish were fed according to appetite, by automatic feeders. Later, feed was supplied according to appetite in one to three meals depending on the length of daylight.

2.3. Sample collection

Samples were taken from the fish at four sampling time points as illustrated in Fig. 1 showing the experimental set-up. The samples were collected once during freshwater period, i.e. two weeks before seawater transfer in May 2016 (FW) and three times during the seawater period, i.e. four weeks after seawater transfer (SW1, June 2016) and two times thereafter (SW2 and SW3, November 2016 and April 2017). Only fish with content along the whole intestine were sampled to be sure that fish were exposed to the diets for several hours before the sampling. Fish were anesthetized with tricaine methanesulfonate (MS222, Argent Chemical Laboratories) and euthanized by cervical dislocation before tissue sampling. Three replicate sampling groups for each treatment, 12 fish per replicate were sampled, i.e. 36 fish per treatment. Length and body weight were measured for all fish, and blood were collected from the caudal vein using heparinized vacutainers for plasma biochemistry analyses. Thereafter, the abdominal cavity was opened, and the digestive organs were taken out and cleared of adipose tissue.

For each replicate, 6 of them were collected for the analysis of digestive functions, i.e. 18 fish per treatment. Specifically, the content from the proximal intestine (PI) divided in two (PI 1 and PI 2), mid intestine (MI) and distal intestine (DI) divided in two (DI 1 and DI 2) were collected and pooled for the analysis of bile acid concentration and trypsin activity. After removing the content, the tissues from PI, MI and DI were weighed for organosomatic indices, respectively, then collected for the evaluation of leucine aminopeptidase (LAP) capacity.

Another 6 fish per replicate were collected for the evaluations of histology and qPCR. More specifically, tissues from DI and pyloric caeca (PC) were rinsed in phosphate buffered saline (PBS) three times to remove traces of remaining chyme before cut into pieces for histological evaluation. These tissue were fixed in 4% phosphate-buffered

Table 1
Formulation and nutrient composition of the two diet series[a].

Feed composition	FW-Ref	FW-Test	SW1-Ref	SW1-Test	SW2-Ref	SW2-Test	SW3-Ref	SW3-Test
Ingredients (%)								
Marine protein sources[b]	40	40	30	30	19	19	19	19
Plant protein sources[c]	35	35	39	39	53	53	53	53
North Atlantic fish oil	9	9	24	24	10	10	10	10
Rapeseed oil	9	9	–	–	7	7	7	7
Binders & Micronutrients	7	7	7	7	11	11	11	11
Sum	100	100	100	100	100	100	100	100
Nutrient composition (%)								
Crude protein	44	44	44	44	46	46	46	46
Crude fat	22	22	28	28	22	22	22	22
Starch	7.5	7.5	8	8	10	10	10	10
Crude fiber	1.5	1.5	3	3	3	3	3	3
Ash	7	7	6	6	5	5	5	5
Functional ingredients[d]								
Essential fatty acids	–	–	–	✓	–	✓	–	✓
Nucleotides	–	✓	–	✓	–	✓	–	✓
Yeast cell walls	–	–	–	–	–	✓	–	–
One prebiotic	–	–	–	–	–	–	–	✓

[a] The composition of four different basic diets varied throughout the time of observation following the strategy developed for this commercial site according to the development and production and health of the fish in the farm. At each observation time, two diets were formulated, one without functional ingredients (Ref diet) and one with functional ingredients (Test diet). FW, sampling point in freshwater (May 2016); SW1, the first seawater sampling point (June 2016); SW2, the second seawater sampling point (November 2016); SW3, the final seawater sampling point (April 2017).
[b] Mix of Scandinavian origin fish meal and, fish protein concentrate (Norway).
[c] Mix of soy protein concentrate, wheat protein concentrate, wheat gluten, sunflower meal.
[d] Inclusion levels were determined according to recommendations from the producers and cannot be disclosed due to commercial interests and production of intellectual rights. These functional ingredients were added in the dry meal mix with other dry ingredients in a homogenous dry mix, then going to the preconditioner and extruder.

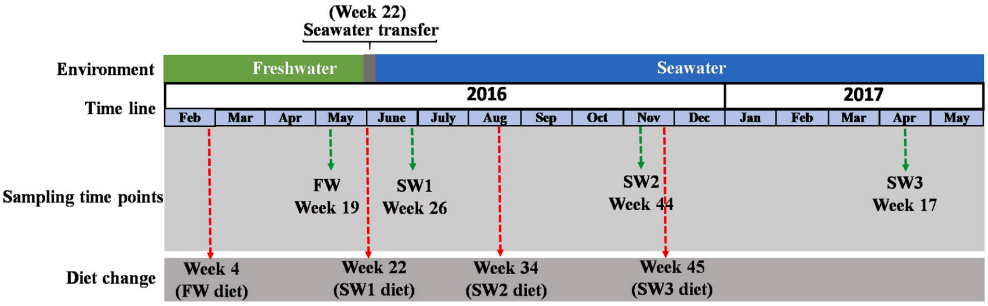

Fig. 1. Outline of the experimental setup. The time for sampling and diet change are shown in green and red dotted arrow, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

formaldehyde solution for 24 h, and then transfer to 70% ethanol for storage. Regarding qPCR, tissues from DI and PC were preserved in RNAlater solution and incubated at 4 °C for 24 h, and then stored at −20 °C before RNA extraction.

3. Analytical procedures

3.1. Plasma biochemistry

Plasma triglyceride, free fatty acid, cholesterol, glucose, chloride and sodium were analyzed according to standard protocols at the Central Laboratory of the NMBU Faculty of Veterinary Medicine (Oslo, Norway).

3.2. Digesta trypsin activity and total bile acids concentration

Trypsin activity was determined according to the description of Kakade et al. [23], using benzoylarginine p-nitroanilide (No. B-4875, Sigma Chemical Co., St. Louis, MO) as the substrate. Bovine trypsin was used to make standard curve. The total bile acid concentration was quantified by the Enzabile test kit (No. 550101, BioStat Diagnostic Systems, Cheshire, U.K.). Taurocholic acid was used to make the standard curve.

3.3. Leucine aminopeptidase (LAP) capacity

The LAP capacity was analyzed according to the description of Bieth et al. [24] using tissue homogenates. The tissue homogenates were prepared using the ice-cold Tris−mannitol buffer (1:20, w/v). The four-(two-aminoethyl)-benzene-sulfonyl fluoride hydrochloride (Pefabloc SC, Basel, Switzerland) was used as a serine proteinase inhibitor. The LAP capacity is expressed as mmol substrate hydrolysed per hour per kg fish.

3.4. Gut mucosa structure

Tissues from DI and PC were processed to produce 3-μm thickness sections that were stained with hematoxylin and eosin (H&E) according to standard histological techniques [25].

Evaluation of DI sections was conducted with light microscopy and focused on the morphological changes of soybean meal-induced enteritis

(SBMIE) in Atlantic salmon that consist of shortening of mucosal fold length, increase in width and cellularity of the submucosa and lamina propria, and reduction in enterocyte supranuclear vacuolization. For the PC, the degree of vacuolization of the enterocytes was evaluated. The morphological characteristics were scored on a scale of 0–4 where 0 represented normal; 1, mild; 2, moderate; 3, marked, and 4, severe changes as described by Bakke et al., 2007 [26].

3.5. Gut mucosa gene expression

Total RNA was extracted from DI and PC tissues using a FastPrep-24 homogenizer, Trizol® reagent and further purified with PureLink (Thermo Fisher Scientific) with an on-column DNase treatment. For PC, samples from FW, SW1 and SW2 were selected for analysis based on histological results, and equal amount of total RNA from three fish per replicate were pooled after RNA extraction. For DI, three individual fish per replicate were processed (i.e. 9 fish per treatment), and all four sampling points were included in the analysis. The integrity of the RNA samples was verified by the 2100 Bioanalyzer using RNA Nano Chips (Agilent Technologies), and RNA purity and concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). The average RNA integrity number (RIN) of all samples was 9.0. Total RNA was stored at −80 °C until use.

The synthesis of cDNA, DNase treatment and qPCR were conducted as previously described [27]. For DI, 32 target genes related to intestinal immune responses, barrier functions, osmoregulation and nutrient metabolism were selected. Primers and assay details are shown in Table 2. For PC, a panel of 16 target genes was profiled, targeting intestinal lipid and sterol metabolism, immune and barrier functions (Table 3). RNA polymerase II (*rnapolii*) and hypoxanthine phosphoribosyl transferase 1 (*hprt1*) for DI and beta-actin (*actb*) and gylceraldehyde-3-phosphate dehydrogenase (*gapdh*) for PC were used as reference genes for the target gene normalization based on their overall coefficient of variation (CV) and interspecific variance [28]. The geometric mean of the reference genes was used as the normalization factor. Mean normalized levels of target genes were calculated from raw quantification cycle (Cq) values [29].

3.6. Calculations

The thermal growth coefficient (TGC) was calculated as follows:

$$TGC = 1000 * [\text{sampling body weight (g)}^{1/3} - \text{initial body weight (g)}^{1/3}] * (\sum \text{day degree})^{-1}$$

Condition factor (CF) was calculated as follows:

$$CF = 100* \text{ sampling body weight (g) / sampling body length (cm)}^{3}$$

Organosomatic indices (OSI) was calculated as follows:

$$OSI\ (\%) = 100* \text{ organ weight / sampling body weight (g)}$$

3.7. Statistical analysis

In order to compare the mean values and clearly identify significant differences between sampling time points, as well as between different diets at each observation time, eight different treatments were defined by combining the sampling time points and diets, i.e. FW-Ref, FW-Test, SW1-Ref, SW1-Test, SW2-Ref, SW2-Test, SW3-Ref and SW3-Test. A two-

Table 2
Details of primer pairs used for qPCR assays in the distal intestine.

Function	Acronym[a]	Primer sequence (5'→3')	Primer sequence (5'→3')	Annealing temp	Product size (bp)	Acc.no
		Forward	Reverse			
Pro-inflammatory	*il17a*	TGGTTGTGTGCTGTGTGTCTATGC	TTTCCCTCTGATTCCTCTGTGGG	60	136	GW574233
	il8	ATTGAGACGGAAAGCAGACG	CGCTGACATCCAGACAAATCT	60	136	NM_001140710
	il1β	GCTGGAGAGTGCTGTGGAAGA	TGCTTCCCTCCTGCTCGTAG	60	73	NM_001123582
	ifnγ	CTAAAGAAGGACAACCGCAG	CACCGTTAGAGGGAGAAATG	60	159	FJ263446
Anti-inflammatory	*tgfβ*	AGTTGCCTTGTGATTGTGGGA	CTCTTCAGTAGTGGTTTGTCG	60	191	EU082211
	il10	CGCTATGGACAGCATCCT	AAGTGGTTGTTCTGCGTT	62	59	EF165028
Adaptive immunity	*myd88*	GACAAAGTTTGCCCTCAGTCTCT	CCGTCAGGAACCTCAGGATACT	60	110	NM_001136545
	gilt	ACGGAAATGCACACGAATCT	GCCTCCATGCAGTAGACGAT	60	148	BT047766
T-cell markers	*cd4α*	GAGTACACCTGCGCTGTGGAAT	GGTTGACCTCCTGACCTACAAAGG	60	121	NM_001123611
	cd8β	CGCACACACCTCAACAACTC	ATTGATGCGCAGTGTGAAAG	59	153	AY693394
	cd3γδ	AAAGGCGCATGGACAGATCT	GCCCGCACAACATTAAAGCT	60	160	NM_001123621
Goblet cell markers	*muc13*	ATTGTCGGCACTGTTCTTGG	GGAGCTCTTCTTGGACGTCT	60	88	DY723445
	muc2	TCTGTCCTGATGGGATGAAAC	GGACTCCAAACAAACGCAAT	60	143	CK885177
Stress response	*cat*	CCCAAGTCTTCATCCAGAAACG	CGTGGGCTCAGTGTTGTTGA	60	101	BG935638
	hsp70	CCCCTGTCCCTGGGTATTG	CACCAGGCTGGTTGTCTGAGT	60	121	BG933934
	sod	CCACGTCCATGCCTTTGG	TCAGCTGCTGCAGTCACGTT	55	140	BG936553
Tissue remodeling	*pcna*	TGAGCTCGTCGGGTATCTCT	GTCCTCATTCCCAGCACACT	55	170	BT056931
Mucosal barrier function	*zo-1*	CAAAGCCAGTGTATGCCCAG	CAGCTTCATACTCGGCCTGA	60	119	XM_014175464.1
	e-cadherin	ACTATGACGAGGAGGGAGGT	TGGAGCGATGTCATTACGGA	60	107	BT058864.1
	claudin-15	GGCACGTCTGAGAAACAACA	TAGGAAGTGGCAGCCTGACT	60	92	BK006395
	claudin-25b	CCTGTAAGAGGGGTCCATCA	TGACACATGTTCTGCCCTGT	60	101	BK006399
	occludin	GACAGTGAGTTCCCCACCAT	ATCTCTCCCTGCAGGTCCTT	60	101	NM_001173656.1
Nutrient transport	*slc6a6*	GGAGGTGGAAGGACAGATCA	ACATGCCACCTTTCGTTACC	60	143	NM_001139800
	fabp2b	TGCCTTCCCCTCATTCTCTA	GGTGATACGGTCTTCATCCAA	60	152	BT046827
	pept	GGCTTTCTGCTCTGTGAAGG	TAGGGGGACACAACAAGACC	55	89	NM_001146682
	slc10a2	CCCTGGGAATCTACGTCAAA	GGTCCAGGAGGACTGGTACA	60	134	XP_014019465
Osmoregulation	*ecc*	GCAGTGTTGCTGCTGGTTTA	TCAGGCACCACTGGGTTAAT	60	104	C081R050
	aqp10	GGTGTTGGTGATCGGAGTCT	CGCCCTAAACACCTCATCC	62	121	DW569041
	aqp1a	CTACCTTCCAGCTGGTCCTG	TGATACCGCAGCCTGTGTAG	62	141	BT046625
	aqp1b	CTGTGGGTCTGGGACATCTT	TAAGGGCTGCTGCTACACCT	62	153	NM_001140000.1
	nkaα1b	CTGCTACATCTCAACCAACAACATT	CACCATCACAGTGTTCATTGGAT	60	81	NM_001124460
	aqp8 ab	GTTGGCATAGTTCTCCTTTGATG	TTTCAACCCTCCCTTCACC	60	148	KC626879.1
Reference genes	*rnapoii*	CCAATACATGACCAAATATGAAAGG	ATGATGATGGGGATCTTCCTGC	60	157	BG936649
	hprt1	CCGCCTCAAGAGCTACTGTAAT	GTCTGGAACCTCAAACCCTATG	60	255	BT043501

[a] For explanation of gene abbreviations see Tables S1–3.

Table 3
Details of primer pairs used for qPCR assay in the pyloric caeca.

Function	Acronym[a]	Primer sequence (5'→3')	Primer sequence (5'→3')	Annealing temp	Product size (bp)	Acc.no
		Forward	Reverse			
Immune	*il1β*	GCTGGAGAGTGCTGTGGAAGA	TGCTTCCCTCCTGCTCGTAG	60	73	NM_001123582
	tgfβ	AGTTGCCTTGTGATTGTGGGA	CTCTTCAGTAGTGGTTTGTCG	60	191	EU082211
	cd3γδ	AAAGGCGCATGGACAGATCT	GCCCGCACAACATTAAAGCT	60	160	NM_001123621
Mucosal barrier function	*Zo-1*	CAAAGCCAGTGTATGCCCAG	CAGCTTCATACTCGGCCTGA	60	119	XM_014175464.1
	claudin-15	GGCACGTCTGAGAAACAACA	TAGGAAGTGGCAGCCTGACT	60	92	BK006395
	claudin-25b	CCTGTAAGAGGGGTCCATCA	TGACACATGTTCTGCCCTGT	60	101	BK006399
Nutrient transport	*pept*	GGCTTTCTGCTCTGTGAAGG	TAGGGGGACACAACAAGACC	55	89	NM_001146682
Osmoregulation	*nka-α1b*	CTGCTACATCTCAACCAACAACATT	CACCATCACAGTGTTCATTGGAT	60	81	NM_001124460
	aqp8 ab	GTTGGCATAGTTCTCCTTTGATG	TTTCAACCCTCCCTTCACC	60	148	KC626879
Stress response	*hsp70*	CCCCTGTCCCTGGGTATTG	CACCAGGCTGGTTGTCTGAGT	60	121	BG933934
Lipid metabolism	*fabp2b*	TGCCTTCCCCTCATTCTCTA	GGTGATACGGTCTTCATCCAA	60	152	BT046827
	hmgcr	CCTTCAGCCATGAACTGGAT	TCCTGTCCACAGGCAATGTA	60	224	NM_001173919
	plin2	CCCAGGTCTACTCCAGCTTC	CAGCGACTCCTTCATCTTGC	60	104	BT072598
	apoa1	CTGGTCCTCGCACTAACCAT	TGGACCTCTGTGCAGTCAAC	60	144	NM_001123663
	cyp51	TGCATTGGGGAGAACTTTGC	ATCTGATGACGGGGTTGTGT	60	148	DY731118
	npc1l1	CCAAAGACCTGATCCTGGAA	CGAAGCACACATCCTTCAGA	60	108	CB505644
Reference genes	*gapdh*	AAGTGAAGCAGGAGGGTGGAA	CAGCCTCACCCCATTTGATG	60	84	BT050045
	actb	CAAAGCCAACAGGGAGAAGATGA	ACCGGAGTCCATGACGATAC	60	88	AF012125

[a] For explanation of gene abbreviations see Tables S4–5.

way ANOVA was considered unsuitable as diet composition varied throughout the observation time both regarding macronutrient and functional ingredients composition. Differences in histological scores were analyzed for statistical significance using the Fisher exact test. Post hoc analysis for significant Fisher Exact Test results was conducted using the Chisq.post.hoc test. Both statistical tests were run in the R statistical package (version 3.4.2; 2017) within the RStudio interphase (version 1.1.383; 2017; RStudio Inc.). Other statistical analyses were performed with JMP Pro 13.0.0 (SAS Institute, United States). One-way ANOVA and Tukey-Kramer HSD multiple comparisons were performed to interpret and compare the mean values and identify significant differences between these eight different treatments. Data were evaluated for homogeneity of variance and normality of residuals using the "residual by predicted" plot and histogram, respectively. When necessary, data were transformed by box-cox power transformation to meet the statistical assumptions, and then refitted for a second evaluation. The level of significance in all analyses was set at $P < 0.05$. In the tables and figures, values with the same superscript letter are not significantly different. Figures were made using GraphPad Prism 8 (GraphPad Software, La Jolla, California, United States).

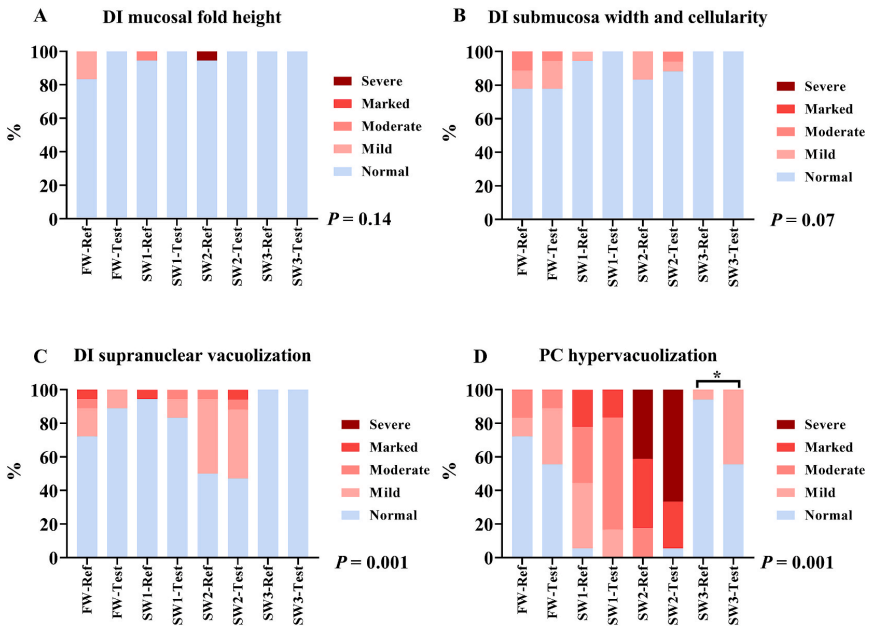

Fig. 2. Contingency charts showing proportions of sampled fish that scored normal, mild, moderate, marked and severe for the selected distal intestine and pyloric caeca morphological characteristics among treatments. *P* values of the Fisher's exact test are shown on the lower right corner of the subplot. Asterisk indicates significant effect of diet ($P < 0.05$, n = 18). DI: distal intestine, SM: submucosa, PC: pyloric caeca.

4. Results

4.1. Histological characteristics of the gut mucosa

The distal intestine of the fish showed normal morphological characteristics for most of the samples based on observations of mucosal fold height, submucosa width and cellularity and enterocyte supranuclear vacuolization. The exception was for samples taken at SW2 for which about 50% of the fish from both Ref and Test diets showed mild to marked reduction in supranuclear vacuolization of the enterocytes (Fig. 2 A, B and C). In pyloric caeca, enterocyte hyper-vacuolization, interpreted as steatosis and indication of lipid malabsorption, was observed (Fig. 2 D). The severity of this hyper-vacuolization increased gradually from FW through SW1, culminated at SW2 and decreased at SW3 (Fig. 2 D). A diet effect was observed for fish sampled at SW3, i.e. a higher proportion of mild steatosis for fish fed the Test diet compared to fish fed Ref diet (Fig. 2 D).

4.2. Gene expression

4.2.1. Gene expression in distal intestine

Overall, expression of genes related to gut immune functions and other health related functions showed clear differences between sampling time points. The results observed at SW1, i.e. just after seawater transfer, revealed substantially suppressed expression of most immune, barrier, stress, goblet cell marker genes as well as some other gut function related gene expressions. However, thereafter, i.e. at SW2 and SW3, most of gene expression levels returned back to the levels observed at FW (Fig. 3 and Tables S1–3).

Also, goblet cell marker mucin-13 (*muc13*) showed lower expression levels in fish at SW1 compared to FW fish, followed by an increase at the later seawater sampling points ($P < 0.05$, Fig. 4 A). Regarding diet effects, only negligible differences were observed.

In detail: Pro-inflammatory cytokines (interleukin-1 beta, *il1β* and interferon gamma, *ifnγ*), showed high expression at FW, a drop after seawater transfer, followed by an increase or no change throughout the rest of the observation period ($P < 0.05$, Fig. 4 B and C). A similar pattern was seen for anti-inflammatory cytokines (transforming growth factor-beta, *tgfβ* and interleukin 10, *il10*) (Fig. 4 D and E), T-cell markers (e.g. cluster of differentiation 3 γδ, *cd3γδ*) (Fig. 4 G) and myeloid differentiation factor 88 (*myd88*) (Fig. 4 I) showing high expression in fish from FW, followed by lower expression of fish from SW1 and higher expression thereafter. For cluster of differentiation 8 beta (*cd8β*), fish showed the lower expression at FW and SW1 but increased at later sampling points (Fig. 4 F). Gamma-interferon inducible lysosomal thiol reductase (*gilt*) showed higher expression at SW3 compared to those from other sampling time points (Fig. 4 H).

Similar to the observations for the immune related genes, genes related to tight junction barrier function (zo-*1*, *claudin-15* and *claudin-25b*) showed lower expression levels in fish from SW1 compared to FW fish, followed by an increase throughout the later seawater sampling points (Fig. 5).

For the intestinal water channel aquaporin-8ab (*aqp8ab*), gene expression levels increased progressively from the first to the last sampling point ($P < 0.05$, Fig. 6 A). The gene expression levels of aquaporin-10 (*aqp-10*) showed a similar trend regarding differences between sampling points but except for a decreased expression at SW2 (Fig. 6 B). Compared to the expression of *aqp-10* and *8adb*, very low expression

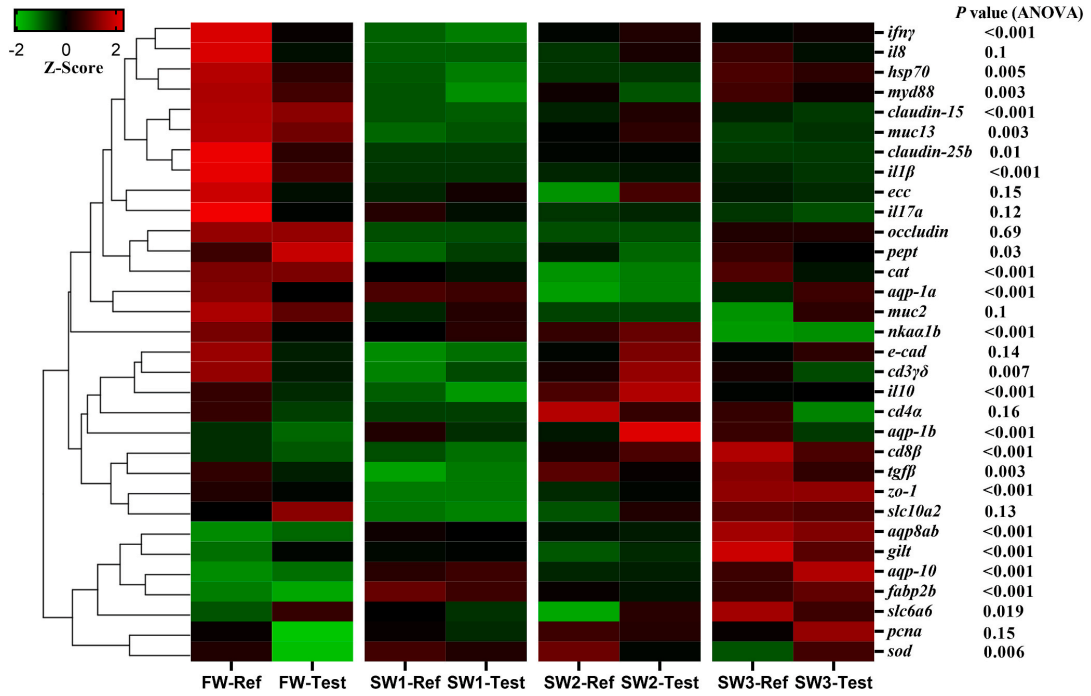

Fig. 3. Heatmap showing the hierarchical clustering of differentially expressed genes in distal intestine of Atlantic salmon fed Ref and Test diets during the observation period. Colors correspond to Z-score (z = (relative expression – mean)/standard deviation) based on the relative gene expression, i.e., red (high positive value) indicates higher relative expressions; Green (low negative value) indicates the lower relative expressions. The level of significance in all analyses was set at $P < 0.05$ between treatments (n = 9). An alternative representation of the data and the explanation of gene abbreviations can be found in Tables S1–3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

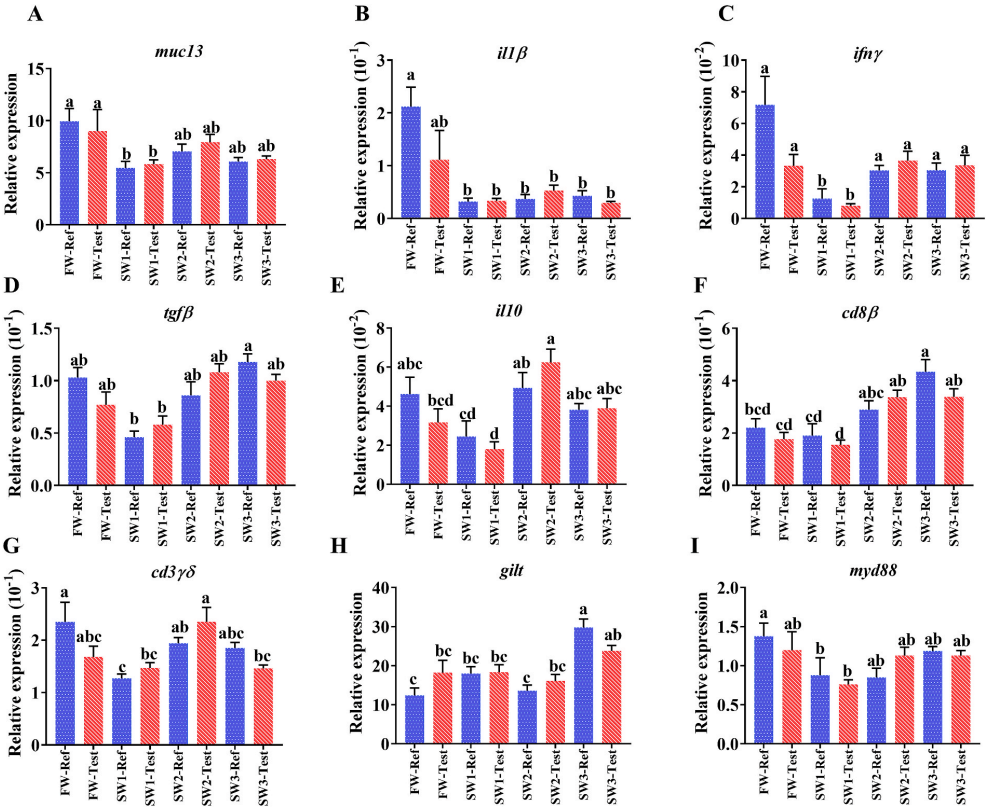

Fig. 4. Expression levels of genes related to immune responses in the distal intestine of Atlantic salmon fed Ref and Test diets during the observation period. For explanation of gene abbreviations, see Table S1. Data are presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 9) and values sharing the same letters are not significantly different.

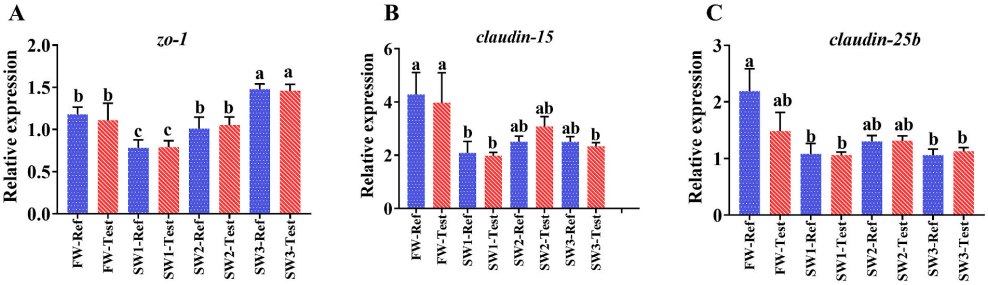

Fig. 5. Expression levels of genes related to barrier function in the distal intestine of Atlantic salmon fed Ref and Test diets during the observation period. For the explanation of gene abbreviations, see Table S1. Data are presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 9) and values sharing the same letters are not significantly different.

levels of aquaporin-1a and 1b (*aqp-1a* and *1b*) were found for all fish without clear difference between sampling points (Table S2).

The peptide transporter (*pept*) showed decreasing expression levels after sea water transfer (Fig. 6 C), while a significant increase was found for fatty acid binding protein 2b (*fabp2b*) ($P < 0.05$, Fig. 6 D).

Regarding the expression profile of other gut health related genes, such as interleukin 17A (*il17a*), interleukin 8 (*il8*), cluster of differentiation 4α (*cd4α*), epithelial chloride channel protein (*ecc*), solute carrier family 10-member 2 a (*slc10a2*), e-cadherin (*e-cad*), occluding, proliferating cell nuclear antigen (*pcna*) and mucin-2 (*muc2*), no significant effects of life stage were found ($P > 0.05$, Tables S1–3). No clear and consistent diet effects were found on the expression level of genes related to intestinal immune responses, barrier functions, osmoregulation and nutrient metabolism at any of the sampling points

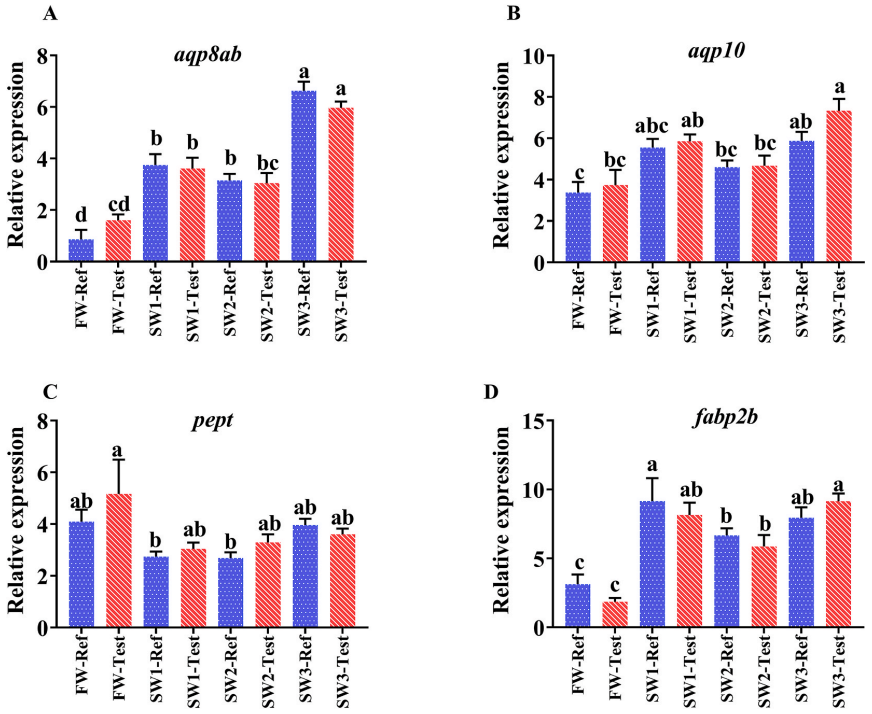

Fig. 6. Expression levels of genes related to water and nutrient transport in the distal intestine of Atlantic salmon fed Ref and Test diets during the observation period. For the explanation of gene abbreviations, see Tables S1–3. Data are presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 9) and values sharing the same letters are not significantly different.

(Tables S1–3).

4.2.2. Gene expression in pyloric caeca

The tissue of PC showed increased expression levels of *aqp8ab* in fish from SW1, followed by a suppression at SW2 (Fig. 7 A). Expression of *pept* decreased from FW throughout the seawater stages ($P < 0.05$, Fig. 7 B). The opposite picture was found for lipid droplet marker perilipin-2 (*plin2*) with higher expression levels observed after seawater transfer ($P < 0.05$, Fig. 7 C). The expression level of cholesterol synthesis gene cytochrome P450 51 (*cyp51*) increased and stabilized after sea transfer ($P < 0.05$, Fig. 7 D). The diet effect was insignificant for these variables at all sampling points. Regarding the gene profile expression of other immune, barrier function and metabolism related genes in PC, no significant effects of life stage nor of diet were found ($P > 0.05$, Tables S4 and 5).

4.3. Growth and body indices

Growth rate, estimated as TGC, was based on the initial weights of the whole population 6 months before FW sampling time point, as well as on the weights of the individual sampled fish. The results showed large differences between the sampling points. In the period of FW and SW1, fish showed the lowest TGC ($P < 0.05$, Fig. 8). In the next period, the growth rate increased significantly to a value about twice as high and stabilized thereafter. In the period between SW2 and SW3, fish fed Test diet tended to have lower growth than the fish fed the Ref diet, but no statistical difference was found (Fig. 8).

The lowest, and similar CF values for the two diets, were observed in fish from FW and SW1, whereas those from SW2 and SW3 showed significantly higher values. At SW2 and SW3, lower CF values were observed for the Test diet compared to the Ref diet (Table 4).

Regarding organo-somatic indices for PI, significant differences were seen between all sampling points ($P < 0.05$, Table 4) with increasing values from FW to SW1 and SW2. At SW3, the values had decreased to levels falling between those of SW1 and SW2. Regarding diet effects, no clear differences between the diets were observed at FW, SW1 or SW2. At the last sampling point (SW3) however, higher PI somatic indices were shown for fish fed Test diet ($P < 0.05$, Table 4). The results for MI and DI somatic indices showed the same trends regarding differences between sampling points, but the differences were less pronounced (Table 4). A significant diet effect was observed in DI at SW3, i.e. higher value in fish fed the Test diet (Table 4).

4.4. Plasma biomarkers

Plasma cholesterol and triglyceride decreased substantially after seawater transfer ($P < 0.05$, Fig. 9 A and B). Thereafter the values returned to a level not significantly different from that of the fish sampled at FW and remained at this level. Regarding plasma free fatty acid, the values decreased slightly after the seawater transfer, for then to increase to values at about the same level as observed in fish in the fresh water. Thereafter the values decreased significantly at SW3 ($P < 0.05$, Fig. 9 C). Fish from the FW sampling point showed markedly higher glucose levels compared to fish from seawater samplings ($P > 0.05$, Fig. 9 D). No significant diet effects were observed for plasma cholesterol, triglyceride, free fatty acid or glucose levels at any of the sampling time points, except for a significantly lower plasma triglyceride level in fish fed the Test diet at the SW2 sampling point ($P < 0.05$, Fig. 9 B).

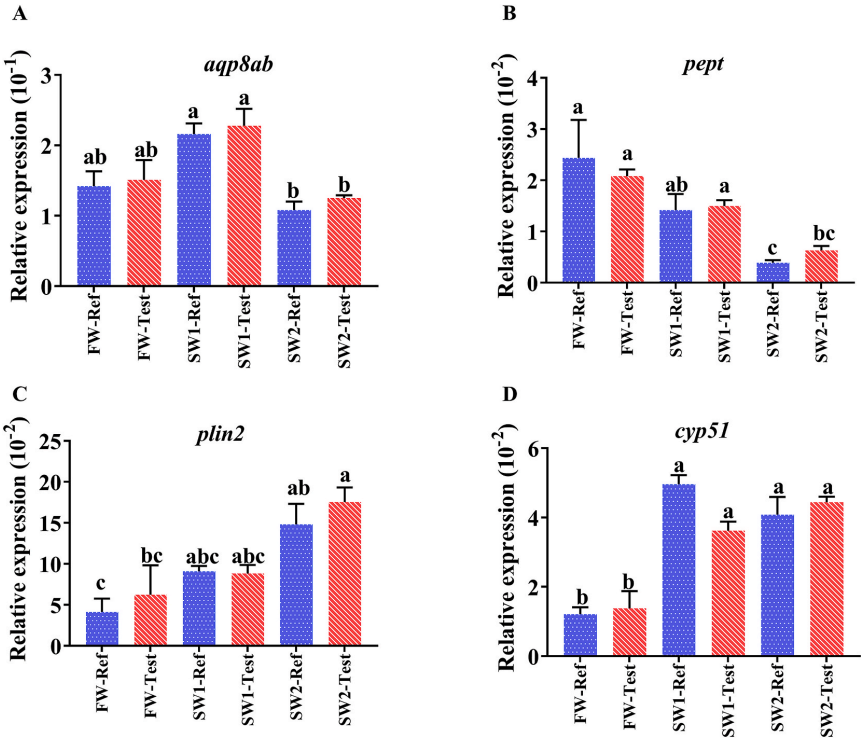

Fig. 7. Expression profile of selected genes in the pyloric caeca of Atlantic salmon fed Ref and Test diets during the observation period. For the explanation of gene abbreviations, see Tables S4–5. Data are presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 3) and values sharing the same letters are not significantly different.

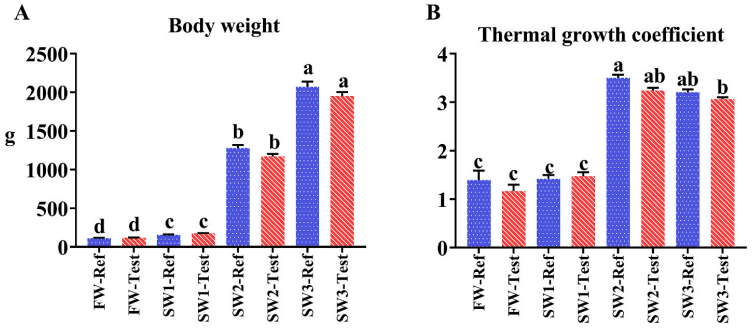

Fig. 8. Growth performance of Atlantic salmon fed Ref and Test diets during the observation period. Data were presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 36) and values sharing the same letters are not significantly different.

Compared to fish from the FW sampling point, fish from the SW had higher plasma chloride and sodium levels ($P < 0.05$, Fig. 9 E and F). A diet effect was only observed at SW2, i.e. higher plasma chloride levels in fish fed the Test diet compared to those fed Ref diet ($P < 0.05$, Fig. 9 E).

4.5. Digesta bile acid concentration and trypsin activity

Compared to fish from other sampling points, fish from SW3 had higher digesta bile salts concentrations in PI 1, PI 2, MI and DI 1 (Table 5). For PI 1 and PI 2, the lowest values were observed in freshwater, while for DI the lowest values were observed right after seawater transfer. In samples from DI 2, no significant differences were observed between life stages. Moreover, no clear diet effect was observed at any of the sampling points (Table 5).

The trypsin activities in intestinal digesta showed no significant differences between sampling points nor between the two diets ($P > 0.05$, Table 5).

Table 4
Condition factor and indices of gut sections in Atlantic salmon fed Ref and Test diets during the observation period[a].

	Condition factor	PI	MI	DI
One-Way ANOVA				
P value	<0.001	<0.001	<0.001	<0.001
Pooled SEM	0.02	0.12	0.01	0.02
Mean values -Tukey-Kramer HSD				
FW-Ref	1.03^{d}	1.6^{e}	0.17^{de}	0.37^{de}
FW-Test	1.04^{d}	1.6^{e}	0.16^{e}	0.32^{e}
SW1-Ref	1.02^{d}	2.4^{d}	0.22^{bcd}	0.49^{bc}
SW1-Test	1.03^{d}	2.2^{d}	0.21^{cde}	0.44^{cd}
SW2-Ref	1.42^{a}	4.8^{a}	0.40^{a}	0.54^{ab}
SW2-Test	1.33^{b}	4.9^{a}	0.38^{a}	0.52^{abc}
SW3-Ref	1.30^{bc}	3.1^{c}	0.25^{bc}	0.49^{bc}
SW3-Test	1.24^{c}	3.7^{b}	0.28^{b}	0.58^{a}

[a] For explanation of sampling point and diet abbreviations see Table 1. PI, proximal intestine; MI, mid intestine; DI, distal intestine. Values with same superscript in a column are not significantly different ($P < 0.05$, condition factor n = 36, organosomatic indices n = 18).

4.6. Brush border leucine aminopeptidase (LAP) capacity

In the PI and DI, the LAP capacity increased significantly after the seawater transfer ($P < 0.05$) and tended to stabilize at a higher level thereafter (Fig. 10). In the MI, the LAP capacity increased slightly and tended to stabilize after the seawater transfer. No clear diet effects on intestinal LAP capacity were observed at any of the sampling points ($P > 0.05$, Fig. 10).

4.7. Management observations

Over the 15-week observation period in freshwater, the body weight in both the fish fed the Ref and Test diet showed average weight of 122 g. The mortality during the freshwater phase was similar for fish fed Ref and Test diets, averaging 9%. The main part of the loss (about 80%) was due to hemorrhagic smolt syndrome (HSS), not uncommonly observed in fish within the smoltification period. At slaughter time, TGC, calculated for all fish in the farm over the whole saltwater phase, showed high values and a trend ($P = 0.085$) towards higher growth for fish fed the Ref diet compared to the Test diet, 3.7 and 3.6, respectively. There was no significant difference between diets regarding FCR ($P = 1.00$). In the seawater period, recorded mortality did not differ significantly ($P = 0.09$) and averaged 8.6% for fish fed the Ref and the Test diet. There were two main events of increased mortality in this period. The first during transport from the freshwater to the seawater site, the second right after seawater transfer which is a frequent observation. Some of this mortality might partly be due to complications from the pre-existing HSS condition. In December, the first winter in seawater, the population was diagnosed with heart and skeletal muscle inflammation (HSMI) which temporarily increased mortality. No outbreaks of parvicapsulosis or teacibaculosis, commonly challenging salmon in the northern regions, occurred in the studied population.

5. Discussion

The main findings of the present work were as follows:

Major, temporary drops were observed in expression of gut immune and barrier function genes four weeks after seawater transfer. Apparent enterocyte hyper-vacuolation in pyloric caeca was observed in samples from FW to SW2 with concurrent increased expression levels of the lipid droplet surface marker *plin2*. No clear indication of beneficial effects was observed throughout the observation period. However, an increased metabolic cost was indicated by lower CF and plasma triglyceride levels, and a tendency to lower growth at SW2 due to the mixture of nucleotides, a prebiotic and essential fatty acids used at this time point.

5.1. Gut immune and barrier functions

Firm conclusions regarding consequences for the fish health, performance of the many alterations in the expression of gut immune and barrier related genes two weeks before seawater transfer and four weeks after, cannot be made. However, existing literature indicates that most

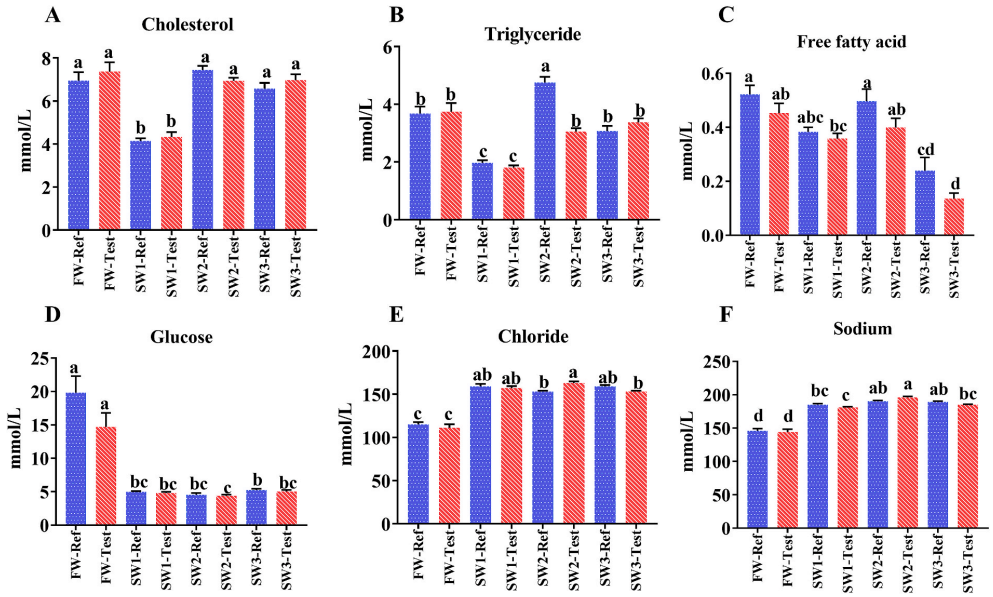

Fig. 9. Plasma biomarkers of Atlantic salmon fed Ref and Test diets during the observation period. Data are presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 36) and values sharing the same letters are not significantly different.

Table 5
The total bile acid concentration and trypsin activity in the digesta of Atlantic salmon fed the Ref and Test diets during the observation period[a].

	Total bile acid levels (mg/g dry matter)					Trypsin activity (U/mg dry matter)				
	PI 1	PI 2	MI	DI 1	DI 2	PI 1	PI 2	MI	DI 1	DI 2
One-Way ANOVA										
P value	<0.001	<0.001	0.002	0.002	0.15	0.05	0.073	0.18	0.46	0.38
Pooled SEM	15.4	12.5	6.9	4.1	2.0	44.8	31.1	24.7	16.6	10.6
Mean values-Tukey-Kramer HSD										
FW-Ref	142^{c}	131^{b}	94^{c}	28^{bc}	9	196	203	172	94	27
FW-Test	152^{c}	126^{b}	106^{bc}	21^{bc}	8	201	203	166	113	49
SW1-Ref	187^{bc}	167^{ab}	108^{abc}	16^{c}	5	252	328	180	61	14
SW1-Test	171^{c}	177^{ab}	107^{bc}	16^{c}	5	334	338	228	74	23
SW2-Ref	174^{c}	144^{b}	97^{c}	29^{b}	14	316	238	107	70	46
SW2-Test	194^{bc}	155^{b}	98^{c}	30^{b}	13	340	246	126	74	46
SW3-Ref	292^{a}	233^{a}	157^{a}	47^{a}	9	401	235	164	105	41
SW3-Test	265^{ab}	229^{a}	133^{ab}	41^{a}	9	373	267	142	100	27

[a] The explanation of sampling point and diet abbreviations see Table 1. PI 1, the proximal half of the proximal intestine; PI 2, the distal half of the proximal intestine; MI, mid intestine; DI 1, the proximal half of the distal intestine; DI 2, the distal half of the distal intestine. Values with same superscript in a column are not significantly different ($P < 0.05$, n = 3).

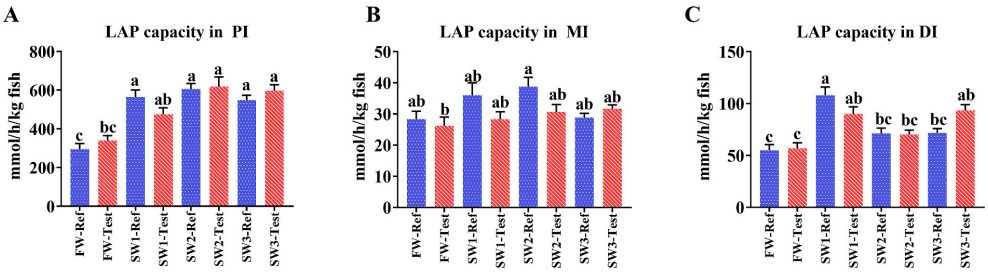

Fig. 10. Leucine aminopeptidase (LAP) capacity of Atlantic salmon fed Ref and Test diets during the observation period. PI, proximal intestine; MI, mid intestine; DI, distal intestine. Data are presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 18) and values sharing the same letters are not significantly different.

of the alternations seen after seawater transfer weaken disease resistance and production [15,30,31]. The mucus layer, with its mucins, acts as the first immune barrier [32]. Downregulation of *muc13* in DI just after seawater transfer, as observed in our study indicates a reduction of goblet cells, since the gene *muc13* codes for the main sialomucin and its downregulation expression has been observed to be accompanied by reduced goblet cells [33,34]. Also, the downregulation of *muc13* might render the tissue more prone to inflammation, as supported by the study in mice that *muc13* prevents intestinal inflammation by inhibiting epithelial cell apoptosis [35].

The observation in our study of down-regulation of expression of pro-inflammatory (*ifnγ* and *il1β*) and anti-inflammatory cytokines genes (*tgfβ* and *il10*) after seawater transfer may have important implications for the disease resistance of fish, since inflammatory cytokines play critical roles in intestinal immune homeostasis [36,37]. Decreased expression of inflammatory cytokines genes may be expected to induce tissue damage due to overreaction of inflammatory response [38–41]. However, this did not appear to be the case in our situation as no clear inflammation signs was observed in the present study. The suppression of pro-inflammatory cytokines genes may be due to the modulation of the *myd88*-independent pathway [42], observed as lower expression of *myd88* in DI after seawater transfer in our study. Regarding anti-inflammatory cytokines, decreased expression of *tgfβ* usually corroborates with *il10* downregulation, also observed in our study, working as a regulatory cytokine in T-cells [43]. T-cell barrier-disrupting effect can be mediated through decreased counteraction of anti-inflammatory cytokines, e.g. *tgfβ* [44,45], as found in our study in which T cell markers, i.e. *cd3γδ* and *cd8β*, revealed lower expression levels at SW1. The activation of T cells may be suppressed by *gilt* indicating that lower *gilt* expression possibly takes place in the course of sensitization of T cells [45,46]. These results are suggested to support the hypothesis that the decreasing gene expression of T cell markers may related to the reduction of T cells or intestinal epithelial cell loss [45]. The lower expression levels of immune-related genes at SW1 may be an unavoidable effect of all the physiological challenges related to stress due to transporting, handling and exposure to new pathogens, as well as great changes in environmental conditions, such as alteration in water temperature and salinity [47–49]. It is not unlikely that the observed alteration in expression of immune genes are important factors in the mechanisms underlying the increased mortality observed soon after seawater transfer [50,51].

The decrease in expression of *zo-1* and claudins after seawater transfer is line with earlier studies [52,53]. Also, the work of [54,55] showed rapidly changing intestinal permeability and translocation rates after seawater transfer which may cause disturbances in barrier functions and suppressed immune defense mechanisms. As mentioned above, the triggers of the changes in immune and barrier functions just after seawater transfer may be both physiological, e.g. related to the smoltification processes, and farming conditions, such as feed deprivation, handling stress, rapidly changing water temperature, water salinity and other environmental factors [55–57]. The dominating claudins, i.e. *claudin-15* and *claudin-25b*, in the intestine of Atlantic salmon are involved in the reorganization of the intestinal epithelium and may affect paracellular permeability during seawater acclimation [58,59]. Hence, the decreased *claudin-15* and *claudin-25b* expression levels after seawater transfer, which is accompanied by increase in paracellular permeability, might also be explained by natural physiological responses for osmoregulation during seawater adaption rather than the

loss of the intestinal immune function with implications for the immune apparatus.

As the fish developed towards SW2 and SW3, most of the biomarkers which were down regulated just after sea transfer, increased to the level observed in fish from FW. This observation suggests that immunological adaption to seawater environment was reached at a stage between SW1 and SW2, i.e. more than four weeks after seawater transfer, as supported by an earlier study [15] that recovery time of the immune-related gene expression to pre-transfer levels should take more than three weeks.

5.2. Variation in PC hyper-vacuolization

The hyper-vacuolization in the PC observed in our study at SW1 and SW2, indicates insufficient lipid transport capacity across the mucosa at these time points [60,61]. These observations corresponded with the increases in *plin2* (adipophilin) expression, a marker for lipid droplets and lipid accumulation, and shunting of lipid into storage vacuoles [62]. Similar correspondence between pyloric caeca hyper-vacuolization and *plin2* expression has been observed in salmon previously [43]. In the current study, we also observed an increased expression of *cyp51*, which is involved in de novo synthesis of cholesterol, another component involved in lipid transport. The apparent hyper-vacuolization of PC observed at the two sampling time points may have different causes. At SW1, the lipid accumulation may be related to the generally slower level of several biomarkers and functions, e.g. the low plasma cholesterol levels, which might lead to reduced capacity for lipid transport, and possibly other factors of importance for lipid transport. Deficiency of nutrients, due to low feed intake, may also be a factor. The hyper-vacuolization observed at the SW2 sampling, one the other hand, may be due to high feed intake and therefore high fat intake concomitant with insufficient supply of components essential for lipid transport, e.g. choline [63]. Until recently, and probably still, commercial salmon feeds, mainly based on plant ingredients, have been deficient in choline for efficient transport of lipid across the mucosa when feed intake is high [63,64].

5.3. Effects of functional ingredients

Several reviews [7,65,66] conclude that inclusion of single or mixtures of functional ingredients, for example, nucleotides, prebiotics and immunostimulants, will improve growth or health of fish during stressful farming conditions, such as seawater transfer and critical life stages. In our study, fish fed the Test diet, with selected functional ingredients, tended to grow slower at SW2 compared to those fed Ref diet. At this sampling point, fish were fed diet with nucleotides, yeast cell walls and essential fatty acids and showed reduced condition factor and plasma triglyceride level, indicating lower lipid content in the body, which would be expected to be due to increased energy demand and consequently reduction in energy efficiency. Overall, the present results indicate that these selected functional ingredients, used during the production cycle, may represent a metabolic cost for the fish. Our findings are in line with previous reports of decreased efficacy of immune-stimulants, including yeast cell walls, after long-term oral administration to fish [67].

The lack of effects of functional ingredients on expression of immune genes and other health indices in our study, was unexpected in light of the results of a multitude of controlled feeding experiments, typically demonstrating strong immune-modulating effects of such components when included in animal feed. It is, however, known that, such effects depend on several factors, including the characteristics of the functional ingredient itself, timing and duration of administration, species and life stage [7,65,66]. One explanation for the apparent discrepancy between our observations and the majority of available scientific literature may therefore be related to the fact that the present experiment was conducted under Arctic conditions, which have never been assessed in controlled laboratory experiments. Another factor may be that the accuracy of the estimates of our study was not as high as in controlled feeding experiments, which in turn may be able to detect smaller differences than the present experiment. The long-term use of functional ingredients throughout the production cycle, is another possible reason for the lack effects on the immune biomarkers [68]. The present experiment reveals a great need for further studies of effects of functional ingredients under commercial conditions in the Arctic region to find whether they are useful or not, and how they should be used if useful.

5.4. General performance

Our observations of low growth performance and body indices at FW and SW1 are in line with previous reports of poor growth performances in shorter time periods before [69,70] and after [71,72] seawater transfer. The poor growth performance is assumed to be related to the demanding smoltification process that takes place in this period as well as stress due to handling and the exposure to new seawater environment, which affect osmoregulation and may reduce feed intake [73,74]. Overall, these adaptation processes imply a higher energy requirement during this developmental stage, for which the fish do not seem to be able to compensate by adjusting their feed intake [10,75,76]. Our observations of lower plasma nutrient concentrations, such as triglyceride and cholesterol, in fish at SW1, support this consideration.

The observation of stable plasma chloride and sodium levels at all the SW sampling time points, at higher levels than observed for FW, are in agreement with the results of previous studies [9,10,77,78]. The results indicate that the fish were well adapted to new seawater environment four weeks after seawater transfer. Aquaporins, especially *aqp8ab*, also play a central role in osmoregulation through trans-epithelial water transport [8,79,80]. One previous study found the expression levels of *aqp8ab* in the intestine was elevated after seawater transfer [8]. These observations were in line with our study that increasing expression levels of *aqp8ab* in DI were observed throughout the production cycle, from the lowest level at FW, to the highest level at SW3, strongly suggesting the importance of *aqp8ab* in regulation of intestinal transcellular uptake of water during seawater acclimation, as also supported by previous studies in Atlantic salmon [81] and Japanese eels [82].

6. Conclusion

The processes taking place from two weeks before to four weeks after seawater transfer caused large reductions in plasma nutrient content and intestinal immune and barrier functions. Apparent enterocyte hyper-vacuolization in pyloric caeca was observed in samples from SW1 to SW2, while there were no clear signs of inflammation of the distal intestine which looked healthy throughout the observation period. Functional ingredients, used throughout the observation period, did not show beneficial effects, but seemed to represent a metabolic cost for the fish.

CRediT authorship contribution statement

Jie Wang: Formal analysis, Experiment design, Sampling, Analyses, Writing, Writing - original draft. **Trond M. Kortner:** Formal analysis, Experiment design, Sampling, Analyses, Writing - review & editing, Supervision. **Elvis M. Chikwati:** Formal analysis, Analyses, Writing - review & editing. **Yanxian Li:** Sampling, Writing - review & editing. **Alexander Jaramillo-Torres:** Sampling, Writing - review & editing. **Jan Vidar Jakobsen:** Writing - review & editing, Funding acquisition. **Jarle Ravndal:** Writing - review & editing, Funding acquisition. **Øyvind Jakobsen Brevik:** Experiment design, Writing - review & editing, Funding acquisition. **Olai Einen:** Writing - review & editing, Funding acquisition. **Åshild Krogdahl:** Experiment design, Sampling, Writing - review & editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.09.019.

References

[1] FAO, The State of World Fisheries and Aquaculture 2016, United Nations Food and Agriculture Organization, 2016.
[2] K.A. Almås, I. Ratvik, OC2017 A-092-Sjøkart Mot 2050-Tiltak for Utvikling Av Biologisk Baserte Marine Næringer Mot 2050, 2017.
[3] J. Irvine, R. Macdonald, R. Brown, L. Godbout, J. Reist, E. Carmack, Salmon in the Arctic and how they avoid lethal low temperatures, North Pac. Anadromous Fish. Comm. Bull. 5 (2009) 39–50.
[4] J.D. Reist, F.J. Wrona, T.D. Prowse, M. Power, J.B. Dempson, J.R. King, R. J. Beamish, An overview of effects of climate change on selected arctic freshwater and anadromous fishes, AMBIO A J. Hum. Environ. 35 (7) (2006) 381–387.
[5] D.a. Rignø, Aquaculture nutrition, in: Gut Health Probiotics and Prebiotics, Book, 2014.
[6] A. Oliva-Teles, Nutrition and health of aquaculture fish, J. Fish. Dis. 35 (2) (2012) 83–108.
[7] V. Kiron, Fish immune system and its nutritional modulation for preventive health care, Anim. Feed Sci. Technol. 173 (1–2) (2012) 111–133.
[8] C.K. Tipsmark, K.J. Sorensen, S.S. Madsen, Aquaporin expression dynamics in osmoregulatory tissues of Atlantic salmon during smoltification and seawater acclimation, J. Exp. Biol. 213 (3) (2010) 368–379.
[9] H. Sundh, T.O. Nilsen, J. Lindstrom, L. Hasselberg-Frank, S.O. Stefansson, S. D. McCormick, K. Sundell, Development of intestinal ion-transporting mechanisms during smoltification and seawater acclimation in Atlantic salmon *Salmo salar*, J. Fish. Biol. 85 (4) (2014) 1227–1252.
[10] N.H. Sissener, M. Sanden, A.M. Bakke, Å. Krogdahl, G.I. Hemre, A long term trial with Atlantic salmon (*Salmo salar L.*) fed genetically modified soy; focusing general health and performance before, during and after the parr–smolt transformation, Aquaculture 294 (1–2) (2009) 108–117.
[11] L.-H. Johansson, G. Timmerhaus, S. Afanasyev, S.M. Jørgensen, A. Krasnov, Smoltification and seawater transfer of Atlantic salmon (*Salmo salar L.*) is associated with systemic repression of the immune transcriptome, Fish Shellfish Immunol. 58 (2016) 33–41.
[12] E.F. Pettersen, M. Ulvenes, G.O. Melingen, H.I. Wergeland, Peripheral blood and head kidney leucocyte populations during out-of-season (0+) parr-smolt transformation and seawater transfer of Atlantic salmon (*Salmo salar L.*), Fish Shellfish Immunol. 15 (5) (2003) 373–385.
[13] G. Melingen, S. Stefansson, A. Berg, H.J.F. Wergeland, S. Immunology, Changes in serum protein and IgM concentration duringsmolting and early post-smolt period in vaccinated and unvaccinated Atlantic salmon (*Salmo salar L.*), Fish Shellfish Immunol. 5 (3) (1995) 211–221.
[14] M. Muona, A.J.A. Soivio, Changes in plasma lysozyme and blood leucocyte levels of hatchery-reared Atlantic salmon (*Salmo salar L.*) and sea trout (*Salmo trutta L.*) during parr-smolt transformation, Aquaculture 106 (1) (1992) 75–87.
[15] L.H. Johansson, G. Timmerhaus, S. Afanasyev, S.M. Jorgensen, A. Krasnov, Smoltification and seawater transfer of Atlantic salmon (*Salmo salar L.*) is associated with systemic repression of the immune transcriptome, Fish Shellfish Immunol. 58 (2016) 33–41.
[16] A.G. Petrie, A.E. Ellis, Evidence of particulate uptake by the gut of Atlantic salmon (*Salmo salar L.*), Fish Shellfish Immunol. 20 (4) (2006) 660–664.
[17] G. Løkka, L. Austbø, K. Falk, E. Bromage, P.G. Fjelldal, T. Hansen, I. Hordvik, E. O. Koppang, Immune parameters in the intestine of wild and reared unvaccinated and vaccinated Atlantic salmon (*Salmo salar L.*), Dev. Comp. Immunol. 47 (1) (2014) 6–16.
[18] L. Tacchi, R. Bickerdike, A. Douglas, C.J. Secombes, S.A. Martin, Transcriptomic responses to functional feeds in Atlantic salmon (*Salmo salar*), Fish Shellfish Immunol. 31 (5) (2011) 704–715.
[19] D. Carbone, C. Faggio, Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of *Sparus aurata* and *Dicentrarchus labrax*, Fish Shellfish Immunol. 54 (2016) 172–178.
[20] S. Torrecillas, D. Montero, M. Izquierdo, Improved health and growth of fish fed mannan oligosaccharides: potential mode of action, Fish Shellfish Immunol. 36 (2) (2014) 525–544.
[21] E. Ringø, Z. Zhou, J.L.G. Vecino, S. Wadsworth, J. Romero, Å. Krogdahl, R.E. Olsen, A. Dimitroglou, A. Foey, S. Davies, M. Owen, H.L. Lauzon, L.L. Martinsen, P. De Schryver, P. Bossier, S. Sperstad, D.L. Merrifield, Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? Aquacult. Nutr. 22 (2) (2016) 219–282.
[22] R. Waagbø, M. Berntssen, T. Danielsen, H. Helberg, A. Kleppa, T. Berg Lea, G. Rosenlund, L. Tvenning, S. Susort, V. Vikeså, Feeding Atlantic salmon diets with plant ingredients during the seawater phase–a full-scale net production of marine protein with focus on biological performance, welfare, product quality and safety, Aquacult. Nutr. 19 (4) (2013) 598–618.
[23] M.L. Kakade, D.E. Hoffa, I.E. Liener, Contribution of trypsin inhibitors to the deleterious effects of unheated soybeans fed to rats, J. Nutr. 103 (12) (1973) 1772–1778.
[24] J. Bieth, B. Spiess, C.G. Wermuth, The synthesis and analytical use of a highly sensitive and convenient substrate of elastase, Biochem. Med. 11 (4) (1974) 350–357.
[25] M.H. Penn, E.Å. Bendiksen, P. Campbell, Å. Krogdahl, High level of dietary pea protein concentrate induces enteropathy in Atlantic salmon (*Salmo salar L.*), Aquaculture 310 (3–4) (2011) 267–273.
[26] A.M. Bakke-McKellep, M.H. Penn, P.M. Salas, S. Refstie, S. Sperstad, T. Landsverk, E. Ringø, Å. Krogdahl, Effects of dietary soyabean meal, inulin and oxytetracycline on intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the teleost Atlantic salmon (*Salmo salar L.*), Br. J. Nutr. 97 (4) (2007) 699–713.
[27] H. Hu, T.M. Kortner, K. Gajardo, E. Chikwati, J. Tinsley, A. Krogdahl, Intestinal fluid permeability in atlantic salmon (*Salmo salar L.*) is affected by dietary protein source, PloS One 11 (12) (2016), e0167515.
[28] T.M. Kortner, E.C. Valen, H. Kortner, I.S. Marjara, A. Krogdahl, A.M. Bakke, Candidate reference genes for quantitative real-time PCR (qPCR) assays during development of a diet-related enteropathy in Atlantic salmon (*Salmo salar L.*) and the potential pitfalls of uncritical use of normalization software tools, Aquaculture 318 (3–4) (2011) 355–363.
[29] P.Y. Muller, H. Janovjak, A.R. Miserez, Z. Dobbie, Processing of gene expression data generated by quantitative real-time RT-PCR, Biotechniques 32 (6) (2002) 1372–1374, 1376, 1378-9.
[30] A. Nylund, H. Hansen, O.J. Brevik, H. Hustoft, T. Markussen, H. Plarre, E. Karlsbakk, Infection dynamics and tissue tropism of Parvicapsula pseudobranchicola (*Myxozoa: myxosporea*) in farmed Atlantic salmon (*Salmo salar*), Parasites Vectors 11 (1) (2018) 17.
[31] L.J. Moore, J. Jarungsriapisit, T.O. Nilsen, S. Stefansson, G.L. Taranger, C. J. Secombes, H.C. Morton, S. Patel, Atlantic salmon adapted to seawater for 9 weeks develop a robust immune response to salmonid alphavirus upon bath challenge, Fish Shellfish Immunol. 74 (2018) 573–583.
[32] B.H. Beck, E. Peatman, Mucosal Health in Aquaculture, Elsevier/AP, Academic Press is an imprint of Elsevier, Amsterdam; New York, 2015.
[33] J. Perez-Sanchez, I. Estensoro, M.J. Redondo, J.A. Calduch-Giner, S. Kaushik, A. Sitja-Bobadilla, Mucins as diagnostic and prognostic biomarkers in a fish-parasite model: transcriptional and functional analysis, PloS One 8 (6) (2013).
[34] I. Estensoro, M.J. Redondo, B. Salesa, S. Kaushik, J. Perez-Sanchez, A. Sitja-Bobadilla, Effect of nutrition and *Enteromyxum leei* infection on gilthead sea bream *Sparus aurata* intestinal carbohydrate distribution, Dis. Aquat. Org. 100 (1) (2012) 29–42.
[35] Y.H. Sheng, R. Lourie, S.K. Linden, P.L. Jeffery, D. Roche, T.V. Tran, C.W. Png, N. Waterhouse, P. Sutton, T.H.J. Florin, M.A. McGuckin, The MUC13 cell-surface mucin protects against intestinal inflammation by inhibiting epithelial cell apoptosis, Gut 60 (12) (2011) 1661–1670.
[36] C.J. Secombes, What's new in fish cytokine research? Fish Shellfish Immunol. 53 (2016) 1–3.
[37] C.J. Secombes, T. Wang, S. Hong, S. Peddie, M. Crampe, K.J. Laing, C. Cunningham, J. Zou, Cytokines and innate immunity of fish, Dev. Comp. Immunol. 25 (8–9) (2001) 713–723.
[38] A. Rymuszka, L. Adaszek, Pro- and anti-inflammatory cytokine expression in carp blood and head kidney leukocytes exposed to cyanotoxin stress - an in vitro study, Fish Shellfish Immunol. 33 (2) (2012) 382–388.
[39] A. Santana, B. Saxena, N.A. Noble, L.I. Gold, B.C. Marshall, Increased expression of transforming growth factor beta isoforms (beta 1, beta 2, beta 3) in bleomycin-induced pulmonary fibrosis, Am. J. Respir. Cell Mol. Biol. 13 (1) (1995) 34–44.
[40] K. Komatsu, S. Tsutsui, K. Hino, K. Araki, Y. Yoshiura, A. Yamamoto, O. Nakamura, T. Watanabe, Expression profiles of cytokines released in intestinal epithelial cells of the rainbow trout, *Oncorhynchus mykiss*, in response to bacterial infection, Dev. Comp. Immunol. 33 (4) (2009) 499–506.
[41] M.D. Fast, S.C. Johnson, S.R. Jones, Differential expression of the pro-inflammatory cytokines IL-1beta-1, TNFalpha-1 and IL-8 in vaccinated pink (*Oncorhynchus gorbuscha*) and chum (*Oncorhynchus keta*) salmon juveniles, Fish Shellfish Immunol. 22 (4) (2007) 403–407.
[42] Z.X. Zhou, B.C. Zhang, L. Sun, Poly(I:C) induces antiviral immune responses in Japanese flounder (*Paralichthys olivaceus*) that require TLR3 and MDA5 and is negatively regulated by Myd88, PloS One 9 (11) (2014), e112918.

[43] Y.X. Li, T.M. Kortner, E.M. Chikwati, H.M. Munang'andu, E.J. Lock, A. Krogdahl, Gut health and vaccination response in pre-smolt Atlantic salmon (*Salmo salar*) fed black soldier fly (*Hermetia illucens*) larvae meal, Fish Shellfish Immunol. 86 (2019) 1106–1113.
[44] S.M. Planchon, C.A. Martins, R.L. Guerrant, J.K. Roche, Regulation of intestinal epithelial barrier function by TGF-beta 1. Evidence for its role in abrogating the effect of a T cell cytokine, J. Immunol. 153 (12) (1994) 5730–5739.
[45] E. Lilleeng, M.H. Penn, Ø. Haugland, C. Xu, A.M. Bakke, Å. Krogdahl, T. Landsverk, M.K.J.F. Frøystad-Saugen, Decreased expression of TGF-β, GILT and T-cell markers in the early stages of soybean enteropathy in Atlantic salmon (*Salmo salar L.*), Fish Shellfish Immunol. 27 (1) (2009) 65–72.
[46] I. Barjaktarevic, A. Rahman, S. Radoja, B. Bogunovic, A. Vollmer, S. Vukmanovic, M. Maric, Inhibitory role of IFN-gamma-inducible lysosomal thiol reductase in T cell activation, J. Immunol. 177 (7) (2006) 4369–4375.
[47] R. Ern, D.T. Huong, N.V. Cong, M. Bayley, T. Wang, Effect of salinity on oxygen consumption in fishes: a review, J. Fish. Biol. 84 (4) (2014) 1210–1220.
[48] Q.H. Abram, B. Dixon, B.A. Katzenback, Impacts of low temperature on the teleost immune system, Biology 6 (4) (2017).
[49] C. Le Morvan, D. Troutaud, P. Deschaux, Differential effects of temperature on specific and nonspecific immune defences in fish, J. Exp. Biol. 201 (Pt 2) (1998) 165–168.
[50] L. Stradmeyer, Survival, growth and feeding of Atlantic salmon, *Salmo salar L.*, smolts after transfer to sea water in relation to the failed smolt syndrome, Aquacult. Res. 25 (1) (1994) 103–112.
[51] E.B. Thorstad, F. Whoriskey, I. Uglem, A. Moore, A.H. Rikardsen, B. Finstad, A critical life stage of the Atlantic salmon *Salmo salar*: behaviour and survival during the smolt and initial post-smolt migration, J. Fish. Biol. 81 (2) (2012) 500–542.
[52] K.R. Groschwitz, S.P. Hogan, Intestinal barrier function: molecular regulation and disease pathogenesis, J. Allergy Clin. Immunol. 124 (1) (2009) 3–20, quiz 21-2.
[53] T. Kinugasa, T. Sakaguchi, X. Gu, H.C. Reinecker, Claudins regulate the intestinal barrier in response to immune mediators, Gastroenterology 118 (6) (2000) 1001–1011.
[54] A.P. Farrell, Encyclopedia of Fish Physiology: from Genome to Environment, Academic Press2011.
[55] F. Jutfelt, R.E. Olsen, B.T. Björnsson, K. Sundell, Parr–smolt transformation and dietary vegetable lipids affect intestinal nutrient uptake, barrier function and plasma cortisol levels in Atlantic salmon, Aquaculture 273 (2–3) (2007) 298–311.
[56] R.C. Cipriano, G.L. Bullock, Furunculosis and Other Diseases Caused by *Aeromonas Salmonicida*, National Fish Health Research Laboratory2001.
[57] H. Sundh, B.O. Kvamme, F. Fridell, R.E. Olsen, T. Ellis, G.L. Taranger, K. Sundell, Intestinal barrier function of Atlantic salmon (*Salmo salar L.*) post smolts is reduced by common sea cage environments and suggested as a possible physiological welfare indicator, BMC Physiol. 10 (2010) 22.
[58] C.M. Van Itallie, J.M. Anderson, Claudins and epithelial paracellular transport, Annu. Rev. Physiol. 68 (2006) 403–429.
[59] C.K. Tipsmark, K.J. Sorensen, K. Hulgard, S.S. Madsen, Claudin-15 and -25b expression in the intestinal tract of Atlantic salmon in response to seawater acclimation, smoltification and hormone treatment, Comp. Biochem. Physiol. Mol. Integr. Physiol. 155 (3) (2010) 361–370.
[60] R.E. Olsen, R. Myklebust, T. Kaino, E. Ringo, Lipid digestibility and ultrastructural changes in the enterocytes of Arctic char (*Salvelinus alpinus L.*) fed linseed oil and soybean lecithin, Fish Physiol. 21 (1) (1999) 35–44.
[61] R.E. Olsen, B.T. Dragnes, R. Myklebust, E. Ringo, Effect of soybean oil and soybean lecithin on intestinal lipid composition and lipid droplet accumulation of rainbow trout, *Oncorhynchus mykiss Walbaum*, Fish Physiol. 29 (3) (2003) 181–192.
[62] H.W. Heid, R. Moll, I. Schwetlick, H.R. Rackwitz, T.W. Keenan, Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases, Cell Tissue Res. 294 (2) (1998) 309–321.
[63] A.K.G. Hansen, T.M. Kortner, A. Krasnov, I. Björkhem, M. Penn, Å.J.B.V. R. Krogdahl, Choline supplementation prevents diet induced gut mucosa lipid accumulation in post-smolt Atlantic salmon (*Salmo salar L.*), BMC Vet. Res. 16 (1) (2020) 32.
[64] A.K. Hansen, T.M. Kortner, V. Denstadli, K. Måsøval, I. Björkhem, H.J. Grav, Å. Krogdahl, Dose-response relationship between dietary choline and lipid accumulation in pyloric enterocytes of Atlantic salmon (*Salmo salar L.*) in seawater, J British Journal of Nutrition (2020) 1–42.
[65] M.S. Hossain, S. Koshio, P. Kestemont, Recent Advances of Nucleotide Nutrition Research in Aquaculture: a review, Reviews in Aquaculture, 2019.
[66] I. Guerreiro, A. Oliva-Teles, P. Enes, Prebiotics as functional ingredients: focus on Mediterranean fish aquaculture, Rev. Aquacult. 10 (4) (2018) 800–832.
[67] M.J.A. Sakai, Current research status of fish immunostimulants, Aquaculture 172 (1–2) (1999) 63–92.
[68] P. Li, D.H. Lewis, D.M. Gatlin 3rd, Dietary oligonucleotides from yeast RNA influence immune responses and resistance of hybrid striped bass (*Morone chrysops x Morone saxatilis*) to *Streptococcus* iniae infection, Fish Shellfish Immunol. 16 (5) (2004) 561–569.
[69] A.I. Berge, A. Berg, H.J. Fyhn, T. Barnung, T. Hansen, S.O. Stefansson, Development of salinity tolerance in underyearling smolts of atlantic salmon (*Salmo-Salar*) reared under different photoperiods, Can. J. Fish. Aquat. Sci. 52 (2) (1995) 243–251.
[70] B.T. Bjornsson, T. Ogasawara, T. Hirano, J.P. Bolton, H.A. Bern, Elevated growth-hormone levels in stunted atlantic salmon, *Salmo-Salar*, Aquaculture 73 (1–4) (1988) 275–281.
[71] G.B. Zydlewski, J. Zydlewski, Na+ Gill, K+-ATPase of Atlantic salmon smolts in freshwater is not a predictor of long-term growth in seawater, Aquaculture 362–363 (2012) 121–126.
[72] M. Usher, C. Talbot, F. Eddy, Effects of transfer to seawater on growth and feeding in Atlantic salmon smolts (*Salmo salar L.*), Aquaculture 94 (4) (1991) 309–326.
[73] B.T. Bjornsson, S.O. Stefansson, S.D. McCormick, Environmental endocrinology of salmon smoltification, Gen. Comp. Endocrinol. 170 (2) (2011) 290–298.
[74] W. Hoar, The Physiology of Smolting Salmonids, Fish physiology, Elsevier1988, pp. 275-343.
[75] M.A. Sheridan, Alterations in lipid-metabolism accompanying smoltification and seawater adaptation of salmonid fish, Aquaculture 82 (1–4) (1989) 191–203.
[76] A. Clarke, N.M. Johnston, Scaling of metabolic rate with body mass and temperature in teleost fish, J. Anim. Ecol. 68 (5) (1999) 893–905.
[77] A. Bergheim, F. Kroglund, D. Vatne, B.J.A. Rosseland, Blood plasma parameters in farmed Atlantic salmon (*Salmo salar L.*), transferred to sea cages at age eight to ten months 84 (2) (1990) 159–165.
[78] C.K. Tipsmark, P. Kiilerich, T.O. Nilsen, L.O. Ebbesson, S.O. Stefansson, S. S. Madsen, Branchial expression patterns of claudin isoforms in Atlantic salmon during seawater acclimation and smoltification, Am J Physiol Regul Integr Comp Physiol. Aquaculture. 294 (5) (2008) R1563–R1574.
[79] K.S. Sundell, H. Sundh, Intestinal fluid absorption in anadromous salmonids: importance of tight junctions and aquaporins, Front. Physiol. 3 (2012) 388.
[80] S.S. Madsen, J.H. Olesen, K. Bedal, M.B. Engelund, Y.M. Velasco-Santamaria, C. K. Tipsmark, Functional characterization of water transport and cellular localization of three aquaporin paralogs in the salmonid intestine, Front. Physiol. 2 (2011) 56.
[81] M.B. Engelund, F. Chauvigne, B.M. Christensen, R.N. Finn, J. Cerda, S.S. Madsen, Differential expression and novel permeability properties of three aquaporin 8 paralogs from seawater-challenged Atlantic salmon smolts, J. Exp. Biol. 216 (Pt 20) (2013) 3873–3885.
[82] Y.K. Kim, S. Watanabe, T. Kaneko, M. Do Huh, S.I. Park, Expression of aquaporins 3, 8 and 10 in the intestines of freshwater- and seawater-acclimated Japanese eels *Anguilla japonica*, Fish. Sci. 76 (4) (2010) 695–702.

II

Gut microbiota of Atlantic salmon (*Salmo salar*), observed from late freshwater stage until one year in seawater, and effects of functional ingredients: A case study from a commercial sized research site in the Arctic region

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Abstract

Background: The importance of the gut microbiota for health and wellbeing is well established for humans and some land animals. The gut microbiota is supposedly as important for fish, but existing knowledge has many gaps, in particular for fish in the northernmost Arctic region. This study addressed the dynamics of Atlantic salmon gut microbiota assemblage and its associations with host responses from freshwater to seawater life stages under large scale, commercial conditions in northernmost Arctic regions of Norway and explored effects of functional ingredients. Microbiota was characterized by 16S rRNA gene sequencing in distal intestinal digesta at four time points: two weeks before seawater transfer (in May, FW); four weeks after seawater transfer (in June, SW1); in November (SW2), and in April (SW3) the following year. Two series of diets were fed, varying throughout the observation time in nutrient composition according to the requirements of fish, one without (Ref diet), and the other with functional ingredients (Test diet). The functional ingredients, i.e. nucleotides, yeast cell walls, a prebiotic and essential fatty acids, were supplemented as single or mixtures based on the strategies from feed company.

Results: Overall, the fish showed higher microbial richness and lactic acid bacteria (LAB) abundance after seawater transfer, while microbial diversity decreased throughout the observation period. At SW1, the gut microbiota was slightly different from those at FW, and was dominated by the genera *Lactobacillus* and *Photobacterium*. As the fish progressed towards SW2 and SW3, the genera *Lactobacillus* and *Mycoplasma* became more prominent, with a corresponding decline in genus *Photobacterium*. The overall bacterial profiles at these time points showed a clear distinction to those at FW. A significant effect of functional ingredients (a mixture of nucleotides, yeast cell walls and essential fatty acids) was observed at SW2, where Test-fed fish showed lower microbial richness, diversity, and LAB

abundance. The multivariate association analysis identified differentially abundant taxa, especially *Megasphaera*, to be significantly associated with gut immune and barrier gene expressions, and plasma nutrients.

Conclusions: The gut microbiota profile varied during the observation period, and the *Mycoplasma* became the dominating bacteria with time. *Megasphaera* levels were associated with gut health markers and plasma nutrients. Functional ingredients induced mild modulations of the gut microbiota profile at an important ongrowing stage.

Keywords: Atlantic salmon; Arctic region; gut microbiota; functional ingredients

Background

The understanding of gut microbiota as a key element for proper function, health and general wellbeing of animals, including fish, has been greatly strengthened in the past decade. Although present knowledge of gut microbiota in fish is still limited [1, 2], it is clear that alterations in gut microbiota profiles may affect enzyme production [3], nutrient digestion and utilization [4, 5] and not at least the immune status which, in turn, may alter disease resistance, for better or worse (reviewed by [6-8]). Based on existing literature, it is apparent that the outcome of alterations in gut microbiota depends on complicated interactions between the host and diet composition, and not at least environmental conditions.

Most studies of gut microbiota in Atlantic salmon (*Salmo salar* L.) to date have described how the bacterial composition of the intestine may be affected by factors such as diet [9-13], environment (e.g. water temperature and salinity) [10, 14-18], disease situation [19], location within the digestive tract [9, 12, 20] and developmental stage [21]. For Atlantic salmon, the freshwater-to-seawater transition phase is a critical period during salmon production. The adaption to the seawater environment, involves a great number of physiological changes, such as increased hypoosmotic-regulatory ability, and alterations in endocrinology,

metabolism, morphology and behavior [22-25]. Recent studies have also demonstrated that the freshwater-to-seawater transition can have major impacts on the microbiota communities of the intestine and skin [12, 26-28].

A consequence of the foreseen increase in demand for Atlantic salmon is search of new production sites. In Norway, northernmost Arctic areas, such as Finnmark, are potential candidates to expand the salmon production sites. However, present knowledge on how extreme variation in photoperiod, low average temperature, long winter period and specific pathogens in the Arctic areas influence gut microbiota is limited. It is highly likely that, due to environmental effects on the fish biology, effect of diet composition on gut health and microbiota may differ in fish produced in the northerly areas from that of fish grown in southerly areas in Norway. The lack of information on how fish in the northernmost Arctic areas might differ in biology and interaction with the environment, has stimulated the feed producers to recommend use functional ingredients to strengthen the fish' capacity to manage harsh environmental conditions and resist site specific pathogens.

Functional ingredients, such as nucleotides, and the so-called immunostimulants and prebiotics, are commonly used with the intention to improve fish health and disease resistance, in particular during stressful farming conditions [29, 30]. They seem to have positive effects on gut health, at least under certain conditions [29, 31-33], and their effects are suggested to be mediated primarily via modulation of the gut microbiota, such as increasing numbers of beneficial bacteria (e.g. lactic acid bacteria, LAB) [6]. However, the effects of functional fish feed ingredients depend on several factors, including the characteristics of the functional ingredient itself, timing and duration of administration, fish species and life stage [6, 34, 35]. It is also likely that functional ingredients may exert different actions under practical farming conditions than what can be expected based on information from controlled tank experiments, since environmental factors possibly have

greater effects than diet on fish health [36]. Yet, how functional ingredients may exert modulatory actions on gut microbiota under large scale, commercial production conditions in the Arctic areas remains relatively unexplored.

Characterizing compositional changes in intestinal bacterial communities during the production cycle, as well as exploring their associations with host responses are fundamental steps to understand the impact of gut microbiota on host function and gut health in practical salmon production. The work presented herein is part of a larger project conducted to gain knowledge on fluctuation in gut function and health of Atlantic salmon from late freshwater stage until one year in seawater farmed under large scale, commercial conditions in Arctic areas. The host response data have been published previously [37]. The present study was conducted with three potential aims, firstly to gain knowledge on the changes in gut microbiota of Atlantic salmon from late freshwater stage until one year in seawater in a large-scale, commercially relevant setting under Arctic conditions, secondly whether use of functional ingredients would modify the microbiota profiles during the observation period, and finally exploring potential relationships between gut microbiota and host response.

Results

The absolute bacterial DNA level in the digesta of the distal intestine was not significantly affected by sampling time point or diet composition ($P > 0.05$, **Figure S1**). From the 16S rRNA gene sequencing, a total number of 10.8 million counts were obtained. The minimum and maximum counts per sample were 16,639 and 159,531, respectively, with an average of 74,972 counts/sample. After sequence quality filtering, trimming and filtering of ASVs, the effective sequences were about 16,000/sample available for further downstream analyses.

Alpha diversity

Compared to fish at FW, fish sampled from seawater showed higher microbial richness (Observed species index), especially at SW1 and SW3 ($P < 0.05$, **Fig. 1a**). The evenness

(Pielou's evenness) (**Fig. 1b**) and diversity (Shannon's index) (**Fig. 1c**), on the other hand, did not show significant differences between the sampling time points. However, the diversity, estimated by Simpson's index, showed a decreasing trend throughout the observation period with the lowest value in fish from SW3 ($P < 0.05$, **Fig. 1d**).

Regarding the effects of functional ingredients, significant differences were observed (Observed species index and Shannon's index) at fish at SW2, but not at any of the other time points. Fish fed the Test diet showed reduced richness and diversity compared to those fed the Ref diet ($P < 0.05$, **Fig. 1 a and c**).

Beta diversity

Results from the permutation multivariate analysis of variance (PERMANOVA) analysis of both weighted and unweighted UniFrac revealed significant differences in gut microbiota among sampling time points ($P < 0.001$, **Table 1**). Compared to fish from SW1, fish sampled at SW2 and SW3, showed more apparent difference from those in fish at FW based on both weighted and unweighted UniFrac ($P < 0.001$, **Table 1**). Regardless dietary treatment, principal coordinate analysis (PCoA) plots based on weighed UniFrac showed that samples from SW2 and SW3 tended to cluster together and seemed to be separated from those at FW and SW1 (**Fig. 1e**). The PCoA plots based on unweighted UniFrac showed that samples within each sampling time point clustered together and tended to separate from samples from other time points (**Fig. 1f**).

Significant effect of dietary treatment, i.e. inclusion of functional ingredients, was observed in fish at SW1 and SW2 according to the PERMANOVA analysis of unweighted UniFrac ($P < 0.01$ and $P < 0.001$, respectively, **Table 1**). The PCoA plots based on unweighted UniFrac metrics showed that at SW2 fish within Ref diet tended to cluster together compared to those in Test-fed fish **(Fig. 1g)**. No clear diet effects were observed in fish at FW or SW3 ($P > 0.05$, **Table 1**). Overall, fish from the SW2 sampling time point

showed the strongest response to diet, where significant effects on both alpha and beta diversity were observed. We therefore chose to present detailed contrast effects of functional ingredients on microbiota assemblage for SW2 only.

Gut microbiota composition

The relative abundance of microbiota in all samples at the genus level (the top 25 genera) is illustrated in **Fig. 2a**. Overall, the most abundant phyla *Firmicutes* and *Proteobacteria* varied among sampling time points, and accounted, in total, for about 80% of the total abundance. As fish progressed towards SW2 and SW3, phyla *Firmicutes* and *Tenericutes* became more prominent corresponding to a decline in phylum *Proteobacteria*. The most abundant genera within the phylum *Firmicutes* were lactic acid bacteria (LAB), mainly *Lactobacillus*, *Leuconostoc* and *Lactococcus*, and the most abundant genera within *Proteobacteria* and *Tenericutes* were *Photobacterium* and *Mycoplasma*, respectively (**Fig. 2a**). Across all samples, 10 genera including *Lactobacillus, Photobacterium, Leuconostoc* and *Lactococcus*, were core microbiota at genus level (above 0.1% relative abundance in 80% of samples) (**Fig. 2b**). Notably, in freshwater stage, genera *Deefgea*, *Flavobacterium* and *Pseudomonas*, as well as family *Ruminococcaceae* were detected sporadically, but when present, they alone or together dominated gut microbiota (**Fig. 2a**). Here, we focused on the three major genera, i.e. *Lactobacillus*, *Photobacterium* and *Mycoplasma*, as they varied among sampling time points. Specifically, after seawater transfer, fish showed an increased relative abundance of *Lactobacillus*, i.e. 29% ± 15%, 48% ± 10% and 50% ± 20% at SW1, SW2 and SW3, respectively, compared to that those at FW (7% ± 6%) (**Fig. 2a and c**). Regarding *Photobacterium*, four weeks after seawater transfer, i.e. at SW1, fish had the highest relative abundance of *Photobacterium* (40% ± 25%) compared to fish from other sampling time points (**Fig. 2a and c**). Higher relative abundance of *Mycoplasma* was observed in fish at SW2 (7% ± 17%) and SW3 (23% ± 31%) while low levels, less than 0.1%, were observed

171 at FW and SW1 (**Fig. 2a and c**). The MaAsLin 2 analysis showed significant differences in
172 69 genera among sampling time points including *Lactobacillus*, *Photobacterium* and
173 *Mycoplasma* (**Fig. 3**).

174 Regarding the effects of functional ingredients at SW2 (**Fig. 4a**), Test-fed fish had a lower
175 relative abundance of *Lactobacillus* (17% ± 19%) than those in Ref-fed fish (48% ± 10%).
176 The relative abundance of *Photobacterium* (12% ± 4%) and *Mycoplasma* (7% ± 17%) were
177 observed in Ref-fed fish, while *Photobacterium* (26% ± 40%) and *Mycoplasma* (27% ± 37%)
178 were found in Test-Fed fish. The MaAsLin 2 analysis showed that significant differences
179 between Ref and Test diets were due to the decrement of 25 genera/family in fish fed Test
180 diet, including LAB, such as *Lactobacillus* and *Leuconostoc* (**Fig. 4b**).

181 **Significant associations between distal intestinal digesta microbiota and metadata of**
182 **interest**

183 In the heatmap (**Fig. 5a**), cells that denote significant associations are colored (red or blue)
184 and overlaid with a plus (+, positive) or minus (-, negative) sign that indicates the direction
185 of association between microbial clade abundance and the PC1 value of PCA of host
186 responses. Of note, the gut immune gene expression were negatively correlated with their
187 PC1 value of the PCA, while gut barrier gene expression and plasma nutrient levels were
188 both positively related with their PC1 value of the PCA (**Table S1**).

189 The multivariate association analysis identified 27 differentially abundant taxa that were
190 significantly correlated with gene expression related to gut barrier function (**Fig. 5a**). Except
191 for *Flavobacterium*, 26 differentially abundant taxa, such as *Photobacterium* and LAB (e.g.
192 *Lactobacillus*), showed a negative correlation with expression levels related to barrier
193 function genes (diagnostic plots of raw data in **Figure S2**). Among 26 taxa, the relative
194 abundance of *Megasphaera* showed a clear negative correlation with expression levels of DI
195 barrier function genes (FDR = 0.032, **Fig. 5b**), which decreased as PC1 of the PCA increased.

196 The relative abundance of *Megasphaera* was positively correlated with expression levels
197 of gut immune genes (FDR = 0.053, **Fig. 5a and c**), which decreased as PC1 of the PCA
198 increased. The relative abundance of *Megasphaera* (FDR = 0.132) and *Bacteoides* (FDR =
199 0.249) showed weak positive correlations with the levels of plasma nutrients (**Fig 5a and d**),
200 which increased as PC1 of the PCA increased.
201

202 # Discussion

203 **Effects of sampling time points on distal intestinal digesta microbiota**

204 The observed changes in gut microbiota composition from freshwater to seawater sampling
205 points were probably related to the adjustments made in commercial diets, different
206 environmental conditions across sampling points, as well as the host physiology, which
207 changed substantially during the observation time, may play important roles in these
208 alterations. Together, these changes likely lead to the competitive distribution by certain
209 microbial species, thereby reorienting the gut microbiota composition of the fish.
210 Environmental conditions, such as alterations in temperature and water salinity, are well
211 documented to influence gut microbiota [14, 38, 39]. The freshwater-to-seawater transition
212 has also profound effects on host physiology, e.g. on osmoregulatory and immune functions
213 in the gut [22, 40-42]. The effects of physiological changes on gut microbiota profiles in our
214 study may be a protracted process taking longer than the four-week timeline examined, and
215 are generally line with recent studies in Atlantic salmon [12, 28].

216 Although previous studies all have reported seawater transfer has profound effects on the
217 microbiota profiles of salmon, the observed changes in intestinal microbiota composition
218 vary inconsistently among studies. Our observation that fish showed an increase in microbial
219 richness in distal intestinal digesta after seawater transfer is in agreement with results of
220 previous studies of gut [27] and skin microbiota [28] of Atlantic salmon. However, other

studies have reported that seawater transfer decrease [26, 43] or maintain [12, 21] microbial richness compared to the freshwater stage. In the present work, the phyla *Firmicutes* (mainly genus *Lactobacillus*) and *Proteobacteria* (mainly genus *Photobacterium*) dominated the gut microbiota four weeks after seawater transfer, which is in accordance with the study of Lokesh and co-workers [21]. Other studies have reported that phylum *Firmicutes* strongly dominated the distal intestinal digesta microbiota, whereas other taxa, including phylum *Proteobacteria*, declined three weeks after seawater transfer [26, 27]. The reasons behind this discrepancy are unclear, but likely caused by the combined variation in environmental conditions, diet composition and sample origin, and possibly also methodology.

The LAB has been identified as a major component of the gut microbiota in Atlantic salmon and is presumed to have beneficial effects on the host through immune regulation, improvement of digestive processes and inhibition of pathogens, at least under certain conditions [44-47]. Our study showing a higher relative abundance of LAB (mainly *Lactobacillus*) in fish during seawater stages than those in FW, is in agreement with the results of Dehler and co-workers [26]. In the present study, the alteration in LAB abundance occurred in parallel to the increase in content of plant ingredients in the diets at SW2 and SW3. The increased dietary content of fermentable plant carbohydrates, serving as substrate for LAB bacteria, may therefore be the cause of the increase in relative abundance of LAB, as also observed in previous studies [9, 20, 48, 49]. However, one recent study found a significant decrease in the relative abundance of LAB in mucosa-associated microbiota of Atlantic salmon six weeks after seawater transfer [12]. Digesta- and mucosa-associated gut microbiota have shown substantial different composition [9, 12, 20] and it is therefore possible that these two gut compartments may contain different LAB levels and/or compositions. Given the potential functional role of LAB for salmon health, additional

studies of both digesta- and mucosa-associated microbiota are recommended to increase our knowledge of LAB roles on salmon health and function.

Compared to fish at SW2 and SW3, fish at SW1 shared more similar bacterial taxa with those in fish at FW, which could be attributed to microbial colonization in historical life processes [50]. The alteration in microbial profile from SW1 to SW2 may have other potential causes. Since the observation period (from June to November) between SW1 and SW2 is an important stage for growth and physiological changes due to increased water temperature and high feed intake [37], the temperature may be the leading environmental factor impacting these alternations, and diet could exert synergistic effects. Day length is another environmental factor which might have influenced gut microbiota in our study, for which documentation is lacking so far.

As Atlantic salmon progressed towards adult, the enrichment of genus *Mycoplasma* at SW2 and SW3 time points compared to FW and SW1 is one of the most striking differences found in our study. Previous studies have found *Mycoplasma*, as a member of the core microbiota, to be one of the most abundant bacteria in both farmed and wild Atlantic salmon during seawater stages [26, 43, 51-55] reaching levels above 70% of total abundance in certain cases [19, 43, 54]. Increased levels of genus *Mycoplasma* may therefore be an important characteristic for gut microbiota of adult salmon. Some increasing bacteria species, such as *Photobacterium* in our study, are widely inhabiting in the surrounding seawater environment [15]. Increased levels of these species in the salmon gut after seawater transfer may therefore be related to increased water intake, in order to prevent the dehydration in hyperosmotic seawater environment. Interestingly, *Mycoplasma* are rarely [50] or not observed [56, 57] in the surrounding seawater. Hence, the reason for their colonization in intestine is uncertain although they seem to be particularly well-adapted to the intestinal environment of Atlantic salmon. On the contrary, one previous study by Lokesh et al. [21]

found changes in *Mycoplasma* between life stages to be minimal, and this taxon was only abundant at the early freshwater life stages and during the smoltification process. Besides differences in sample origin, this discrepancy could also be due to the differences in environmental factors including geographic location and dietary differences that could influence the relative abundance of *Mycoplasma*. Anyhow, given the important symbiotic relationship between *Mycoplasma* and host [58], more studies are warranted to increase our understanding of their ecological and functional significance.

Effects of functional ingredients on distal intestinal digesta microbiota

Available research indicate that functional ingredients, when included in fish diets, such as prebiotics, nucleotides and immunostimulants, may affect gut microbiota through direct or indirect modulatory effects (reviewed by [6, 34-36, 59, 60]). However, except at SW2 in our study, the applied functional ingredients were unable to produce significant alterations in microbial profile of the distal intestine. How dietary functional ingredients may modulate gut microbiota composition in fish will clearly depend on various important factors, such as the specific composition of the functional ingredients, timing and duration of administration, fish life stage, fish physiology, as well as environment factors [34, 35]. The lack of effects of functional ingredients could be explained by low feed intake, since our study was conducted under Arctic conditions with low average temperature during most of the observation period. Another explanation is that compared to the small-scale experimental trials of limited duration in the majority of available scientific literature, it is likely that, the complicated and changeable environmental conditions in the current study may have resulted in diminished impact of the functional ingredients on gut microbiota [36].

As mentioned above, the observation period from SW1 to SW2 is an important ongrowing stage due to high temperature and long daylight, and thereby high feed intake. The decreased microbial richness and diversity and the relative abundance of LAB, observed at SW2 for

Test-fed fish could therefore be attributed to the high ingestion of a mixture of nucleotides, yeast cell walls and essential fatty acids. Similar results have been observed in rainbow trout previously [61]. Unexpectedly, these findings seem to be in contrast to the positive effects often ascribed to functional ingredients on abundance of presumed beneficial bacteria, such as LAB, in intestine and other mucosal surfaces [6, 35, 62-64]. One potential explanation for the discrepancy may be linked to the duration of administration as long-term oral administration of immunostimulants have been reported to cause decreased efficacy in fish [65]. This assumption is also supported by an apparent increased metabolic cost in fish fed these functional ingredients at SW2, i.e. lower condition factor and plasma triglyceride levels, and a tendency to lower growth [37]. It is therefore possible that the ten-week continuous oral administration of these selected functional ingredients before the sampling at SW2, may have resulted in less favorable changes in gut microbiota composition, and may offer an explanation for the reduced richness, diversity and relative LAB abundance. Despite that the functional ingredients decreased the relative abundance of LAB, it is still far away to conclude that Test-fed fish showed an "unhealthier" gut microbiota profile compared to Ref-fed fish. The interaction between gut microbiota and host is too complex to generalize which kind of microbiota profile may benefit the host physiology [3]. As an example, certain LAB, such as the probiotic strain *Lactobacillus plantarum*, can disrupt healthy intestinal tissues in mammal [66, 67] and induce gut dysbiosis in fish [68]. Our findings call into question of the effects of long-term administration of these functional ingredients, and also suggest the need for caution when and how using them to regulate gut microbiota.

Associations between distal intestinal digesta microbiota and host response

Very recently, attempts have been made to start shedding light on the potential physiological functions of the gut microbiota in Atlantic salmon. For example, the salmon gut bacterial communities can be closely associated to flesh pigmentation [69, 70], lipid metabolism [71]

and immunostimulation [72]. A recent study from our group demonstrated that mucosa-enriched taxa, *Brevinema andersonii* and unclassified *Spirochaetaceae* were found to be correlated with expression levels of gut immune and barrier genes, respectively [73]. In the present work, 26 differentially abundant taxa including *Photobacterium*, LAB (e.g. *Lactobacillus*) and *Megasphaera* were negatively correlated with gut barrier gene expression, and *Megasphaera* was positively associated with gut immune gene expression and plasma nutrient levels. The mechanism behind and implications of these relationships remain unknown. Possibly, certain bacterial taxa shape intestinal barrier and immune functions, and could thereby regulate metabolic functions [74]. However, knowledge on the interaction between gut microbiota and host responses is still a largely unexplored area, and future studies are clearly warranted.

Conclusions

This study provides new information on the dynamics of salmon gut microbiota assemblage and its associations with host responses from late freshwater stage until one year in seawater during large scale, commercial farming conditions in Arctic regions. The core microbiota genera *Lactobacillus* and *Photobacterium* varied among sampling time points. As fish progressed towards adult, the genera *Lactobacillus* and *Mycoplasma* became more prominent corresponding to a decline in genus *Photobacterium* indicating more apparent separation with fish from freshwater. Significant effect of functional ingredients on gut microbiota was observed at fish after a rapid growth period showing that inclusion of a mixture of nucleotides, yeast cell walls and essential fatty acids reduced microbial richness and diversity, as well as the relative abundance of LAB. The differentially abundant taxa including *Photobacterium,* LAB (e.g. *Lactobacillus*) and *Megasphaera* were found to be negatively correlated with gut barrier gene expression, while the relative abundance of

Megasphaera were positively correlated with the levels both in gut immune gene expression and plasma nutrients.

Materials and methods

Experimental fish

The experimental setup is shown in **Fig. 6**. Atlantic salmon hatched in the spring of 2015 were raised in two large, closed aluminum flow-through tanks for spring smolt production at Hopen, near Bodø (N67° – E14°). The tanks were supplied with freshwater from a nearby lake. When the fish were ready to be transferred to seawater, they were transported by a well-boat to Sommarbukt (N70° – E22°), near Alta, in Finnmark of Norway, where the fish from each tank was split into triplicated sea cages, i.e. three replicates for each dietary treatment in seawater, each holding about 55,000 fish. The temperature followed natural fluctuations in the water intake, ranging from 1 to 14 °C for the entire period. Oxygen and salinity levels fluctuated from 8 to 15 mg/L and from 11 to 44 ‰ throughout the experimental period, respectively (**Figure S3**).

Diet composition and sampling

The macronutrient composition of the diet series varied throughout the observation time according to the requirements of the fish. At each observation time, two series of diets were fed, one without functional ingredients (Ref diet) and one with functional ingredients (Test diet). The functional ingredients, e.g. nucleotides, yeast cell walls, a prebiotic and essential fatty acids, were supplemented to the diets either as single ingredient or as mixtures following the strategy developed for this particular commercial site and according to the development and production stage of the fish in the farm. The inclusion levels of these functional ingredients were not listed due to commercial interests and production of intellectual rights. The samples were collected at four sampling time points as the experimental set-up in **Fig. 6**: two weeks before seawater transfer (May 2016, FW) and three

times during the seawater period, i.e. four weeks after seawater transfer (June 2016, SW1) and two times thereafter (November 2016 and April 2017, SW2 and SW3, respectively). Eight treatments were defined by sampling life stages and dietary treatment, i.e. FW-Ref, FW-Test, SW1-Ref, SW1-Test, SW2-Ref, SW2-Test, SW3-Ref and SW3-Test. The formulations and nutrient compositions of the diets among treatments are presented in **Table 2**.

Only fish with digesta throughout the distal intestine were selected to ensure exposure to the diet at the time of sampling. At each sampling time point, 3 times 3 fish were sampled for each dietary treatment. Regarding the freshwater sampling, three groups of fish came from the same tank as the facility's tanks were too big, each holding 180 000 fish, and the facility did not allow replicate tanks for each diet. This approach was considered to be suitable and included in the statistical evaluation as independent replicates for observation of diet effects. The results of our study confirmed that this approach was acceptable, as the means of the fish in the two tanks did not differ significantly, and the variances were similar, indicating no important tank variation [37]. For the sampling in seawater, the three groups of fish per diet came from three sea cages. A total of 72 fish were collected for DNA extraction. All tools were cleaned and decontaminated by an ethanol spray and flaming during each sampling fish. The digesta from the distal intestinal region as previously defined [75] was collected into 1.5 mL skirted sterile centrifuge tubes, then mixed thoroughly using a spatula before frozen in liquid N_2, thereafter stored at -80 °C before DNA extraction.

DNA extraction

One fish was randomly selected from per treatment to divide 72 samples into 9 batches for DNA extraction. About 100 mg of digesta of distal intestine from each sample was used for DNA extraction and processed according to the protocol in the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany), except for an additional heating step following the

bead beating step at 95 °C for 7 min before proceeding according to the standard procedure suggested by [76]. At each DNA extraction batch, a blank negative control and a positive mock control (ZymoBIOMICS Mock Community Standard, Zymo Research Corp, Irvine, CA, USA) were included and processed in parallel with the experimental samples.

PCR amplification

PCR amplification of about 300bp amplicons from the V1-V2 region of the 16S rRNA was carried out using the bacterial universal primers 27F (5′ AGA GTTTGA TCM TGG CTC AG 3′) and 338R-I (5′ GCW GCC TCC CGT AGG AGT 3′) and 338R-II (5′ GCW GCCACC CGT AGG TGT 3′). The PCR was carried out as previously described by Gajardo et al. [20] using 25 µl sample volume in duplication with 2 µl of DNA template, 22.4 µl Phusion® High-Fidelity PCR Master Mix (Thermo Scientific, CA, United States of America) and 0.6 µl of forward (27F) and reverse (pooled 338R-I and II) primers (50 pM). The PCR was run in duplicate and negative PCR controls using molecular grade water as a template were included. The duplicate PCR products were pooled and analyzed in 1.5% agarose gels and samples with bright bands between 300 and 350bp were considered suitable for further processing. Since samples from one of 9 batches showed low quality of PCR products, we removed these samples for further analysis. Hence, there were 8 samples per treatment left for final sequencing (n = 8).

DNA quantification

The 16S rRNA gene quantity in the diluted DNA templates used for the amplicon PCR was measured by qPCR. The qPCR assays were performed using a universal primer set (forward, 5'-CCA TGA AGT CGG AAT CGC TAG-3'; reverse, 5'-GCT TGA CGG GCG GTG T-3') used for bacterial DNA quantification as the description in previous studies [77, 78].

PCR cleanup, library preparation and sequencing

PCR cleanup, library preparation and sequencing were performed using the protocol provided by Illumina (part #15044223 Rev B). Briefly, the PCR products were cleaned using AMPure beads followed by index PCR using Nextera XT Index kit (Illumina, California, USA; catalog no., FC-131-1096) and subsequently another round of purification with the AMPure beads. After the cleanup, the representative libraries were analyzed using the Agilent DNA 1000 Kit (Agilent Technologies, California, USA; catalog no., 5067-1505) to verify the library size. The cleaned libraries were quantified using Qubit fluorometer (Thermo Scientific, CA, United States of America). The library was then denatured and diluted to 6 pM, 20% of 6 pM PhiX control was added before finally being sequenced on an Illumina MiSeq platform. 300bp paired-end reads were generated.

Data analysis

Raw sequence data was analyzed using the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) software version 2019.4 (https://qiime2.org/) [79]. Sequence analysis was performed by QIIME2. First, sequences were demultiplexed using QIIME2. The sequences were then denoised, pair-ended, trimmed and quality filtered using the DADA2 algorithm in QIIME2 to generate amplicon sequence variants (ASVs) [80]. The taxonomy was assigned in QIIME2 against the SILVA database (version 132) [81] trained with a scikit-learn naive Bayes machine-learning classifier [82]. The contaminant sequences were removed based on their prevalence and abundance in the samples according to the description of [83]. The majority of removed sequences were classified as *Pseudomonas, Acinetobacter, Leptothrix, Aeromonas,* an unclassified bacterium of *Betaproteobacteriales* order, three kinds of genera *Flavobacterium*, an unclassified bacterium of *Chitinophagales* order and *Cutibacterium*. *Streptophyta* filtering is usually performed to remove chloroplast sequences which are assumed to reflect non-bacterial-associated taxa [84]. The other sequences considered as

contamination were sequences found in the negative controls from both de DNA extraction and PCR amplification.

Phylogenetic classification, richness and diversity parameters

All ASVs were aligned with MAFFT [85] and then phylogeny was constructed with FastTree 2 [86]. In order to compute alpha and beta diversity, the ASVs table was rarefied at 16,000 reads to have an even number of reads across all the samples. Differences in alpha diversity (observations within sampling time points and dietary treatment) were evaluated by four indices: 1) Observed species index, which counts the numbers of ASV in each sample, also called richness; 2) Pielou's evenness, which refers to the abundances of the species; 3) Shannon's index which takes into account richness as well as how many of each ASV are observed (abundance), also called diversity; 4) Simpson's index, which describes the diversity of a community. Two indices were used also for evaluation of beta diversity, which estimates phylogenetic difference between bacteria communities: 1) Unweighted UniFrac Distance, indicating number of different ASV and their phylogenetic distance; 2) Weighted UniFrac Distance, which takes into account the number of different ASV, their phylogenetic distance as well as the number of similar ASV.

Statistical analysis and graphics

To evaluate the effect of the sampling time points through freshwater to seawater on gut microbiota composition and exclude the potential effect of functional ingredients, only fish fed Ref diets among sampling time points were analyzed and compared. At each sampling time point, statistical comparisons between Ref and Test diets were conducted to explore the effect of the functional ingredients. In order to assess differences in microbiota composition between the different treatments, Kruskal-Wallis test followed by multiple comparisons was performed to compare the alpha diversity using GraphPad Prism 7 (GraphPad Software, La Jolla, California, United States). Regarding the dietary functional ingredients effect at SW2,

the data of gut microbiota composition at phylum level was subjected to multiple t-tests using GraphPad Prism 7. In addition, Primer 7 (version, 7.0.13) was used to perform beta diversity analysis followed PERMANOVA [87]. The raw data generated by QIIME2 was also used to make core microbiota of all samples at genus levels (above 0.1% relative abundance in 80% of samples) using MicrobiomeAnalyst [88]. The graphs of alpha diversity, heatmaps and gut microbiota composition were made by GraphPad Prism 7 basing on the raw data generated by QIIME2.

Microbiome Multivariable Association with Linear Models (MaAsLin2)

Differentially abundant taxa (genus level) among the sampling time points and between the dietary treatments at SW2 were identified by the MaAsLin2 (version, 0.99.12) (https://huttenhower.sph.harvard.edu/maaslin2) in R, using the default program parameters. Bacterial taxa of very low abundance (< 0.01%) or low prevalence (present in < 25% of samples) were removed before running the differential abundance testing. The difference in the taxa abundance was considered significant when the *q*-value (FDR) was below 0.05.

Regarding the multivariate association analysis, the distal intestinal digesta microbiota were tested for the associations with metadata of interest (from the same individual fish) (**Table S1**) using the MaAsLin2. Bacterial taxa of more than 0.1% abundance and 25% prevalence of samples were selected for association testing. The significant association was set at *q*-value less than 0.25. The metadata of interest, i.e. gut immune and barrier functions (gene expression in distal intestine), as well as plasma nutrients (plasma cholesterol and triglyceride) were selected to run the multivariate association testing with fixed factor, i.e. treatment, since these gut immune and barrier functions, and plasma nutrients varied greatly among sampling time points with clearly decreasing values in fish at SW1 [37]. The gut immune functions related genes were selected for the association testing including the goblet cell marker (*muc13*), pro-inflammatory cytokines (interleukin-1 beta, *il1β* and interferon

gamma, *ifnγ*), anti-inflammatory cytokines (i.e. transforming growth factor-beta, *tgfβ* and interleukin 10, *il10*), T-cell markers (i.e. cluster of differentiation 3γδ and 8β, *cd3γδ* and *cd8β*), as well as the myeloid differentiation factor 88 (*myd88*). The gut barrier functions related genes were selected for the association testing including *zo-1*, *claudin-15* and *claudin-25b*. Since the expression levels of immune related genes were highly correlated, we ran a principal component analysis (PCA) and extracted the first principle component (PC1) for the association testing to avoid multicollinearity and reduce the number of association testing. Similarly, gut barrier functions related genes were highly correlated, their extracted PC1 of the PCA was used for the association testing. The plasma nutrients were also highly correlated. Their extracted PC1 of the PCA was used for the association testing.

Abbreviations

LAB: Lactic acid bacteria; PERMANOVA: permutation multivariate analysis of variance; PCoA: Principal coordinate analysis; 16S rRNA: 16 Svedberg ribosomal ribonucleic acid; QIIME 2: Quantitative Insights Into Microbial Ecology 2; ASVs: amplicon sequence variants. ANOVA: analysis of molecular variance; il1β: interleukin-1 beta; ifnγ: interferon gamma; tgfβ: transforming growth factor-beta; il10: interleukin 10; cd3γδ: cluster of differentiation 3γδ; cd8β: cluster of differentiation 8β; myd88: myeloid differentiation factor 88; PCA: principal component analysis; PC1: extracted the first principle component.

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Authors' contributions

Experiment design: JW, TMK, ÅK and ØJB. Sampling: JW, TMK, ÅK, YL, KG and AJT. Analyses: JW, AJT, KG and YL. Supervision: TMK and ÅK. Writing, original draft: JW. Writing, review and editing: JW, TMK, ÅK, YL, AJT, KG, JVJ and ØJB.

Availability of data and material

Sequence data have been deposited at NCBI SRA database under BioProject accession ID: PRJNA660116.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Competing interests

The authors have no conflict of interest to declare.

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References

[1] Wang AR, Ran C, Ringø E, Zhou ZG. Progress in fish gastrointestinal microbiota research. Reviews in Aquaculture 2017.

[2] Tarnecki AM, Burgos FA, Ray CL, Arias CRJJoam. Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. 2017;123(1):2-17.

[3] Ray A, Ghosh K, Ringø EJAN. Enzyme-producing bacteria isolated from fish gut: a review. 2012;18(5):465-92.

[4] Falcinelli S, Picchietti S, Rodiles A, Cossignani L, Merrifield DL, Taddei AR, et al. Lactobacillus rhamnosus lowers zebrafish lipid content by changing gut microbiota and host transcription of genes involved in lipid metabolism. Sci Rep 2015;5:9336.

[5] Semova I, Carten JD, Stombaugh J, Mackey LC, Knight R, Farber SA, et al. Microbiota Regulate Intestinal Absorption and Metabolism of Fatty Acids in the Zebrafish. Cell Host & Microbe 2012;12(3):277-88.

[6] Ringø E, Zhou Z, Vecino JLG, Wadsworth S, Romero J, Krogdahl Å, et al. Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? Aquaculture Nutrition 2016;22(2):219-82.

[7] Merrifield DL, Ringø E. Aquaculture nutrition: gut health, probiotics and prebiotics. John Wiley & Sons; 2014.

[8] Austin B. The bacterial microflora of fish, revised. ScientificWorldJournal 2006;6:931-45.

[9] Gajardo K, Jaramillo-Torres A, Kortner TM, Merrifield DL, Tinsley J, Bakke AM, et al. Alternative Protein Sources in the Diet Modulate Microbiota and

Functionality in the Distal Intestine of Atlantic Salmon (Salmo salar). Appl Environ Microbiol 2017;83(5).
[10] Zarkasi KZ, Taylor RS, Abell GC, Tamplin ML, Glencross BD, Bowman JP. Atlantic Salmon (Salmo salar L.) Gastrointestinal Microbial Community Dynamics in Relation to Digesta Properties and Diet. Microb Ecol 2016;71(3):589-603.
[11] Catalan N, Villasante A, Wacyk J, Ramirez C, Romero J. Fermented Soybean Meal Increases Lactic Acid Bacteria in Gut Microbiota of Atlantic Salmon (Salmo salar). Probiotics Antimicrob Proteins 2018;10(3):566-76.
[12] Jaramillo-Torres A, Rawling M, Rodiles A, Mikalsen HE, Johansen LH, Tinsley J, et al. Influence of dietary supplementation of probiotic Pediococcus acidilactici MA18/5M during the transition from freshwater to seawater on intestinal health and microbiota of Atlantic salmon (Salmo salar L.). 2019;10:2243.
[13] Krogdahl Å, Kortner TM, Jaramillo-Torres A, Gamil AAA, Chikwati E, Li Y, et al. Removal of three proteinaceous antinutrients from soybean does not mitigate soybean-induced enteritis in Atlantic salmon (Salmo salar, L). 2020;514:734495.
[14] Dehler CE, Secombes CJ, Martin SA. Environmental and physiological factors shape the gut microbiota of Atlantic salmon parr (Salmo salar L.). Aquaculture 2017;467:149-57.
[15] Rud I, Kolarevic J, Holan AB, Berget I, Calabrese S, Terjesen BF. Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production. Aquacultural Engineering 2017;78:50-62.
[16] Fogarty C, Burgess CM, Cotter PD, Cabrera-Rubio R, Whyte P, Smyth C, et al. Diversity and composition of the gut microbiota of Atlantic salmon (Salmo salar) farmed in Irish waters. J Appl Microbiol 2019.
[17] Hovda MB, Fontanillas R, McGurk C, Obach A, Rosnes JT. Seasonal variations in the intestinal microbiota of farmed Atlantic salmon (Salmo salar L.). Aquaculture Research 2012;43(1):154-9.
[18] Zarkasi KZ, Abell GC, Taylor RS, Neuman C, Hatje E, Tamplin ML, et al. Pyrosequencing-based characterization of gastrointestinal bacteria of Atlantic salmon (Salmo salar L.) within a commercial mariculture system. J Appl Microbiol 2014;117(1):18-27.
[19] Karlsen C, Ottem KF, Brevik OJ, Davey M, Sorum H, Winther-Larsen HC. The environmental and host-associated bacterial microbiota of Arctic seawater-farmed Atlantic salmon with ulcerative disorders. Journal of Fish Diseases 2017;40(11):1645-63.
[20] Gajardo K, Rodiles A, Kortner TM, Krogdahl A, Bakke AM, Merrifield DL, et al. A high-resolution map of the gut microbiota in Atlantic salmon (Salmo salar): A basis for comparative gut microbial research. Sci Rep 2016;6:30893.
[21] Lokesh J, Kiron V, Sipkema D, Fernandes JMO, Moum T. Succession of embryonic and the intestinal bacterial communities of Atlantic salmon (Salmo salar) reveals stage-specific microbial signatures. Microbiologyopen 2019;8(4):e00672.
[22] Bjornsson BT, Stefansson SO, McCormick SD. Environmental endocrinology of salmon smoltification. Gen Comp Endocrinol 2011;170(2):290-8.
[23] Sundh H, Nilsen TO, Lindstrom J, Hasselberg-Frank L, Stefansson SO, McCormick SD, et al. Development of intestinal ion-transporting mechanisms during smoltification and seawater acclimation in Atlantic salmon Salmo salar. J Fish Biol 2014;85(4):1227-52.

[24] Handeland SO, Imsland AK, Bjornsson BT, Stefansson SO, Porter M. Physiology during smoltification in Atlantic salmon: effect of melatonin implants. Fish Physiol Biochem 2013;39(5):1079-88.
[25] Tipsmark CK, Sorensen KJ, Hulgard K, Madsen SS. Claudin-15 and -25b expression in the intestinal tract of Atlantic salmon in response to seawater acclimation, smoltification and hormone treatment. Comp Biochem Physiol A Mol Integr Physiol 2010;155(3):361-70.
[26] Dehler CE, Secombes CJ, Martin SAM. Seawater transfer alters the intestinal microbiota profiles of Atlantic salmon (Salmo salar L.). Sci Rep 2017;7(1):13877.
[27] Rudi K, Angell IL, Pope PB, Vik JO, Sandve SR, Snipen LG. Stable Core Gut Microbiota across the Freshwater-to-Saltwater Transition for Farmed Atlantic Salmon. Appl Environ Microbiol 2018;84(2).
[28] Lokesh J, Kiron V. Transition from freshwater to seawater reshapes the skin-associated microbiota of Atlantic salmon. Sci Rep 2016;6:19707.
[29] Tacchi L, Bickerdike R, Douglas A, Secombes CJ, Martin SA. Transcriptomic responses to functional feeds in Atlantic salmon (Salmo salar). Fish Shellfish Immunol 2011;31(5):704-15.
[30] Kiron V. Fish immune system and its nutritional modulation for preventive health care. Animal Feed Science and Technology 2012;173(1-2):111-33.
[31] Carbone D, Faggio C. Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of Sparus aurata and Dicentrarchus labrax. Fish Shellfish Immunol 2016;54:172-8.
[32] Torrecillas S, Montero D, Izquierdo M. Improved health and growth of fish fed mannan oligosaccharides: potential mode of action. Fish Shellfish Immunol 2014;36(2):525-44.
[33] Wang J, Lei P, Gamil AAA, Lagos L, Yue Y, Schirmer K, et al. Rainbow trout (Oncorhynchus mykiss) intestinal epithelial cells as a model for studying gut immune function and effects of functional feed ingredients. Frontiers in immunology 2019;10:152.
[34] Hossain MS, Koshio S, Kestemont P. Recent advances of nucleotide nutrition research in aquaculture: a review. Reviews in Aquaculture 2019.
[35] Guerreiro I, Oliva-Teles A, Enes P. Prebiotics as functional ingredients: focus on Mediterranean fish aquaculture. Reviews in Aquaculture 2018;10(4):800-32.
[36] Ringø E, Olsen RE, Gifstad TO, Dalmo RA, Amlund H, Hemre GI, et al. Prebiotics in aquaculture: a review. Aquaculture Nutrition 2010;16(2):117-36.
[37] Wang J, Kortner TM, Chikwati EM, Li Y, Jaramillo-Torres A, Jakobsen JV, et al. Gut immune functions and health in Atlantic salmon (Salmo salar) from late freshwater stage until one year in seawater and effects of functional ingredients: A case study from a commercial sized research site in the Arctic region. Fish & Shellfish Immunology 2020;106:1106-19.
[38] Herlemann DPR, Labrenz M, Jurgens K, Bertilsson S, Waniek JJ, Andersson AF. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. Isme Journal 2011;5(10):1571-9.
[39] Legrand TP, Wynne JW, Weyrich LS, Oxley AP. A microbial sea of possibilities: current knowledge and prospects for an improved understanding of the fish microbiome. Reviews in Aquaculture 2020;12(2):1101-34.
[40] Hoar W. The Physiology of Smolting Salmonids. Fish physiology. Elsevier; 1988, p. 275-343.
[41] Johansson LH, Timmerhaus G, Afanasyev S, Jorgensen SM, Krasnov A. Smoltification and seawater transfer of Atlantic salmon (Salmo salar L.) is

associated with systemic repression of the immune transcriptome. Fish Shellfish Immunol 2016;58:33-41.
[42] Karlsen C, Ytteborg E, Timmerhaus G, Host V, Handeland S, Jorgensen SM, et al. Atlantic salmon skin barrier functions gradually enhance after seawater transfer. Sci Rep-Uk 2018;8.
[43] Jin Y, Angell IL, Rod Sandve S, Snipen LG, Olsen Y, Rudi K. Atlantic salmon raised with diets low in long-chain polyunsaturated n-3 fatty acids in freshwater have a Mycoplasma-dominated gut microbiota at sea. Aquacult Env Interac 2019;11:31-9.
[44] Ringø E, Gatesoupe FJ. Lactic acid bacteria in fish: a review. Aquaculture 1998;160(3-4):177-203.
[45] Ringø E, Lovmo L, Kristiansen M, Bakken Y, Salinas I, Myklebust R, et al. Lactic acid bacteria vs. pathogens in the gastrointestinal tract of fish: a review. Aquaculture Research 2010;41(4):451-67.
[46] Balcázar JL, De Blas I, Ruiz-Zarzuela I, Vendrell D, Gironés O, Muzquiz JLJFI, et al. Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (Oncorhynchus mykiss). 2007;51(1):185-93.
[47] Askarian F, Zhou Z, Olsen RE, Sperstad S, Ringø E. Culturable autochthonous gut bacteria in Atlantic salmon (Salmo salar L.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes and in vitro growth inhibition of four fish pathogens. Aquaculture 2012;326-329:1-8.
[48] Reveco FE, Øverland M, Romarheim OH, Mydland LTJA. Intestinal bacterial community structure differs between healthy and inflamed intestines in Atlantic salmon (Salmo salar L.). 2014;420:262-9.
[49] Schmidt V, Amaral-Zettler L, Davidson J, Summerfelt S, Good C. Influence of Fishmeal-Free Diets on Microbial Communities in Atlantic Salmon (Salmo salar) Recirculation Aquaculture Systems. Appl Environ Microbiol 2016;82(15):4470-81.
[50] Webster TMU, Rodriguez-Barreto D, Castaldo G, Gough P, Consuegra S, Garcia de Leaniz C. Environmental plasticity and colonisation history in the Atlantic salmon microbiome: a translocation experiment. Molecular ecology 2020;29(5):886-98.
[51] Holben WE, Williams P, Gilbert M, Saarinen M, Sarkilahti LK, Apajalahti JH. Phylogenetic analysis of intestinal microflora indicates a novel Mycoplasma phylotype in farmed and wild salmon. Microb Ecol 2002;44(2):175-85.
[52] Llewellyn MS, McGinnity P, Dionne M, Letourneau J, Thonier F, Carvalho GR, et al. The biogeography of the atlantic salmon (Salmo salar) gut microbiome. ISME J 2016;10(5):1280-4.
[53] Gupta S, Feckaninova A, Lokesh J, Koscova J, Sorensen M, Fernandes J, et al. Lactobacillus Dominate in the Intestine of Atlantic Salmon Fed Dietary Probiotics. Front Microbiol 2019;9:3247.
[54] Gupta S, Lokesh J, Abdelhafiz Y, Siriyappagouder P, Pierre R, Sorensen M, et al. Macroalga-Derived Alginate Oligosaccharide Alters Intestinal Bacteria of Atlantic Salmon. Front Microbiol 2019;10:2037.
[55] Webster TMU, Consuegra S, Hitchings M, Garcia de Leaniz C. Interpopulation Variation in the Atlantic Salmon Microbiome Reflects Environmental and Genetic Diversity. Appl Environ Microbiol 2018;84(16).
[56] Karlsen C, Ottem K, Brevik ØJ, Davey M, Sørum H, Winther-Larsen H. The environmental and host-associated bacterial microbiota of Arctic seawater-farmed Atlantic salmon with ulcerative disorders. J Fish Dis 2017;40(11):1645-63.

[57] Webster TMU, Consuegra S, Hitchings M, de Leaniz CG. Interpopulation variation in the Atlantic salmon microbiome reflects environmental and genetic diversity. Appl Environ Microbiol 2018;84(16):e00691-18.
[58] Cheaib B, Yang P, Kazlauskaite R, Lindsay E, Heys C, De Noa M, et al. Unpicking the mysterious symbiosis of Mycoplasma in salmonids. bioRxiv 2020.
[59] Song SK, Beck BR, Kim D, Park J, Kim J, Kim HD, et al. Prebiotics as immunostimulants in aquaculture: a review. Fish Shellfish Immunol 2014;40(1):40-8.
[60] Mehdinejad N, Imanpour MR, Jafari V. Combined or Individual Effects of Dietary Probiotic Pedicoccus acidilactici and Nucleotide on Growth Performance, Intestinal Microbiota, Hemato-biochemical Parameters, and Innate Immune Response in Goldfish (Carassius auratus). Probiotics Antimicrob Proteins 2018;10(3):558-65.
[61] Dimitroglou A, Merrifield DL, Moate R, Davies SJ, Spring P, Sweetman J, et al. Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, Oncorhynchus mykiss (Walbaum). J Anim Sci 2009;87(10):3226-34.
[62] Ringø E, Erik Olsen R, Gonzalez Vecino JL, Wadsworth S. Use of Immunostimulants and Nucleotides in Aquaculture: A Review. Journal of Marine Science: Research & Development 2012;02(01).
[63] Adel M, Lazado CC, Safari R, Yeganeh S, Zorriehzahra MJJAr. Aqualase®, a yeast-based in-feed probiotic, modulates intestinal microbiota, immunity and growth of rainbow trout Oncorhynchus mykiss. 2017;48(4):1815-26.
[64] Rufchaie R, Hoseinifar SHJCJoF. Effects of dietary commercial yeast glucan on innate immune response, hematological parameters, intestinal microbiota and growth performance of white fish (Rutilus frisii kutum) fry. 2014;72(4):156-63.
[65] Sakai MJA. Current research status of fish immunostimulants. 1999;172(1-2):63-92.
[66] Tsilingiri K, Barbosa T, Penna G, Caprioli F, Sonzogni A, Viale G, et al. Probiotic and postbiotic activity in health and disease: comparison on a novel polarised ex-vivo organ culture model. Gut 2012;61(7):1007-15.
[67] Mileti E, Matteoli G, Iliev ID, Rescigno M. Comparison of the immunomodulatory properties of three probiotic strains of Lactobacilli using complex culture systems: prediction for in vivo efficacy. PLoS One 2009;4(9):e7056.
[68] Liu Z, Liu W, Ran C, Hu J, Zhou Z. Abrupt suspension of probiotics administration may increase host pathogen susceptibility by inducing gut dysbiosis. Sci Rep 2016;6:23214.
[69] Nguyen CDH, Amoroso G, Ventura T, Minich JJ, Elizur A. Atlantic Salmon (Salmo salar L., 1758) Gut Microbiota Profile Correlates with Flesh Pigmentation: Cause or Effect? Mar Biotechnol (NY) 2020.
[70] Nguyen CDH, Amoroso G, Ventura T, Elizur A. Assessing the Pyloric Caeca and Distal Gut Microbiota Correlation with Flesh Color in Atlantic Salmon (Salmo salar L., 1758). Microorganisms 2020;8(8).
[71] Dvergedal H, Sandve SR, Klemetsdal G, Rudi K, Angell IL. Association of gut microbiota with metabolism in juvenile Atlantic Salmon. BioRxiv 2020.
[72] Parra M, Espinoza D, Valdes N, Vargas R, Gonzalez A, Modak B, et al. Microbiota Modulates the Immunomodulatory Effects of Filifolinone on Atlantic Salmon. Microorganisms 2020;8(9).
[73] Li Y, Bruni L, Jaramillo-Torres A, Gajardo K, Kortner TM, Krogdahl Å. Differential Response of Digesta-and Mucosa-Associated Intestinal Microbiota to

Dietary Black Soldier Fly (Hermetia illucens) Larvae Meal in Seawater Phase Atlantic Salmon (Salmo salar). BioRxiv 2020.
[74] Maynard CL, Elson CO, Hatton RD, Weaver CT. Reciprocal interactions of the intestinal microbiota and immune system. Nature 2012;489(7415):231-41.
[75] Bakke-McKellep AM, Nordrum S, Krogdahl Å, Buddington R. Absorption of glucose, amino acids, and dipeptides by the intestines of Atlantic salmon (Salmo salar L.). Fish Physiology and Biochemistry 2000;22(1):33-44.
[76] Knudsen BE, Bergmark L, Munk P, Lukjancenko O, Prieme A, Aarestrup FM, et al. Impact of Sample Type and DNA Isolation Procedure on Genomic Inference of Microbiome Composition. mSystems 2016;1(5).
[77] Vandeputte D, Kathagen G, D'hoe K, Vieira-Silva S, Valles-Colomer M, Sabino J, et al. Quantitative microbiome profiling links gut community variation to microbial load. Nature 2017;551(7681):507.
[78] Ramseier CA, Kinney JS, Herr AE, Braun T, Sugai JV, Shelburne CA, et al. Identification of pathogen and host-response markers correlated with periodontal disease. Journal of periodontology 2009;80(3):436-46.
[79] Bolyen E, Rideout JR, Dillon MR, Bokulich N, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 2019;37(8):852-7.
[80] Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods 2016;13(7):581-+.
[81] Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 2013;41(Database issue):D590-6.
[82] Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, et al. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. 2018;6(1):90.
[83] Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 2018;6.
[84] Parris DJ, Brooker RM, Morgan MA, Dixson DL, Stewart FJ. Whole gut microbiome composition of damselfish and cardinalfish before and after reef settlement. Peerj 2016;4.
[85] Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 2002;30(14):3059-66.
[86] Price MN, Dehal PS, Arkin APJPo. FastTree 2–approximately maximum-likelihood trees for large alignments. 2010;5(3):e9490.
[87] Clarke KR, Clarke K, Gorley K, Clarke K, Gorley R. PRIMER v6: user manual/tutorial. 2006.
[88] Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res 2017;45(W1):W180-W8.

802 **Figure legends**

803 **Fig. 1** Alpha diversity and beta diversity of distal intestinal digesta microbiota of Atlantic
804 salmon. **a** Microbial richness in distal intestinal digesta microbiota of Atlantic salmon
805 between treatments, as measured using the Observed species index. **b** Microbial evenness in
806 distal intestinal digesta microbiota of Atlantic salmon between treatments, as measured using
807 the Pielou's evenness. **c** Microbial diversity in distal intestinal digesta microbiota of Atlantic
808 salmon between treatments, as measured using the Shannon's index. **d** Microbial diversity
809 in distal intestinal digesta microbiota of Atlantic salmon between treatments, as measured
810 using the Simpson's index. **e** PCoA plots based on weighted UniFrac show the clustering
811 between treatments. **f** PCoA plots based unweighted UniFrac show the clustering between
812 treatments. **g** PCoA plots based on unweighted UniFrac show the clustering of dietary
813 treatment at SW2. For alpha diversity, asterisks indicate significant effect of diet among
814 treatments (* $P < 0.05$, ** $P < 0.01$, n = 8). For beta diversity, each dot represents one sample.

815 **Fig. 2** Gut microbiota composition of distal intestinal digesta microbiota of Atlantic salmon.
816 **a** Top 25 most abundant taxa at genus level of all samples and mean (right side) relative
817 abundance of each taxon between fish fed Ref diet among sampling time points. The top 25
818 genera were selected accounted for more than 80% of the total abundance in each treatment.
819 f__, family. **b** The core microbiota between samples at genus level. The figures showing the
820 bacteria were selected above 0.1% relative abundance in 80% of samples. **c** Balloon plot
821 showing the relative abundance of five major genera between treatments (n = 8). The five
822 major genera were selected based on MaAsLin 2 and core microbiota analysis.

823 **Fig. 3** Heatmap of bacterial abundance in fish fed the Ref diets based on MaAsLin 2 analysis.
824 Rows indicate results for 69 bacteria at genus level (*q-value* < 0.05), columns depict the
825 results for the 8 samples at each of the four sampling time points. Color differences indicate

differences in normalized relative abundances at genus level, i.e., red (high positive value) indicates the maximum relative abundance; Green (low negative value) indicates the minimum relative abundance. o__: order; f__: family.

Fig. 4 The effect of dietary functional ingredients on distal intestinal digesta microbiota of Atlantic salmon at SW2. **a** Mean relative abundance (Top 25) of each taxon at genus level in fish at SW2 fed Ref and Test diets. The top 25 genera were selected accounted for more than 80% of the total abundance in each treatment. **b** The heatmap of bacterial abundance at SW2 based on MaAsLin 2 analysis. Rows depict results for 25 bacteria (*q-value* < 0.05), columns depict the results from the 8 samples from fish fed each of the two diets. Colors correspond with normalized relative abundances, i.e., red (high positive value) indicates the maximum relative abundance; Green (low negative value) indicates the minimum relative abundance. f__: family.

Fig. 5 Significant associations between microbial clades with sample metadata. **a** Heatmap summarizing all the significant associations between microbial clades and sample metadata. Color key: -log (q-value) * sign (coefficient). Cells that denote significant associations are colored (red or blue) and overlaid with a plus (+) or minus (-) sign that indicates the direction of association: qPCR_barrier_function (-), negative correlation between microbial clade abundance and qPCR_barrier_function (PC1 of PCA); qPCR_immune_response (-), negative correlation between microbial clade abundance and qPCR_immune_function (PC1 of PCA); plasma_nutrients (+), positive correlation between microbial clade abundance and the levels of plasma_nutrients (PC1 of PCA). **b** The negative correlation between the relative abundance of *Megasphaera* and qPCR_barrier_function (PC1 of PCA). **c** The negative correlation between the relative abundance of *Megasphaera* and qPCR_barrier_function (PC1 of PCA). Of note, the gut immune gene expression was negatively correlated with PC1 of the PCA, which decreased as PC1 of the PCA increased (Table S1). Hence, the relative

abundance of *Megasphaera* shows a positive correlation with immune gene expression level. **d** The positive correlation between the relative abundance of *Megasphaera* and *Bacteoides* and plasma_nutrients (PC1 of PCA), respectively. FDR, false discovery rate; f__, family. The significant association was set at FDR < 0.25.

Fig. 6 Outline of the sampling program. The four diet series were changed at week 4, week 22, week 34 and week 45, respectively (Diet composition see Table 2).

Table 1 Result of the PERMANOVA analysis of the weighted and unweighted UniFrac[1].

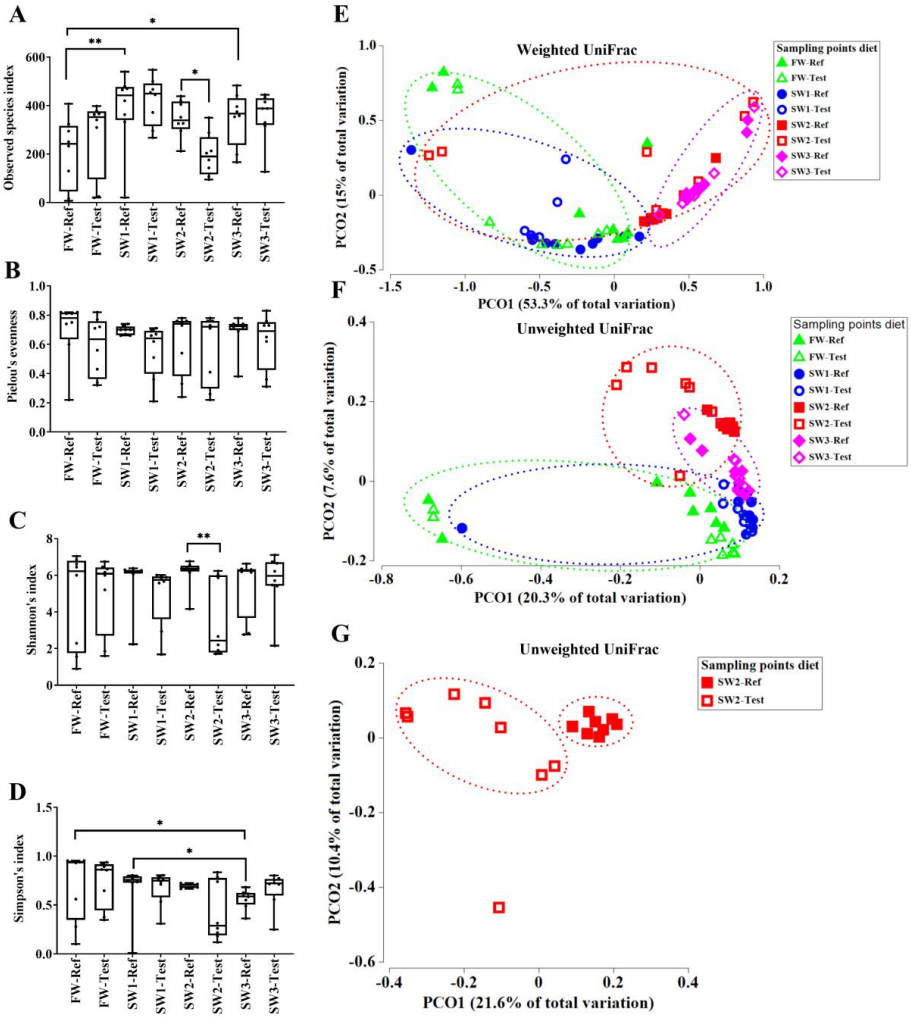
PERMANOVA	Weighted UniFrac		Unweighted UniFrac	
	Pseudo-F	*P*	Pseudo-F	*P*
Sampling time points	7.518	0.001	3.008	0.001
Pairwise comparison				
FW-Ref vs SW1-Ref		0.005		0.025
FW-Ref vs SW2-Ref		0.001		0.001
FW-Ref vs SW3-Ref		0.001		0.001
SW1-Ref vs SW2-Ref		0.001		0.001
SW1-Ref vs SW3-Ref		0.001		0.002
SW2-Ref vs SW3-Ref		0.001		0.001
Dietary effect at each sampling time point				
FW-Ref vs FW-Test	0.949	0.409	1.056	0.347
SW1-Ref vs SW1-Test	2.331	0.05	1.271	0.01
SW2-Ref vs SW2-Test	2.099	0.129	2.937	0.001
SW3-Ref vs SW3-Test	0.698	0.406	1.176	0.146

[1]FW, sampling time point in freshwater (May 2016); SW1, the first seawater sampling time point (June 2016); SW2 the second seawater sampling time point (November 2016); SW3, the final seawater sampling time point (April 2017); Ref: diet without functional ingredients; Test, diet with functional ingredients.

877 **Table 2** Feed composition and formulation of diets during the feeding trial[1].

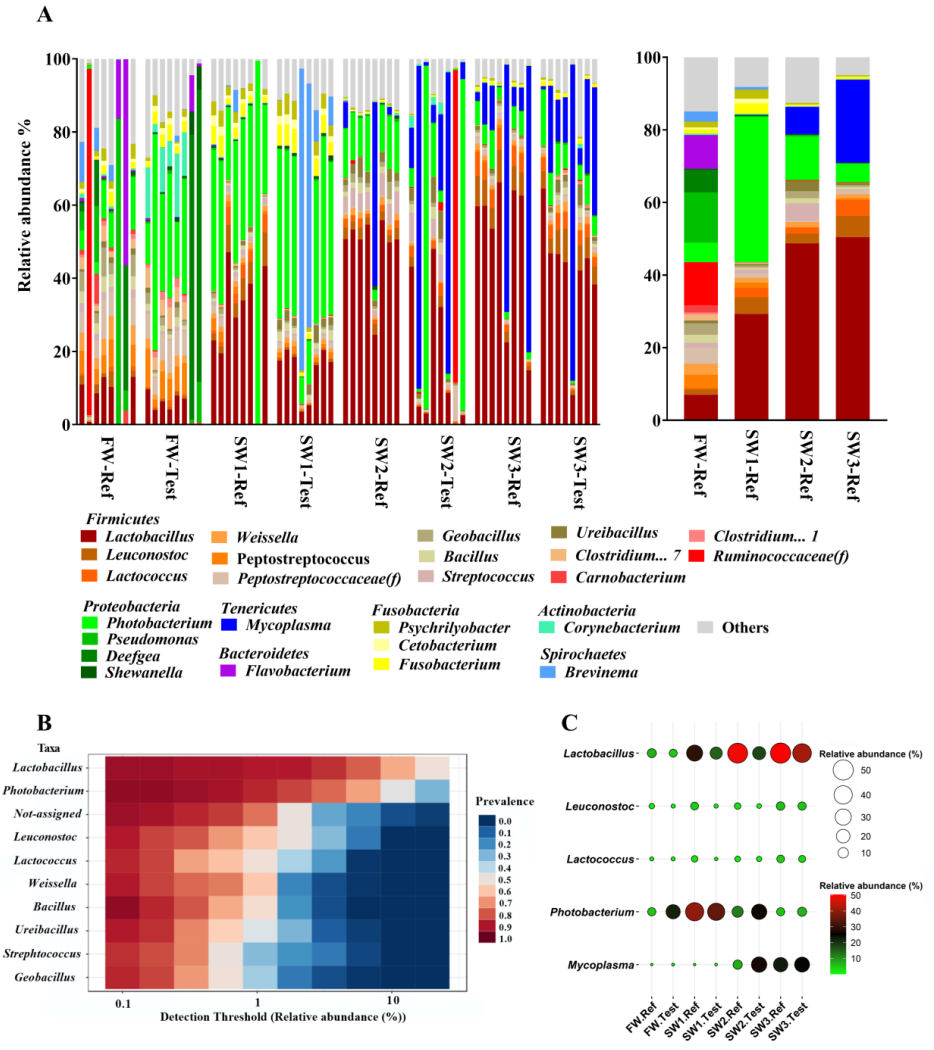
Feed composition	FW-Ref	FW-Test	SW1-Ref	SW1-Test	SW2-Ref	SW2-Test	SW3-Ref	SW3-Test
Ingredients (%)								
Marine protein sources[2]	40	40	30	30	19	19	19	19
Plant protein sources[3]	35	35	39	39	53	53	53	53
North Atlantic fish oil	9	9	24	24	10	10	10	10
Rapeseed oil	9	9	-	-	7	7	7	7
Binders & Micronutrients	7	7	7	7	11	11	11	11
Sum	100	100	100	100	100	100	100	100
Nutrient composition (%)								
Crude protein	44	44	44	44	46	46	46	46
Crude fat	22	22	28	28	22	22	22	22
Starch	7.5	7.5	8	8	10	10	10	10
Crude fiber	1.5	1.5	3	3	3	3	3	3
Ash	7	7	6	6	5	5	5	5
Functional ingredients[4]								
Essential fatty acids	-	-	-	√	-	√	-	√
Nucleotides	-	√	-	√	-	√	-	√
Yeast cell walls	-	-	-	-	-	√	-	-
A prebiotic	-	-	-	-	-	-	-	√

878 [1]The composition of four different basic diets varied throughout the time of observation following the strategy developed for this commercial
879 site according to the development and production and health of the fish in the farm. At each observation time, two series of diets were fed, one
880 without functional ingredients (Ref diet) and one with functional ingredients (Test diet). FW, sampling time point in freshwater (May 2016);
881 SW1, the first seawater sampling time point (June 2016); SW2 the second seawater sampling time point (November 2016); SW3, the final
882 seawater sampling time point (April 2017). [2]Mix of Scandinavian origin fish meal and, Fish protein concentrate (Norway). [3]Mix of soy protein
883 concentrate, wheat protein concentrate, wheat gluten, sunflower meal. [4]Inclusion levels were determined according to recommendations from
884 the producers and cannot be disclosed due to commercial interests and intellectual rights.


A
Observed species index
600
400
200
0
*
**
*
FW-Ref
FW-Test
SW1-Ref
SW1-Test
SW2-Ref
SW2-Test
SW3-Ref
SW3-Test
B
Pielou's evenness
1.0
0.8
0.6
0.4
0.2
0.0
C
Shannon's index
8
6
4
2
0
**
D
Simpson's index
1.5
1.0
0.5
0.0
*
*
E
Weighted UniFrac
PCO2 (15% of total variation)
1.0
0.5
0
-0.5
-1.5
-1.0
-0.5
0
0.5
1.0
PCO1 (53.3% of total variation)
Sampling points diet
FW-Ref
FW-Test
SW1-Ref
SW1-Test
SW2-Ref
SW2-Test
SW3-Ref
SW3-Test
F
Unweighted UniFrac
PCO2 (7.6% of total variation)
0.4
0.2
0
-0.2
-0.8
-0.6
-0.4
-0.2
0
0.2
PCO1 (20.3% of total variation)
Sampling points diet
FW-Ref
FW-Test
SW1-Ref
SW1-Test
SW2-Ref
SW2-Test
SW3-Ref
SW3-Test
G
Unweighted UniFrac
PCO2 (10.4% of total variation)
0.2
0
-0.2
-0.4
-0.6
-0.4
-0.2
0
0.2
0.4
PCO1 (21.6% of total variation)
Sampling points diet
SW2-Ref
SW2-Test

885

886 **Fig. 1**



887 **Fig. 2**

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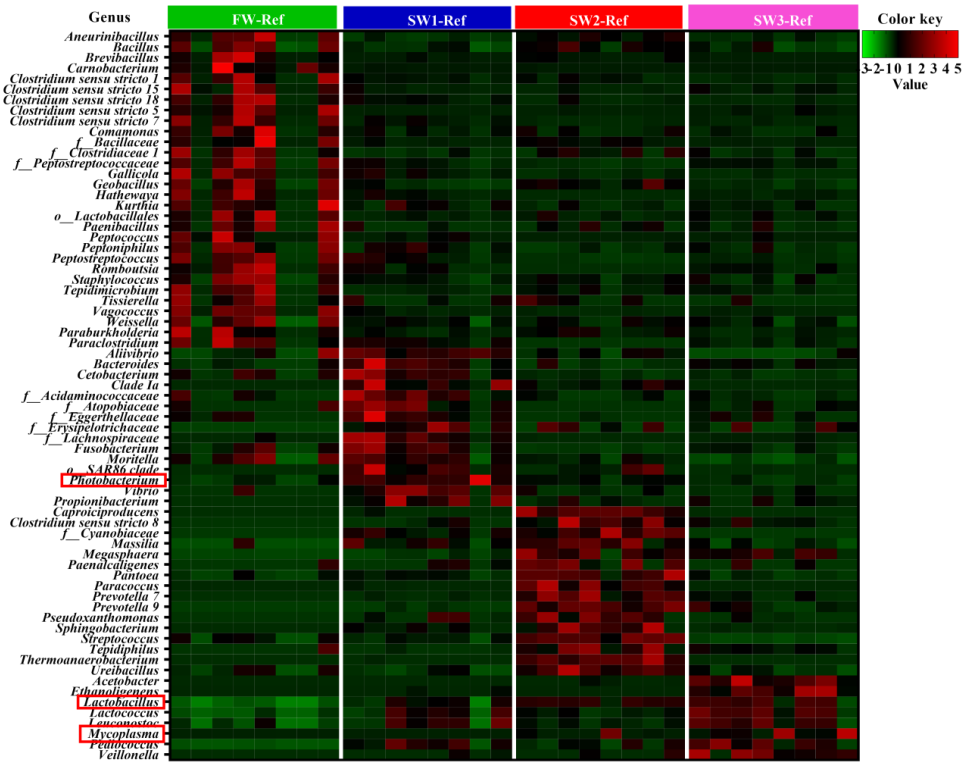

Genus
FW-Ref
SW1-Ref
SW2-Ref
SW3-Ref
Color key
-3 -2 -1 0 1 2 3 4 5
Value
Aneurinibacillus
Bacillus
Brevibacillus
Carnobacterium
Clostridium sensu stricto 1
Clostridium sensu stricto 15
Clostridium sensu stricto 18
Clostridium sensu stricto 5
Clostridium sensu stricto 7
Comamonas
f__Bacillaceae
f__Clostridiaceae 1
f__Peptostreptococcaceae
Gallicola
Geobacillus
Hathewaya
Kurthia
o__Lactobacillales
Paenibacillus
Peptococcus
Peptoniphilus
Peptostreptococcus
Romboutsia
Staphylococcus
Tepidimicrobium
Tissierella
Vagococcus
Weissella
Paraburkholderia
Paraclostridium
Aliivibrio
Bacteroides
Cetobacterium
Clade Ia
f__Acidaminococcaceae
f__Atopobiaceae
f__Eggerthellaceae
f__Erysipelotrichaceae
f__Lachnospiraceae
Fusobacterium
Moritella
o__SAR86 clade
Photobacterium
Vibrio
Propionibacterium
Caproiciproducens
Clostridium sensu stricto 8
f__Cyanobiaceae
Massilia
Megasphaera
Paenalcaligenes
Pantoea
Paracoccus
Prevotella 7
Prevotella 9
Pseudoxanthomonas
Sphingobacterium
Streptococcus
Tepidiphilus
Thermoanaerobacterium
Ureibacillus
Acetobacter
Ethanoligenens
Lactobacillus
Lactococcus
Lanzonotus
Mycoplasma
Pediococcus
Veillonella

893

894 **Fig. 3**

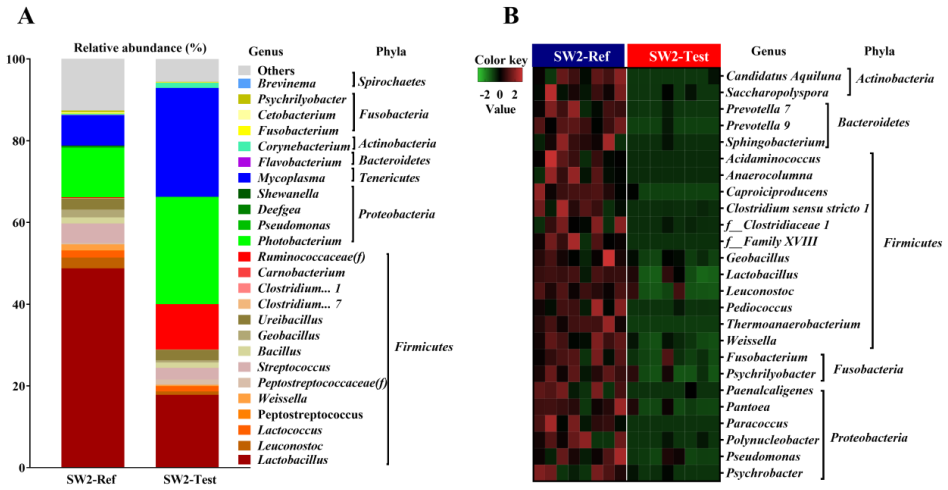

A
Relative abundance (%)
100
80
60
40
20
0
SW2-Ref
SW2-Test
Genus
Phyla
Others
Brevinema
Psychrilyobacter
Cetobacterium
Fusobacterium
Corynebacterium
Flavobacterium
Mycoplasma
Shewanella
Deefgea
Pseudomonas
Photobacterium
Ruminococcaceae(f)
Carnobacterium
Clostridium... 1
Clostridium... 7
Ureibacillus
Geobacillus
Bacillus
Streptococcus
Peptostreptococcaceae(f)
Weissella
Peptostreptococcus
Lactococcus
Leuconostoc
Lactobacillus
Spirochaetes
Fusobacteria
Actinobacteria
Bacteroidetes
Tenericutes
Proteobacteria
Firmicutes
B
Color key
-2 0 2
Value
SW2-Ref
SW2-Test
Genus
Phyla
Candidatus Aquiluna
Saccharopolyspora
Prevotella 7
Prevotella 9
Sphingobacterium
Acidaminococcus
Anaerocolumna
Caproiciproducens
Clostridium sensu stricto 1
f__Clostridiaceae 1
f__Family XVIII
Geobacillus
Lactobacillus
Leuconostoc
Pediococcus
Thermoanaerobacterium
Weissella
Fusobacterium
Psychrilyobacter
Paenalcaligenes
Pantoea
Paracoccus
Polynucleobacter
Pseudomonas
Psychrobacter
Actinobacteria
Bacteroidetes
Firmicutes
Fusobacteria
Proteobacteria

Fig. 4

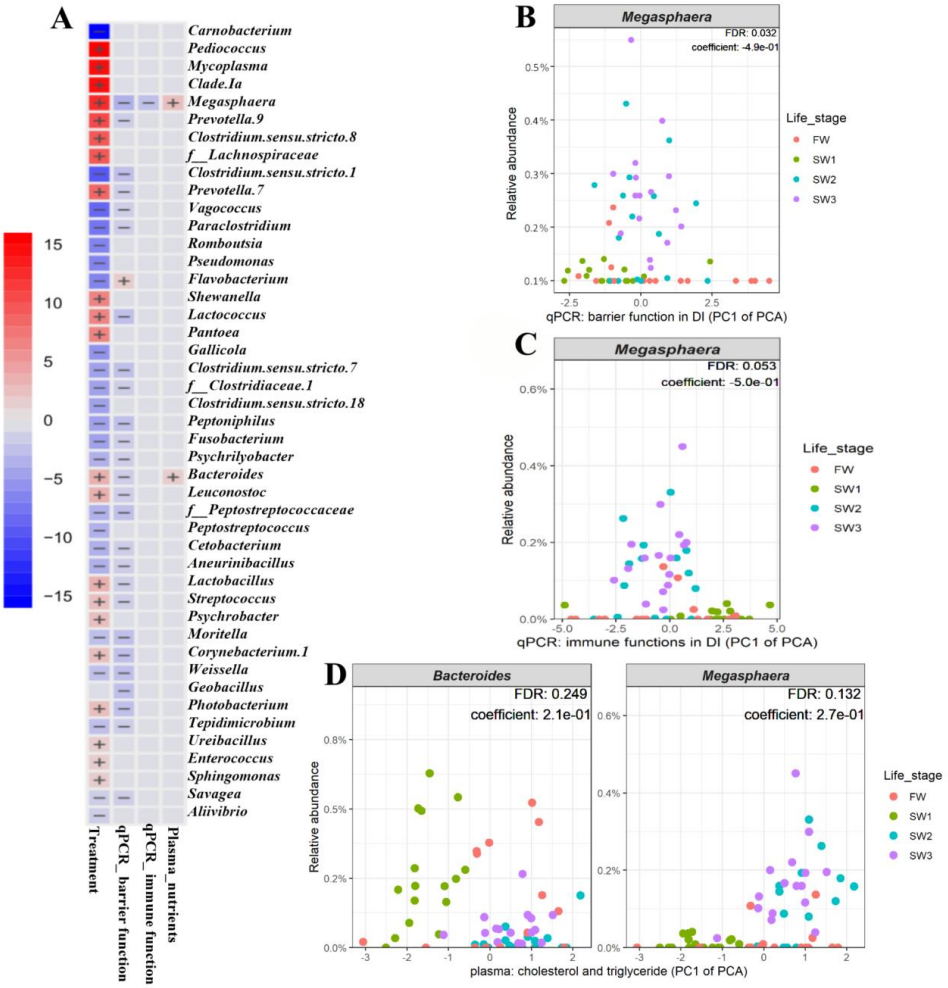

A
Carnobacterium
Pediococcus
Mycoplasma
Clade.Ia
Megasphaera
Prevotella.9
Clostridium.sensu.stricto.8
f__Lachnospiraceae
Clostridium.sensu.stricto.1
Prevotella.7
Vagococcus
Paraclostridium
Romboutsia
Pseudomonas
Flavobacterium
Shewanella
Lactococcus
Pantoea
Gallicola
Clostridium.sensu.stricto.7
f__Clostridiaceae.1
Clostridium.sensu.stricto.18
Peptoniphilus
Fusobacterium
Psychrilyobacter
Bacteroides
Leuconostoc
f__Peptostreptococcaceae
Peptostreptococcus
Cetobacterium
Aneurinibacillus
Lactobacillus
Streptococcus
Psychrobacter
Moritella
Corynebacterium.1
Weissella
Geobacillus
Photobacterium
Tepidimicrobium
Ureibacillus
Enterococcus
Sphingomonas
Savagea
Aliivibrio
15
10
5
0
−5
−10
−15
Treatment
qPCR_barrier function
qPCR_immune function
Plasma_nutrients
B
Megasphaera
FDR: 0.032
coefficient: -4.9e-01
Relative abundance
qPCR: barrier function in DI (PC1 of PCA)
Life_stage
FW
SW1
SW2
SW3
C
Megasphaera
FDR: 0.053
coefficient: -5.0e-01
Relative abundance
qPCR: immune functions in DI (PC1 of PCA)
Life_stage
FW
SW1
SW2
SW3
D
Bacteroides
FDR: 0.249
coefficient: 2.1e-01
Megasphaera
FDR: 0.132
coefficient: 2.7e-01
Relative abundance
plasma: cholesterol and triglyceride (PC1 of PCA)
Life_stage
FW
SW1
SW2
SW3

Fig. 5

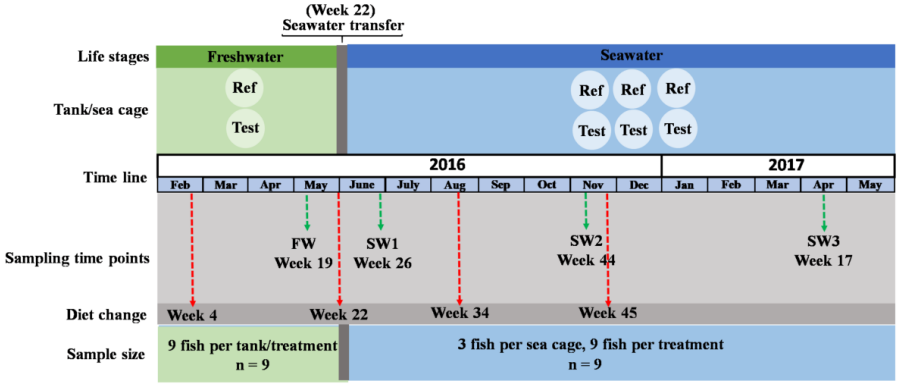

(Week 22)
Seawater transfer
Life stages
Freshwater
Seawater
Tank/sea cage
Ref
Test
Ref
Ref
Ref
Test
Test
Test
Time line
2016
2017
Feb
Mar
Apr
May
June
July
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Sampling time points
FW
Week 19
SW1
Week 26
SW2
Week 44
SW3
Week 17
Diet change
Week 4
Week 22
Week 34
Week 45
Sample size
9 fish per tank/treatment
n = 9
3 fish per sea cage, 9 fish per treatment
n = 9

Fig. 6

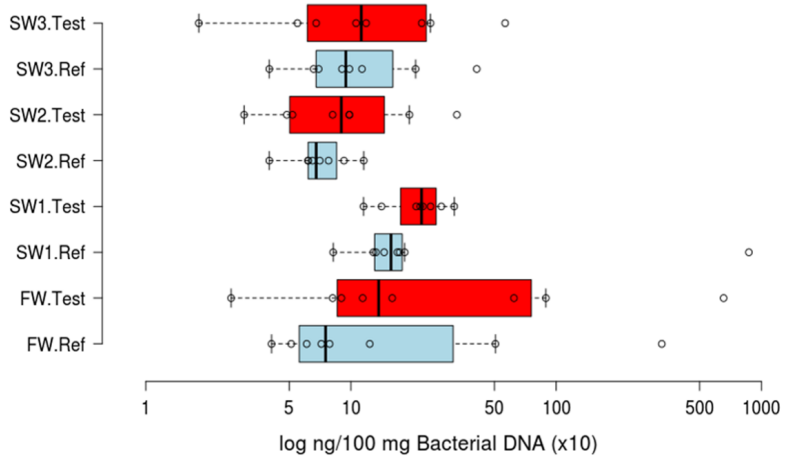
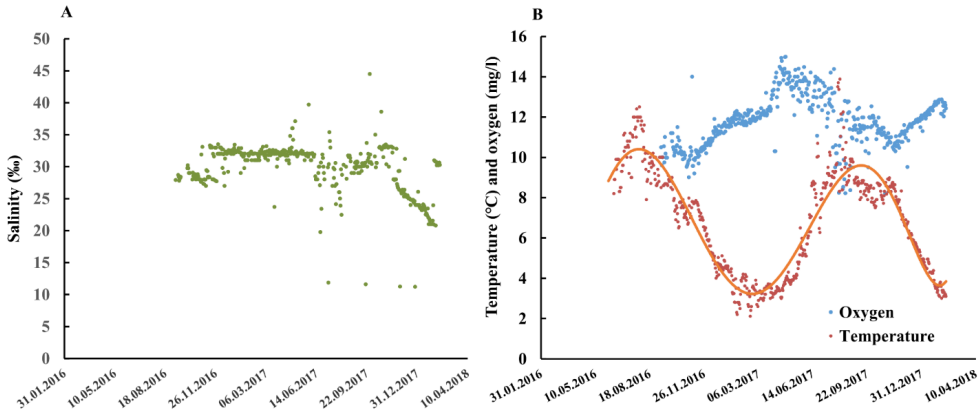


Figure S1. The bacterial DNA quantification among treatments.

947 **Figure S2.** Microbial clades showing significant associations with expressions of barrier
948 function related genes in the distal gut. Since the expression levels of barrier function related
949 genes were highly correlated, we ran a principle component analysis (PCA) and used the first
950 principle component (PC1) for the association testing to avoid multicollinearity and reduce the
951 number of association testing. Except *Flavobacterium*, 26 differentially abundant taxa showed
952 a clear negative correlation with expression levels of gut barrier function genes, which
953 decreased as PC1 of the PCA increased. FDR, false discovery rate.



954

955 **Figure S3.** The experimental conditions of salinity (A), temperature and oxygen (B) in water
956 through the production cycle.

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971 **Table S1: The metadata of interest for multivariate association analysis**

Treatment	Plamma nutrients		qPCR_Immune fucntions								qPCR_Barrier fucntions			PC1 of PCA		
	Cholesterol	Triglyceride	*muc13*	*il1b*	*ifnr*	*il10*	*tgfb*	*cd8b*	*cd3*	*myd88*	*zo-1*	*claudin15*	*claudin-25b*	plasma_ nutrients	qPCR_ immune	qPCR_ barrier
FW-Ref	3	1.3	9.26	0.14	0.05	0.05	0.06	0.16	0.24	1.32	0.96	2.79	1.29	-3.06	-1.54	0.09
FW-Ref	5.4	5.8	7.06	0.11	0.07	0.04	0.12	0.20	0.21	0.74	0.89	2.69	1.92	1.07	-1.09	0.51
FW-Ref	6.3	4.3	5.72	0.16	0.03	0.01	0.07	0.12	0.11	0.80	0.88	2.68	0.72	0.90	1.31	-0.94
FW-Ref	4.1	3.8	11.75	0.11	0.08	0.07	0.12	0.31	0.25	2.02	1.50	5.01	4.46	-0.36	-3.29	3.33
FW-Ref	5.3	1.4	8.24	0.42	0.20	0.09	0.08	0.40	0.51	2.04	1.16	3.63	2.45	-1.55	-4.58	1.65
FW-Ref	9.6	4	17.57	0.30	0.08	0.03	0.13	0.14	0.24	1.85	1.44	10.57	3.60	1.79	-3.01	3.99
FW-Ref	8.9	4.1	10.21	0.14	0.02	0.03	0.08	0.34	0.18	1.38	1.48	3.85	1.54	1.65	-1.30	1.40
FW-Test	7.7	3.5	7.31	0.09	0.02	0.01	0.05	0.14	0.09	0.88	0.96	4.03	1.06	1.02	1.75	0.31
FW-Test	5.4	2.7	5.75	0.02	0.01	0.02	0.04	0.15	0.09	0.75	0.62	1.83	0.95	-0.31	2.76	-1.57
FW-Test	8.6	3.3	5.79	0.05	0.03	0.04	0.02	0.13	0.21	1.00	0.73	2.11	1.01	1.18	1.11	-1.03
FW-Test	6.1	2.7	4.01	0.02	0.01	0.01	0.07	0.11	0.11	0.70	0.68	1.50	0.68	-0.02	3.05	-2.18
FW-Test	5.1	2.9	5.48	0.06	0.03	0.04	0.07	0.13	0.17	1.04	0.90	1.82	0.90	-0.32	0.38	-1.11
FW-Test	8.5	3.5	6.65	0.03	0.04	0.03	0.13	0.24	0.18	0.90	0.87	1.50	1.24	1.26	-0.29	-0.97
FW-Test	8	1.8	20.29	0.14	0.03	0.03	0.11	0.34	0.25	1.97	2.17	10.03	2.32	-0.10	-3.03	3.87
FW-Test	7.7	2.3	19.18	0.54	0.06	0.08	0.10	0.23	0.25	2.77	2.14	9.48	3.84	0.26	-4.37	4.50
SW1-Ref	3.4	2.2	5.82	0.03	0.01	0.01	0.05	0.15	0.09	0.72	0.87	2.27	1.33	-1.80	3.08	-0.29
SW1-Ref	3.8	2.3	4.81	0.02	0.00	0.03	0.04	0.17	0.11	0.76	0.81	1.70	1.06	-1.45	2.53	-1.12
SW1-Ref	5.7	1.9	4.35	0.05	0.06	0.03	0.02	0.11	0.12	0.60	0.41	1.86	0.71	-0.82	2.20	-2.55
SW1-Ref	4.1	1.7	4.91	0.02	0.01	0.01	0.06	0.12	0.13	0.57	0.68	1.27	1.03	-1.82	2.81	-1.81
SW1-Ref	4.4	2.3	5.78	0.02	0.00	0.01	0.08	0.22	0.14	0.79	0.98	1.58	0.96	-1.10	2.13	-1.10
SW1-Ref	3.5	2.1	4.81	0.01	0.00	0.01	0.02	0.12	0.11	0.67	0.63	1.63	0.74	-1.82	4.66	-2.05
SW1-Ref	4	1.2	3.48	0.03	0.00	0.01	0.05	0.10	0.16	0.42	0.53	1.29	0.71	-2.51	3.70	-2.69
SW1-Ref	3	2.4	10.20	0.07	0.01	0.08	0.54	0.55	0.63	2.65	1.36	5.39	2.45	-1.95	-4.89	2.42
SW1-Test	5.6	2.2	4.18	0.03	0.01	0.01	0.06	0.16	0.12	0.59	0.43	1.72	1.12	-0.60	3.00	-1.90
SW1-Test	4.6	1.6	7.29	0.04	0.01	0.04	0.08	0.17	0.17	1.11	1.10	2.37	1.34	-1.65	0.50	0.11
SW1-Test	5.8	1.9	5.59	0.03	0.01	0.01	0.05	0.20	0.16	0.83	0.84	2.58	1.01	-0.78	1.96	-0.57

Continued Table S1

SW1-Test	4.3	2.2	5.43	0.06	0.00	0.01	0.04	0.12	0.12	0.67	0.57	2.16	0.99	-1.23	3.32	-1.39
SW1-Test	3.9	1.4	7.44	0.04	0.01	0.02	0.06	0.10	0.13	0.84	0.95	2.15	1.14	-2.29	1.88	-0.47
SW1-Test	6.5	1.4	6.01	0.03	0.01	0.02	0.02	0.13	0.18	0.71	0.78	1.86	0.87	-1.05	2.30	-1.35
SW1-Test	5.2	1.3	5.09	0.02	0.00	0.01	0.06	0.23	0.16	0.62	0.94	1.57	0.88	-1.73	2.65	-1.29
SW1-Test	3.2	1.9	7.18	0.03	0.01	0.04	0.11	0.21	0.19	0.89	0.94	1.91	1.24	-2.21	0.04	-0.52
SW2-Ref	8.7	3.1	6.16	0.02	0.03	0.05	0.08	0.44	0.20	0.50	0.81	2.43	1.14	1.09	0.04	-0.52
SW2-Ref	6.7	8	8.61	0.03	0.03	0.07	0.08	0.35	0.22	1.09	1.10	2.75	1.47	2.18	-1.29	0.45
SW2-Ref	8.3	4.7	5.44	0.01	0.03	0.02	0.06	0.25	0.21	0.87	0.87	2.28	1.31	1.73	0.88	-0.30
SW2-Ref	7.7	4.3	10.70	0.09	0.04	0.05	0.13	0.27	0.23	1.31	1.24	2.85	1.84	1.39	-2.17	1.00
SW2-Ref	7.3	3.3	8.10	0.06	0.02	0.03	0.11	0.18	0.17	0.91	0.79	1.86	1.25	0.78	-0.16	-0.79
SW2-Ref	8.7	4.7	5.42	0.05	0.04	0.03	0.10	0.27	0.22	0.25	0.72	1.73	0.82	1.85	0.77	-1.62
SW2-Ref	6.9	4.2	5.35	0.03	0.02	0.03	0.06	0.20	0.15	0.69	0.74	2.18	1.17	1.08	1.21	-0.77
SW2-Ref	5.9	3.5	9.02	0.02	0.04	0.09	0.09	0.45	0.22	1.30	1.99	3.83	1.67	0.37	-1.89	1.93
SW2-Test	5.9	3.5	4.93	0.02	0.02	0.05	0.08	0.32	0.18	0.90	0.83	2.24	1.13	0.37	0.31	-0.63
SW2-Test	6.4	2.5	9.34	0.08	0.03	0.06	0.16	0.35	0.24	1.44	1.18	3.17	1.66	-0.04	-2.42	0.93
SW2-Test	5.6	2.5	7.44	0.08	0.02	0.04	0.10	0.27	0.19	1.03	0.86	2.84	1.33	-0.36	-0.68	0.00
SW2-Test	7.7	3.3	6.46	0.04	0.05	0.06	0.09	0.38	0.25	0.77	0.89	2.35	1.17	0.91	-1.21	-0.40
SW2-Test	7.3	2.8	8.28	0.03	0.07	0.07	0.09	0.48	0.27	1.14	1.17	3.26	1.33	0.48	-2.12	0.63
SW2-Test	6.5	2.3	7.47	0.04	0.05	0.09	0.09	0.21	0.13	1.01	0.91	2.60	1.26	-0.15	-0.69	-0.13
SW2-Test	6.9	3	6.10	0.01	0.02	0.04	0.06	0.28	0.15	0.78	0.85	2.13	0.85	0.47	1.00	-1.07
SW2-Test	7	3.1	12.69	0.08	0.05	0.10	0.13	0.41	0.29	1.69	1.75	5.73	1.70	0.57	-3.53	2.34
SW3-Ref	5.5	2.9	7.54	0.09	0.05	0.05	0.15	0.67	0.24	1.43	1.62	3.08	1.76	-0.14	-2.59	1.42
SW3-Ref	8.1	2.1	4.02	0.02	0.02	0.03	0.10	0.61	0.22	0.98	1.45	2.00	0.69	0.21	-0.09	-0.70
SW3-Ref	7.8	3.4	5.94	0.07	0.02	0.03	0.11	0.31	0.15	0.99	1.27	2.17	1.13	1.00	-0.02	-0.05
SW3-Ref	5.9	3.1	4.75	0.02	0.01	0.03	0.09	0.34	0.14	1.04	1.30	1.70	0.75	0.15	0.77	-0.96
SW3-Ref	7	4.5	6.34	0.04	0.05	0.05	0.11	0.33	0.18	1.25	1.62	2.65	0.96	1.24	-1.11	0.32
SW3-Ref	7.7	4.6	7.55	0.03	0.04	0.05	0.16	0.46	0.19	1.38	1.80	3.09	1.18	1.51	-1.78	0.99
SW3-Ref	6.5	3.3	5.84	0.03	0.03	0.04	0.11	0.34	0.18	1.13	1.39	2.68	1.13	0.50	-0.51	0.36
SW3-Ref	4.5	2.2	6.33	0.02	0.03	0.03	0.11	0.31	0.17	1.25	1.51	3.29	0.85	-1.12	-0.30	0.35

Continued Table S1

SW3-Test	7.7	3.3	6.41	0.02	0.07	0.04	0.09	0.17	0.13	1.15	1.22	2.27	1.14	0.91	0.04	-0.04
SW3-Test	7.6	3.1	5.80	0.02	0.02	0.02	0.09	0.32	0.15	0.89	1.24	2.38	0.87	0.77	0.59	-0.34
SW3-Test	7.5	3.6	6.04	0.04	0.01	0.02	0.08	0.33	0.13	0.99	1.29	2.11	1.05	1.00	0.66	-0.18
SW3-Test	7	3.5	7.01	0.02	0.04	0.06	0.13	0.35	0.16	1.23	1.47	1.90	1.01	0.79	-1.16	-0.19
SW3-Test	5.7	3.3	7.65	0.03	0.03	0.03	0.10	0.30	0.13	1.36	1.60	2.64	1.48	0.18	-0.31	0.93
SW3-Test	7.6	3.7	5.55	0.04	0.03	0.04	0.10	0.37	0.15	1.17	1.68	2.67	1.23	1.09	-0.43	0.75
SW3-Test	6.2	2.5	7.50	0.04	0.05	0.06	0.12	0.47	0.19	1.45	1.90	3.16	1.30	-0.12	-1.93	1.23
SW3-Test	6.3	3.8	5.69	0.02	0.03	0.04	0.10	0.27	0.13	0.95	1.30	1.89	1.15	0.68	0.43	-0.20

III

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ORIGINAL RESEARCH
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Rainbow Trout (*Oncorhynchus Mykiss*) Intestinal Epithelial Cells as a Model for Studying Gut Immune Function and Effects of Functional Feed Ingredients

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The objective of this study was to evaluate the suitability of the rainbow trout intestinal epithelial cell line (RTgutGC) as an *in vitro* model for studies of gut immune function and effects of functional feed ingredients. Effects of lipopolysaccharide (LPS) and three functional feed ingredients [nucleotides, mannanoligosaccharides (MOS), and beta-glucans] were evaluated in RTgutGC cells grown on conventional culture plates and transwell membranes. Permeation of fluorescently-labeled albumin, transepithelial electrical resistance (TEER), and tight junction protein expression confirmed the barrier function of the cells. Brush border membrane enzyme activities [leucine aminopeptidase (LAP) and maltase] were detected in the RTgutGC cells but activity levels were not modulated by any of the exposures. Immune related genes were expressed at comparable relative basal levels as these in rainbow trout distal intestine. LPS produced markedly elevated gene expression levels of the pro-inflammatory cytokines *il1b*, *il6*, *il8*, and *tnfa* but had no effect on ROS production. Immunostaining demonstrated increased F-actin contents after LPS exposure. Among the functional feed ingredients, MOS seemed to be the most potent modulator of RTgutGC immune and barrier function. MOS significantly increased albumin permeation and *il1b*, *il6*, *il8*, *tnfa*, and *tgfb* expression, but suppressed ROS production, cell proliferation and *myd88* expression. Induced levels of *il1b* and *il8* were also observed after treatment with nucleotides and beta-glucans. For barrier function related genes, all treatments up-regulated the expression of *cldn3* and suppressed *cdh1* levels. Beta-glucans increased TEER levels and F-actin content. Collectively, the present study has provided new information on how functional ingredients commonly applied in aquafeeds can affect intestinal epithelial function in fish. Our findings

suggest that RTgutGC cells possess characteristic features of functional intestinal epithelial cells indicating a potential for use as an efficient *in vitro* model to evaluate effects of bioactive feed ingredients on gut immune and barrier functions and their underlying cellular mechanisms.

Keywords: RTgutGC, *in vitro* model, lipopolysaccharide, functional feed ingredients, mucosal immune responses, gut barrier

INTRODUCTION

The fish intestine is a multifunctional organ responsible for key physiological processes such as digestion, absorption of nutrients, and osmoregulation (1). Furthermore, the intestine has an important immunological role and constitutes a physical barrier against pathogens (1). In order to secure optimal gut health and function in farmed fish, there is now particular focus on various feed additives including functional feed ingredients that are branded not only in terms of their nutritional value, but also based on their health promoting and disease preventing properties. These functional feed ingredients could include intact microbes (e.g., probiotic organisms), mixed or purified extracts from microbial or plant structural components [e.g., mannanoligosaccarides (MOS), beta-glucans], metabolites (e.g., nucleotides) or even conventional nutrients, if their dietary inclusion is higher than the animal's requirement. Functional feeds are typically applied during predicted stressful events or challenging farming conditions, such as grading, sea water transfer, vaccination, and during critical life stages to help the animal ward off pathogens and secure good health (2). Functional feed ingredients are generally believed to exert their main actions locally within the gut, and may have direct modulatory effects on gut microbiota (3), gut barrier, immune, and/or metabolic functions (4–7). For example, nucleotides are of crucial importance for a whole range of normal intestinal functions, such as growth, nutrient metabolism, immune system, tissue repair, and development (8). Beta-glucans can increase cellular and humoral immune responses in immune cells and epithelial tissues of fish (9–11). MOS as an immune modulator has a close relation to pathogen colonization blocking and immune system regulation, as well as improving intestinal morphology and the epithelial brush border (10, 12, 13).

Current knowledge regarding mechanisms underlying effects of functional feed ingredients on fish gut health and function is, however, limited largely due to a lack of targeted research tools. The use of *in vitro* approaches, such as appropriate cell lines, would facilitate further research on basic functions of the digestive tract and effects of functional feed ingredients on host intestinal immune, barrier and digestive function. It would also reduce the current dependence on large-scale feeding trials, thus contributing to a shift toward 3R studies within fish nutrition research. In mammalian research, intestinal cell lines have proven to be valuable tools for exploration of basic mechanisms of gut function and health and interactions with dietary components. For example, nucleotide supplements in human Caco-2 and rat IEC-6 cell lines have been observed to strengthen intestinal maturation and growth (14). Beta-glucans and plant flavonoids can suppress nuclear factor-kB transactivation, regulate immune response, and strengthen intestinal epithelial barrier function in human Caco-2 cells (15–17).

Until recently, no relevant intestinal cell lines from fish have been available, but promising cultures have been established based on the rainbow trout (*Oncorhynchus mykiss*) intestinal derived cell line RTgutGC (18). Since their initial isolation, RTgutGC cells have been relatively well-characterized and are now routinely grown as monolayers on permeable supports, leading to a two-compartment intestinal barrier model consisting of a polarized epithelium. The system is divided into an upper (apical) and a lower (basolateral) compartment, thereby mimicking the intestinal lumen and the portal blood, respectively. Reported structural and functional features of the RTgutGC cells include tight junction and desmosome formation between adjacent cells, development of transepithelial resistance and polarization over time to exhibit epithelial and brush border characteristics (18–20). The cell line has, as such, been proposed as a physiologically adequate fish intestinal epithelial model, equivalent to the Caco-2 cell line for human intestinal epithelium (20, 21), and has been used recently in studies on fish intestinal immune and barrier function (18, 22, 23).

The objective of this study was to evaluate the suitability of the RTgutGC cells as an *in vitro* model for studies of gut immune function and effects of functional feed ingredients. Effects of a prototype pathogen-associated molecular pattern (PAMP), lipopolysaccharide (LPS), and three functional ingredients commonly applied in commercial fish feeds (nucleotides, MOS, and beta-glucans) were evaluated by diverse analyses, including cell viability measurements and proliferation, brush border digestive enzyme activity, barrier function, ROS production, morphology, and relevant gene and protein expression.

MATERIALS AND METHODS

RTgutGC Cell Culture

Routine RTgutGC cell cultivation was based on the description by Kawano et al. (18). Briefly, RTgutGC cells were cultured in 75-cm^2 flasks (TPP, Trasadingen, Switzerland). L-15 complete medium (L-15/C), i.e., Leibovitz's L-15 medium without Phenolred (21083, Gibco Invitrogen, Basel, Switzerland) supplemented with 10% bovine serum (F7524, Sigma Aldrich, Buchs, Switzerland) and gentamicin (15710-049, Invitrogen, Basel, Switzerland) with a final concentration of 100 μg/mL, was used to culture cells in a 20°C incubator under normal atmosphere. Cells were split in a 1:2 ratio using trypsin (0.25% in PBS w/o Ca^{2+},

Mg^{2+}; L0910; Biowest; Nuaillé, France) after reaching confluency.

For cells grown on conventional culture plates without inserts, 1 mL or 3.5 mL cell suspensions (1.5×10^5 cells/mL, 78,947 cells per cm^2 for 24-well plates and 54,688 cells per cm^2 for 6-well plates) were seeded in 24-well (No.662160, Greiner-Bio-one, Frickenhausen, Germany) or 6-well plates (No. 657960, Greiner-Bio-one, Frickenhausen, Germany), respectively, and were cultured to reach at least 80 % confluency before use (3–4 days).

For the two-compartment intestinal barrier model, RTgutGC cells were cultured as described previously (19, 20). Briefly, 24-well plates with 33.6 mm^2 transwell inserts (No. 662 630, Greiner-Bio-one, Frickenhausen, Germany) and 6-well plates with 425.4 mm^2 transwell inserts (No. 657 630, Greiner-Bio-one, Frickenhausen, Germany) with pore sizes of 3 μm were used to simulate gut lumen (apical /upper chamber) and portal blood (basolateral /lower chamber). Cells were seeded adding 300 μL or 3.5 mL cell suspension (8×10^4 cells/mL, 71,429 cells per cm^2 for 24-well plates and 65,820 cells per cm^2 for 6-well plates) in the apical chamber of 24-well or 6-well plates, respectively. Then, 1 or 3.5 mL of L-15/C were added into the basolateral chamber of 24-well or 6-well plates, respectively. The apical and basolateral medium was changed once per week for a total of 28 days.

Exposure Design

Stock solutions were prepared for LPS and the functional ingredients. LPS (L2630, Sigma, Norway) stock solution was prepared to 1 mg/mL in L15/ex medium. The L15/ex medium contains only the inorganic salts, galactose, and pyruvate concentrations of L-15 (24). Nucleotides (T25-1KT, Sigma, Norway) stock solution was prepared to 10 mg/mL using milliQ water. MOS (Active MOS extracted from yeast, Biorigin, São Paulo, Brazil) stock solution was prepared to 20 mg/mL using sterile PBS, and then sonicated in a water bath (30 s/3 times) and centrifuged (500 × g/ 5 min). The supernatant was subsequently transferred into new vials and stored at −20°C according to previous descriptions (13). Beta-glucans (G5011, Sigma, Norway) stock solution was prepared to 2 mg/mL in sterile PBS according to previous reports (23).

For all exposure tests performed with the two-compartment intestinal barrier model, the stock solutions of LPS and the functional ingredients were diluted in mucosal saline, prepared according to Genz et al. (25) (**Supplementary Table 1**), and added to the apical chamber in order to mimic intestinal lumen conditions. Before performing the exposure tests with LPS and the functional ingredients, L-15/C and mucosal saline acted as exposure medium for RTgutGC cells to evaluate whether the mucosal saline affected cell viability. For exposure tests performed with conventional plates, working solutions were prepared by diluting in mucosal saline or L15 medium, depending on the analytical assay as specified below. To select final working concentrations for further analysis, LPS and the functional ingredients were tested at a range of different concentrations in 6 h exposures in 24-well-conventional plates without inserts (**Supplementary Table 2**).

Assessment of Cell Viability

Alamar Blue (AB, DAL1025, Invitrogen, Basel, Switzerland) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM, C1345, Invitrogen, Basel, Switzerland) were used to measure cell viability (24, 26). AB was used to measure cell metabolic activity, whereas CFDA-AM was used to measure cell membrane integrity. After 6 h of incubation, stimulant working solutions were discarded, cells were washed twice using 1 mL PBS and subsequently, a volume of 400 μL of fresh AB and CFDA-AM were added to each well. The plates were then incubated at 20°C for 30 min in the dark before measurement. The Cytation 3 plate reader (Bio Tek Instruments, Winooski, USA) was used to measure the fluorescence of AB ($\lambda ex = 530$ nm; $\lambda ex = 595$ nm) and CFDA-AM ($\lambda ex = 493$ nm; $\lambda ex = 541$ nm).

Measurement of Transepithelial Electrical Resistance (TEER)

As a quality measure of monolayer formation, TEER was measured in RTgutGC cells grown in 24-well-culture plates with membrane inserts at day 1, 7, 14, and 28. Additionally, TEER was measured in RTgutGC cells exposed to LPS and the functional ingredients for 6 h after 28 days of culture on transwell membrane inserts in 6-well plates. TEER levels were measured using an EVOM Voltohmmeter with STX2 electrode and Endohm-6 electrode (World Precision Instruments, Berlin, Germany) as described by Geppert et al. (19). TEER was calculated by subtracting the values without cells from the values with cells. TEER values were given as $\Omega \times cm^2$.

Brush Border Membrane Enzyme Activity

After 3–4 days of culture on conventional 24-well plates, RTgutGC cells were exposed to LPS and the functional ingredients for 6 h. After discarding the mucosal saline with LPS or functional ingredients, cells were harvested by trypsination and centrifugation. Cell pellets were reconstituted in 1 mL ice-cold 2 mM Tris/50 mM mannitol pH 7.1, containing phenyl-methyl-sulphonyl fluoride (P-7626, Sigma, Norway) as serine protease inhibitor. Brush border membrane enzyme activities, i.e., leucine amino peptidase (LAP) and maltase, were subsequently measured. LAP activity was analyzed colorimetrically with a commercial kit (NO. 251, Sigma, Norway) using L-leucine-β-naphthylamide as substrate according to the methods described by Krogdahl et al. (27). Maltase activity was measured using maltose as substrate according to the description of Dahlquist (28). Total protein concentrations were determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany). Enzyme activities were expressed as mol substrate hydrolysed per hour per mg protein.

Albumin Translocation Assay

After 28 days of culture on transwell-membrane inserts in 6-well plates, RTgutGC cells with an initial seeding density of 8×10^4 cells/mL (65,820 cells per cm^2) were exposed to LPS and the functional ingredients for 6 h. The permeation of fluorescent-labeled albumin was then used to evaluate the barrier potential of the cells. 20 μL albumin (Alexa FluorTM 488 Bovine Serum Albumin, Thermo Fisher Scientific, USA) was added into the

apical chamber of each well, and 250 μL of culture medium was collected from the basolateral chamber at the following intervals: 10, 30, 45, 60, and 90 min and temporary stored in the dark at 20 °C. After collecting all the samples, 100 μL of each sample was added to a 96- well black plate (M5061-40EA, Sigma, Norway) in duplicate, and fluorescence was measured using a Cytation 3 plate reader (Bio Tek Instruments, Winooski, USA) equipped with a 490 excitation and 525 emission filter.

Quantitative Real Time PCR (qPCR)

After 28 days of culture on transwell membrane inserts in 6-well plates, RTgutGC cells were exposed to LPS and the functional ingredients for 6 h, and subsequently harvested for gene expression profiling. After discarding the mucosal saline with LPS or functional ingredients, 1 mL of TRIzol (Invitrogen, Thermo Fisher Scientific, USA) was added to each apical chamber. Cells were collected by scarping and flushing the membrane inserts 10 times with the TRIzol solution. The cell homogenate was transferred into a 1.5 mL Eppendorf tube, snap frozen in liquid N_2 and subsequently stored at −80 °C until RNA extraction. Gene expression levels in RTgutGC cells were compared with those of rainbow trout tissues by using total RNA samples from liver, pyloric, mid and distal intestine obtained from a fresh-water stage female rainbow trout as previously described (29). RNA was subsequently purified using a PureLink RNA mini Kit (Invitrogen, Thermo Fisher Scientific, USA). RNA purity and concentration were measured using an Epoch Microplate Spectrophotometer (BioTeK Instruments, Winooski, USA). The RNA integrity was verified using a 2100 Bioanalyzer in combination with RNA Nano Chip (Agilent Technologies, Santa Clara, USA). First-strand complementary DNA was synthesized from 1.0 μg total RNA from all samples using SuperScript IV VILO Master Mix (Invitrogen™, ThermoFisher Scientific). Negative controls were performed in parallel by omitting RNA or enzyme.

Twelve target genes with important functions related to immunity, barrier function and metabolism were profiled. The qPCR primers were designed using Primer3web software version 4.0.0 (http://primer3.ut.ee/) or obtained from the literature. Primer details are shown in **Supplementary Table 3**. All primer pairs were first used in gradient reactions in order to determine optimal annealing temperatures. To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and visual inspection of the PCR products by agarose gel electrophoresis. PCR eﬃciency for each gene assay was determined using 2-fold serial dilutions of randomly pooled complementary DNA. The expressions of individual gene targets were analyzed using the LightCycler 96 (Roche Diagnostics, Basel, Switzerland). Each 10 μl DNA amplification reaction contained 2 μl PCR grade water, 2 μl of 1:10 diluted complementary DNA template, 5 μl LightCycler 480 SYBR Green I Master (Roche Diagnostics) and 0.5 μl (10 mM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no-template control. The three-step qPCR run included an enzyme activation step at 95°C (5 min), forty to forty-five cycles at 95°C (10 s), 60°C (10 s), and 72°C (15 s) and a melting curve step. Target gene expression was normalized to the geometric average of beta-actin (*actb*) and ribosomal protein s20 (*rps20*) after confirming reference gene intra- and interspecific stability (30). Mean normalized expression of the target genes was calculated from raw Cq values by relative quantification (31).

Cell Proliferation Assay

The ability of RTgutGC cells to close a gap during exposure to LPS, MOS and beta-glucans was investigated in a cell proliferation assay by using 2-well-culture inserts (80241, Ibidi GmbH, Martinsried, Germany). The inserts were placed on a conventional cell culture surface, i.e., a μ-Dish 35 mm (81156, Ibidi GmbH, Martinsried, Germany) creating two wells, which were separated by a rubber partition. Approximately 10,000 cells in 70 μL L-15/C were seeded into each well. The cultures were incubated at 20 °C for 2 days until confluence. Then, the rubber partition was removed to create a 500 μm gap between the cells. Immediately, LPS (50 μg/mL), MOS (4 mg/mL), beta-glucans (100 μg/mL) and PBS (control), all dissolved in L-15 medium, were added to the cultures and phase contrast pictures were captured at day 0, 1, 2, and 4 (or until the gap was closed) using a ZEISS Axio microscope (with Axiocam 105 color). The image were processed using ImageJ (32). In brief, all images were first adjusted using Adjust tool for achieving a clear contrast between the cell-free area and area covered by cells. Subsequently, the cell-free area was measured using Analyze particles tool. The cell proliferation rate was calculated by dividing the cell-free area at each time point with the cell-free area at day 0.

Oxidative Stress Detection and Substrate Uptake Assay

After 3–4 days of culture on conventional 6-well plates, RTgutGC cells were exposed to LPS and the functional ingredients for 6 h. After discarding the mucosal saline with LPS or functional ingredients, cells were harvested by trypsination and centrifugation. Cell pellets were reconstituted in 5% FBS in PBS for ROS generation measurement. CellROX® (C10444, Thermo Fisher, Waltham, USA) reagent was added to the cell suspensions at a final concentration of 5 mM, followed by incubation at room temperature for 30 min. After the incubation, cells were washed three times with ice-cold phosphate buﬀered saline (PBS) and ROS generation was analyzed by flow cytometry (Beckman Coulter Gallios). At least 10,000 events were collected for each sample. Data were analyzed using Kaluza software v.2.1 (Beckman Coulter) and gated using Side scatter (SSC) (granularity) and Forward scatter (FSC) (size) parameters. Discrimination of aggregates from single cells was performed using side scatter-W (SSC-W) vs. side scatter (SSC). ROS was measured at 650/675 nm (FL3).

Fluorescence conjugated Zymosan (Z23373, Thermo Fisher Scientific, USA) and albumin (Alexa FluorTM 488 Bovine Serum Albumin, Thermo Fisher Scientific, USA) were added into culture media at 20 and 12.5 μg/ml, respectively for cells growing in 6-well-conventional plates. Cells were trypsinized and centrifuged after 1, 1.5, and 3 h after adding substrates, respectively. Cell pellets were reconstituted in 5% FBS in PBS before flow cytometry (Beckman Coulter Gallios) was performed to analyze the cells with or without fluorescence at 495/519 nm.

Immunocytochemistry of F-actin Content

For morphological characterization, confocal laser microscopy was used for imaging. RTgutGC cells in L-15/C were seeded in an 8 chamber tissue cultured treated glass Falcon CultureSlide® (Corning, New York, USA) at a density of 150,000 cells per chamber. When reaching 80% confluence, cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS) and treated with LPS (50 μg/mL), MOS (4 mg/mL), beta-glucans (100 μg/mL), and PBS (control), all dissolved in L-15 medium. After 6 h, cells were washed with DPBS and fixed with 3% paraformaldehyde (Sigma-Aldrich) for 20 min at 4°C. Following fixation, the cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, USA) for 10 min at room temperature. Cells were then incubated in blocking buffer (BB) (10% goat serum, 3% bovine serum albumin, and 0.1% Triton X-100 in DPBS) for 1 h at room temperature. Afterwards, cells were incubated with phalloidin (R425, Thermo Fisher) for F-acin staining according to the manufacturer's instruction. After staining, cells were washed three times for 3 min with DBPS and left to air dry. Once dry, plastic chambers were removed from the slides. Three drops of mounting medium, Fluoroshield (Sigma-Aldrich), containing DAPI were added to the slides, followed by covering with coverslip. The image was analyzed by ImageJ software to investigate the morphology change of the cells under different treatments. Three random pictures were taken from cells under respective treatments. Individual cell numbers were counted based on DAPI-stained nuclear numbers manually. F-actin contents were subsequently calculated by the total fluorescence intensity of phalloidin divided by number of the cells.

Protein Expression of E-cadherin, Aquaporin 8, and Hsp70 by Western Blot Analysis

RTgutGC cells were seeded on 6-well plates and grown until confluence before 6 h exposure to LPS (50 μg/mL), MOS (4 mg/mL), beta-glucans (100 μg/mL), and PBS (control), all dissolved in L-15 medium. Cells were harvested by trypsinization and centrifugation and protein was extracted using PARIS Kit (AM 1921, Thermo Fisher) according to the manual. Protein concentrations were measured using Bradford protein assay kit (Bio-Rad, Hercules, California, United States) and 10 or 20 μg of the protein were loaded on SDS page gels. After 40 min electrophoresis at 100 voltage, proteins were transferred to PVDF membranes, blocked with 5% dry milk for 1 h at room temperature, and incubated consecutively with E-cadherin monoclonal antibody (#701134, Thermo Fisher), Heat shock protein 70 (Hsp70) monoclonal antibody (MA3-008, Thermo Fisher), or Aquaporin 8 (Aqp8) polyclonal antibody (kindly provided by Prof. Steffen S. Madsen, Institute of Biology, University of Southern Denmark). After 3 times washing in PBS and incubation of HRP conjugated secondary antibody, the signal was visualized with Bio-Rad Gel Doc system after adding ECL detection reagents (GERPN2209, Sigma-Aldrich) to the membrane. Due to the potential influence of treatments on candidate reference protein expression, the total membrane protein content was visualized with Ponceau S (P3504, Sigma) and used as a qualitative loading control.

Statistical Analysis

All data were tested for normality and variance homogeneity using histogram and "residual by predicted" plot, respectively, using JMP Pro 13.0.0 (SAS Institute, United States). When necessary, the data were transformed to achieve normal distribution. Further statistical analyses and graphics were made using GraphPad Prism 7 (GraphPad Software, La Jolla, California, United States). The flow cytometry figures were made by Kaluza (Beckman Coulter). Data of albumin translocation and cell proliferation rate were analyzed using two-way ANOVA using time and treatment as class variables followed by Dunnett multiple comparisons tests. Other data were analyzed using one-way ANOVA followed by Dunnett multiple comparisons tests. Data were calculated as mean ± SEM of two or three independent experiments with 3 or 4 technical well or insert replicates (depending on analytical assays, see specifications in figure legends). Asterisks denote the level of statistical significance ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

RESULTS

Key Features of RTgutGC Cells Exposed in Mucosal Saline in Conventional Culture Plates and Transwell Membranes

Compared with RTgutGC cells cultivated in L-15/C medium, cells cultivated in mucosal saline maintained above 80% cell viability after 12 h exposure (**Figure 1A**). When RTgutGC cells were grown on transwell membrane inserts in 24-well plates, TEER levels increased steadily and reached about 26 Ω × cm^2 after 4 weeks of culture (**Figure 1B**). After addition of fluorescent albumin to mucosal saline solution in the apical chamber, basolateral fluorescence levels increased steadily with time when no cells were seeded on the membrane whereas with cells, low fluorescence was observed over the 90 min observation period, demonstrating that the RTgutGC cells formed a barrier and strongly attenuated albumin translocation from the apical to the basolateral chamber (**Figure 1C**). Confocal fluorescence microscopy images of RTgutGC cells grown on conventional culture plates illustrated presence of the tight junction protein Claudin 3 (red) and the nuclei (blue) (**Figure 1D**).We also investigated the uptake of albumin and zymozan in RTgutGC cells, grown on conventional support, as a character of functional enterocytes. During a time course of 3 h, albumin uptake into RTgutGC cells increased (**Figure 1E**). However, RTgutGC cells did not take up zymosan as shown in **Figure 1F**.

Effects of LPS and Functional Ingredients

Cell Viability

Using a cell viability cut-off level of 80% compared to control cells, 6 h of exposure to 50 μg/mL LPS (**Figure 2A**), 75 μg/mL nucleotides (**Figure 2B**), 4 mg/mL MOS (**Figure 2C**) and 100 μg/mL beta-glucans (**Figure 2D**) were selected as final working concentration for further analysis.

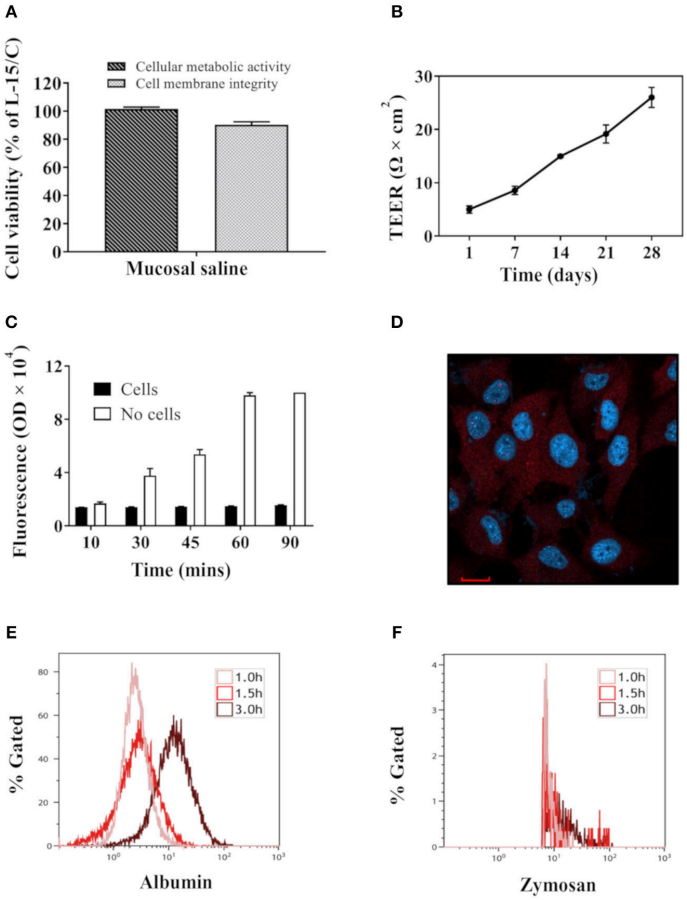

FIGURE 1 | Key features of RTgutGC cells grown on conventional culture plates or transwell membranes. **(A)** Viability of RTgutGC cells in 24-well-culture plates with 1.5×10^5 cells/mL (78,947 cells per cm^2) exposed to mucosal saline for 12 h. **(B)** TEER of RTgutGC cells grown up to 4 weeks in 24-well-culture plates with membrane inserts at initial density of 8×10^4 cells/mL (71,429 cells per cm^2). **(C)** Fluorescent levels in basolateral media after fluorescent albumin exposure into apical chamber in 24-well-transwell membrane plates with or without RTgutGC cells. **(D)** Confocal fluorescence microscopy images of the tight junction protein claudin 3 (red) and the nuclei (blue) in RTgutGC cells grown on conventional culture plates. **(E,F)** Uptake of albumin **(E)** and zymosan **(F)** during the 3 h exposure time with cells cultured in conventional 6-well plates. For both panels **(E,F)**, X axis shows the fluorescence signal from albumin or zymosan in cells. Y axis shows the percentage of albumin/zymosan positive cells out of total live cell population. Data represent mean ± SEM of two independent experiments with 3–4 technical replicates each (wells or inserts). Scale bar = 100 μm.

TEER and Albumin Translocation

After 6 h of exposure to beta-glucans, TEER levels increased significantly compared to control (**Figure 3**, $P < 0.05$). Other treatments had no effect on TEER levels (**Figure 3**, original TEER values seen in **Supplemental Figure 1**).

After 6 h of exposure to MOS increases in basolateral albumin fluorescent levels were observed compared to control cells, significantly at 30 and 60 min time points ($P < 0.05$). No significant effects on the fluorescent level were observed for other treatments (**Figure 4**, $P > 0.05$).

Brush Border Membrane Enzymatic Activity

Brush border membrane enzyme activities (LAP and maltase) were detected in the RTgutGC cells. There were no significant effects of LPS or any of the functional ingredients on LAP (**Figure 5A**) or maltase (**Figure 5B**) activities ($P > 0.05$).

Gene Expression

LPS exposure resulted in markedly increased mRNA levels of several pro-inflammatory cytokines, including interleukin 1β (*il1b*), interleukin 6 (*il6*), interleukin 8 (*il8*), and tumor

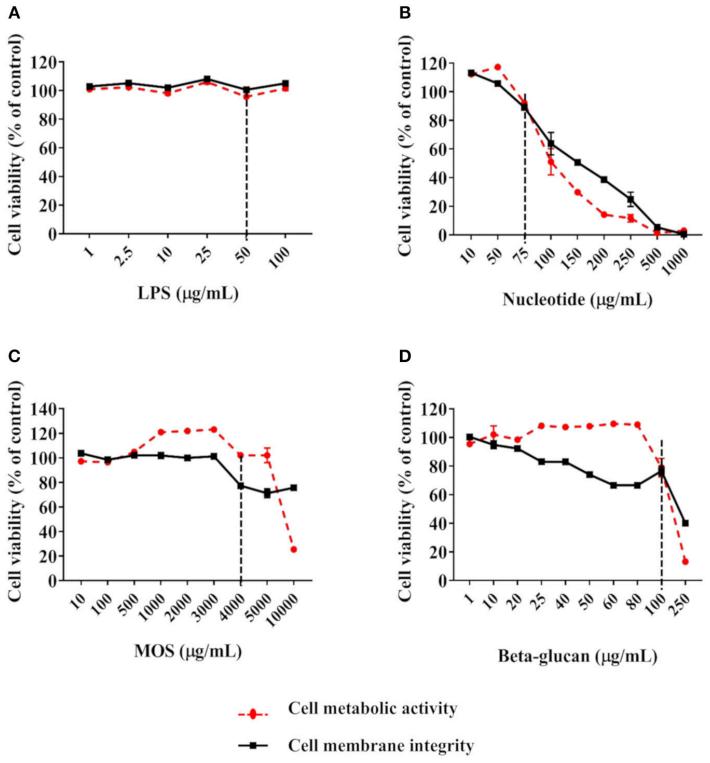

FIGURE 2 | Viability of RTgutGC cells exposed to LPS **(A)**, nucleotides **(B)**, MOS **(C)**, and beta-glucan **(D)**. Breaking lines indicate cut-off level of about 80% cell viability compared to control cells. For LPS, data represent mean ± SEM of two independent experiments with 3 technical well-replicates. For nucleotides, MOS and beta-glucan, two independent experiments with 3 technical well-replicates each and concentrations as shown in **Supplementary Table 1** were conducted. For concentrations evaluated in both experiments, data represent mean ± SEM of the two experiments. For concentrations only evaluated in one of the experiments, single mean values are plotted.

necrosis factor alpha (*tnfa*). Furthermore, LPS up-regulated the expression of the tight junction gene Claudin 3 (*cldn3*, $P < 0.001$), but suppressed the intestinal alkaline phosphatase (*ialp)* expression (**Figure 6**, $P < 0.05$).

Pro-inflammatory cytokine genes (*il1b* and *il8*) were significantly increased after exposing cells to functional ingredients, especially MOS ($P < 0.01$). MOS also produced a significant up-regulation of transforming growth factor beta (*tgfb*) following 6 h of exposure ($P < 0.05$) while expression of myeloid differentiation factor 88 (*myd88*) and proliferating cell nuclear antigen (*pcna*) were significantly decreased (**Figure 6**, $P < 0.01$).

Compared to control, MOS and beta-glucans up-regulated the expression of *cldn3* ($P < 0.01$) while the expression of *ialp* and Na/K-ATPase (*nkaα1b*) decreased significantly following exposure to MOS (**Figure 6**, $P < 0.05$). There was also a significant decrease in the expression of E-cadherin (*cdh1*) after exposure to the different functional ingredients ($P < 0.05$). Gene expression levels of the bile acid transporter solute carrier family 10 member 2 (*slc10a2*) in RTgutGC cells increased significantly after exposure to nucleotides and MOS ($P < 0.05$).

In general, immune genes were expressed at comparable relative basal levels in RTgutGC cells as in rainbow trout distal intestinal tissue, whereas most genes related to barrier function and metabolism showed lower relative expression (**Supplementary Table 3**). Overall, nucleotides produced little or no effect on analytical endpoints related to barrier function and gene expression. In order to reduce costs, we therefore chose to omit nucleotide exposures in the additional analyses outlined below.

Cell Proliferation

In control cells, the gap area of the culture wells was fully closed by day 4 (**Figure 7**). When treated with LPS or beta-glucans, cells were able to close the gap in a similar pace as in the control cells.

In contrast, MOS treatment reduced the cell proliferation and consequently the gap closure rate to <50% at day 4 as shown in **Figure 7**.

ROS Generation

As shown in **Figures 8A–C**, viable cell numbers were not affected by treatments, while MOS diminished ROS positive cells markedly (96% decreased, $P < 0.001$). Moreover, mean fluorescence intensity of ROS in cells were significantly smaller than in other groups ($P < 0.001$).

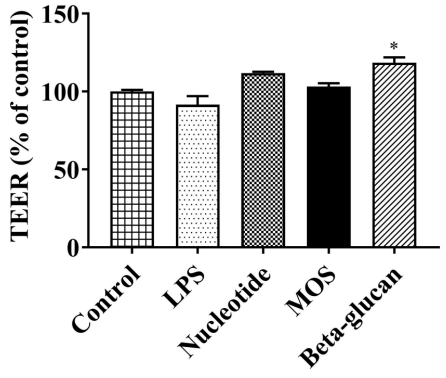

FIGURE 3 | TEER levels of RTgutGC cultures exposed to LPS and functional ingredients in 6-well-transwell membrane inserts for 6 h. Data are expressed as percent of control cells and represent mean + SEM of two independent experiments with 3 technical insert replicates each. Asterisks denote treatment groups statistically different to the control (*$P < 0.05$).

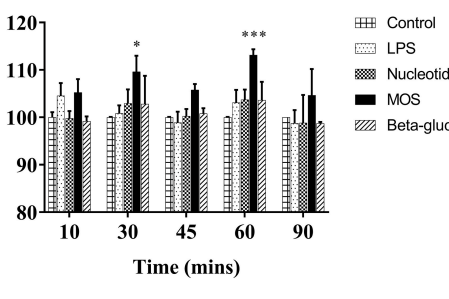

FIGURE 4 | Fluorescent levels in basolateral media after fluorescent albumin exposure into apical chamber in 24-well transwell membrane with RTgutGC cells exposed to LPS and functional ingredients for 6 h. Data are expressed as percent of control cells and represent mean + SEM of two independent experiments with 3 technical insert replicates each. Asterisks denote treatment groups statistically different to the control at the same time point (*$P < 0.05$, ***$P < 0.001$).

F-actin Content and E-cadherin, Aquaporin 8, and Hsp70 Protein Expression

As shown in **Figures 9A,B**, intracellular F-actin contents were significantly increased in LPS and beta-glucan groups ($P < 0.01$), while MOS treated cells remained at control levels. Western blot analyses demonstrated that expressions levels of Aqp8 and Hsp70 were not influenced by any of the treatments. E-cadherin expression was increased in cells treated with LPS, but decreased in beta-glucan and MOS treated groups ($P < 0.01$) (**Figure 10**).

DISCUSSION

Well-characterized *in vitro* model systems offer many benefits for screening purposes, given their simplicity and relative inexpensiveness compared to experiments using live animals. They could also serve as essential tools to increase the knowledge of cellular and molecular mechanisms underlying effects observed in animal trials. In the present work, we have continued the ongoing characterization of the first established intestinal epithelial cell line from fish, RTgutGC (18) and evaluated its suitability as an *in vitro* model for studies of effects of LPS and functional feed ingredients.

Functional Characterization of RTgutGC Cells

Based on previously established RTgutGC cell features (18–23, 33), we first confirmed the viability and barrier function of the RTgutGC cells when grown on transwell membranes. Barrier formation was assessed by TEER measurements and fluorescent albumin translocation from the apical to basolateral cell chamber. TEER levels in the present study were comparable to those reported previously (19, 20). We also observed a strong and time-dependent increase in basolateral fluorescence of albumin in wells without cells, whereas low and stable values were observed for wells with cells. Thus, our observations confirmed earlier reports (20, 33) demonstrating that RTgutGC cells grown on permeable inserts strongly attenuate fluorescent model molecules' translocation from apical to basolateral chamber. RTgutGC barrier function was further supported by related gene and protein expression (*cldn3*, *cdh1*, Claudin 3) as previously demonstrated (19, 20, 22). We also confirmed the findings by Minghetti and co-workers (20) by demonstrating the viability of the RTgutGC cells when exposed to a buffer designed to mimic the intestinal lumen (25), i.e., mucosal saline. Another indication that the RTgutGC cells function as enterocytes is the presence of brush border membrane enzymatic activity. Previous studies have demonstrated that RTgutGC cells possess alkaline phosphatase activity (18). In the current work, we continued to explore RTgutGC brush border features by measuring activity levels of two important brush border digestive enzymes, i.e., LAP and maltase. Activity of both these enzymes were detected in the RTgutGC cells. Higher LAP activity, but very low maltase activity, were found compared to the results of *in vivo* tests (34). Altogether, the current re-establishment of key barrier and brush border features demonstrates the robustness

of the RTgutGC transwell system and shows that RTgutGC cells develop certain intestinal functions similar to the *in vivo* situation.

Effects of LPS Exposure on RTgutGC

We continued to explore RTgutGC cell immune function by detailed exposures to a prototype PAMP, i.e., LPS. LPS showing no effect on cell viability at concentrations up to 100 μg/mL is in line with previous reports suggesting that fish cells without TLR4/CD14 signaling system may be less responsive to LPS compared to mammalian cells (18, 35, 36). The LPS used in the present study was derived from *E. coli*, and it is possible that LPS isolated from a fish pathogen could be more potent in RTgutGC cells. Anyhow, the final working concentration (50 μg/mL LPS) was clearly sufficient to induce immune-related gene expression responses and influence cell proliferation and F-actin contents of RTgutGC cells in this study.

The epithelial cells of the intestinal tract are in direct contact with the external environment of the gut lumen and must be prepared to mount an immune response against antigens and infections agents of dietary origin. It is well-known that intestinal epithelial cells of teleost fish produce several innate immune defense factors, and they can over-express pro-inflammatory cytokines following a bacterial infection (23). In the present study, LPS produced markedly elevated levels of pro-inflammatory cytokine gene expression (*il1b*, *il6*, *il8,* and *tnfa*). The data point toward RTgutGC immunocompetence, and demonstrate that RTgutGC cells possess the ability and transcriptional apparatus to mount an innate immune response against LPS, a common model PAMP. There are, to our knowledge, no published studies on spatial immune gene expression patterns along the rainbow trout intestinal tract. Given that the RTgutGC cell line was initially isolated from the distal intestine (18), which is believed to be a specific intestinal region for certain mucosal immune functions (29), it is interesting to note that RTgutGC relative immune gene expression were found at comparable levels as in the distal intestine of rainbow trout (**Supplementary Table 3**). Induced immune transcriptional responses to pathogen infection have previously been observed in human intestinal epithelial cells (37). In fish, similar effects of LPS on innate immune related gene expression have been observed also in head kidney leukocytes of rainbow trout (9). Moreover, LPS has been reported to up-regulate *tnfa* gene expression in RTgutGC cells grown on conventional culture plates (18). Intestinal alkaline phosphatase (Ialp) is an important apical brush border enzyme, which has been found to lower the expression of pro-inflammatory cytokines by inhibiting the activation and translocation of their master transcription factor NF-κB (38). LPS is a reported substrate for Ialp (38), and in the current work, LPS suppressed *ialp* expression. This response could reflect the interplay between LPS, Ialp and pro-inflammatory cytokine signaling.

Effects of Functional Feed Ingredients on RTgutGC

Our strategy for determining the final exposure concentrations of the functional ingredients was based on measurements of cell viability. When applied at high concentration, all functional ingredients significantly reduced cell viability in RTgutGC cells. We chose our final exposure concentrations at levels that maintained 80% cell viability as compared to control cells, with the underlying assumption that these cells were in a healthy state and could exert true physiological responses to the functional ingredients. It should be noted that the cell viability assays were performed with cells grown on conventional plates, and we therefore assume similar responses to the stimulants in cell grown on membrane inserts.

In two-compartment epithelial cell *in vitro* systems, increased TEER levels are interpreted as an increase in epithelial barrier tightness. In the present study, beta-glucans increased TEER values, whereas no significant effects were observed for nucleotides or MOS exposure. In contrast, MOS treatment increased albumin translocation across the RTgutGC monolayer, indicative of a reduced barrier function that could be attributed

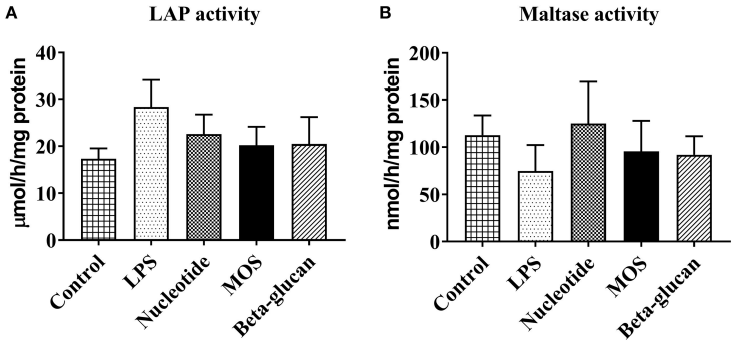

FIGURE 5 | Brush border membrane leucine aminopeptidase (LAP, **A**) and maltase **(B)** activity of RTgutGC cells grown on 24-well-conventional culture plates exposed to LPS and functional ingredients. Data represent mean + SEM of two independent experiments with 3 technical well replicates each.

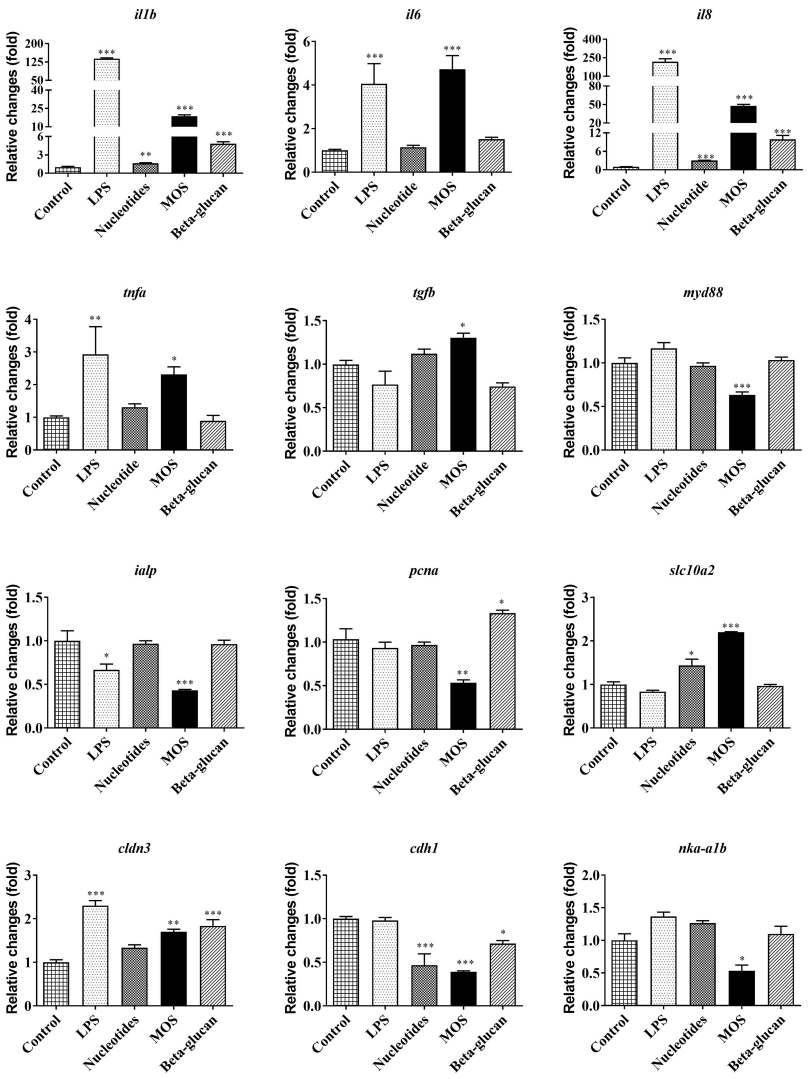

FIGURE 6 | Immune, barrier, and metabolic gene expression in RTgutGC cells grown on 6-well transwell membranes exposed to LPS and functional ingredients. Data are expressed relative to control cell levels and represent mean + SEM of two independent experiments with 3 technical insert replicates each. Asterisks denote treatment groups statistically different to the control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

to alterations in both transcellular and paracellular routes. The relative proportion of trans- and paracellular translocation of albumin remains unknown, and should be explored in future studies, for example by detailed studies of albumin uptake kinetics into RTgutGC cells grown on permeable supports. Of note, we demonstrated that albumin was indeed taken up by the RTgutGC cells when grown on conventional supports, whereas no uptake of the larger molecule zymosan was detected. For junction barrier related gene expression, all functional ingredients suppressed *cdh1* levels and all ingredients except nucleotides increased *cldn3*. The suppressed *cdh1* levels in cells treated with MOS and beta-glucan were also mirrored by decreases at the protein expression level. In Caco-2 cells, decreases in *cldn3* mRNA levels were observed

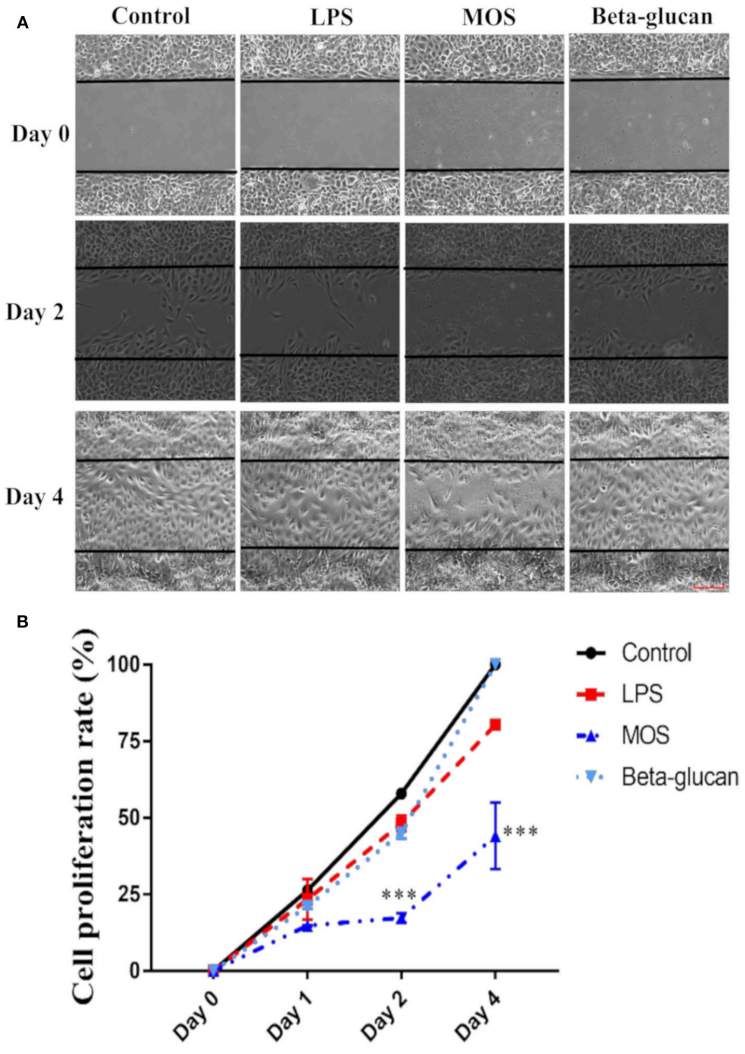

FIGURE 7 | Cell proliferation assay of RTgutGC cells. **(A)** Cells treated with LPS, MOS and beta-glucan at day 0, 2, and 4 after removal of the gap insert. Representative images of three independent experiments are shown. **(B)** Quantification of cell proliferation rate at different time points during treatment. Data represent mean ± SEM of three independent experiments with 3 technical well-replicates each. Asterisks denote treatment groups statistically significant different to the control at the same time point (***$P < 0.001$). Scale bar = 100 μm.

in concert with increase in paracellular permeability and a reduction in TEER (39). Similarly, the observed decreases in adherence junction-related *cdh1* expression would be expected to loosen the junction barrier and increase paracellular permeability. *In vivo*, MOS supplementation to fish has in several independent studies been found to improve microvilli integrity in terms of microvilli density (12) and length (12, 40, 41). In European seabass, MOS treatment enlarged intestinal fold height and reduced gut bacterial translocation, demonstrative of MOS effects on epithelial barrier function (42). Furthermore, beneficial physiological effects on epithelial cells of fish fed MOS could be a result of increasing mucus secretion (43), viscoelasticity of the mucus (44) or induced tight junction closure (ZO-1, occluding or E-cadherin) (45).

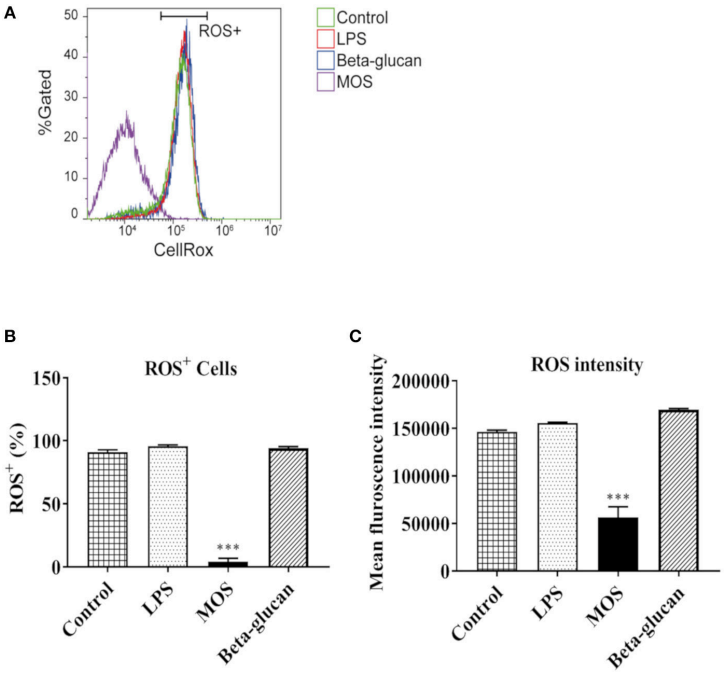

FIGURE 8 | Flow cytometry analysis of reactive oxygen species (ROS) production in RTgutGC cells grown on 6-well conventional plates and exposed to LPS, MOS and beta-glucan for 6 h. **(A)** Representative histograms of ROS dye fluorescence intensity. X axis shows the CellRox fluorescence signal in cells. Y axis shows the percentage of total live cell population. Horizontal bar indicate ROS positive (ROS^+) cells. **(B)** Statistical analysis of ROS^+ cell number under different treatments **(C)** Mean Fluorescent Intensities of ROS dye in cells under different treatments. Data represent mean + SEM of two independent experiments with 3 technical well-replicates each. Asterisks denote treatment groups statistically significant different to the control (***$P < 0.001$).

To our knowledge, there are no published studies of effects of MOS on gut epithelial barrier or tight junction function in rainbow trout. The findings of TEER, albumin translocation, and junction barrier related gene and protein expression in the present study may point to how MOS can act as homeostatic balancer of barrier function *in vivo* and *in vitro* (46).

RTgutGC cell proliferation was assessed by a previously established cell proliferation assay (22). RTgutGC cells had the ability to close the cell free gap in 4 days in this study, which was faster than in a previous report, possibly due to different culture conditions (22). During the 4-day period, MOS strongly reduced the cell proliferation speed compared to control. In addition, MOS significantly suppressed ROS production compared with control cells. ROS plays important roles in homeostasis and cell signaling, and ROS levels typically increase during periods of environmental stress and may cause significant damage to cell structures (47). Whether the MOS-induced decrease in RTgutGC proliferation ability could be a result of reduction in stress fibers and suppressed ROS production as previously reported (48, 49) warrants further investigation. Pcna plays an important role in cell proliferation (50). MOS also down-regulated *pcna* gene expression in the present study, confirming the cell proliferation assay results indicating that MOS inhibited cell proliferation (50).

Functional ingredients are expected to exert immune-modulatory effects in the intestine by regulating the expression of cytokines (2, 7, 10, 51). Among the functional ingredients evaluated in the present work, MOS seemed to be the most potent modulator of RTgutGC immune responses. Specifically, MOS treatment induced levels of pro-inflammatory (*il1b*, *il6*, *il8*, and *tnfa*) and *tgfb* cytokine transcripts, but suppressed *myd88* expression. In particular, the alterations of pro-inflammatory cytokine gene expression and the suppression of *ialp* mirrored the effect of LPS. *In vivo*, dietary MOS in European sea bass can provide protection against *Vibrio alginolyticus* infection (52) and counteract the side effects of soybean meal oil by increasing the mucus cell density and area in the distal intestine and regulating GALT-related genes (i.e. *il6*, *il10*, and *tgfb*) (46). MOS supplementation to rainbow trout was also found

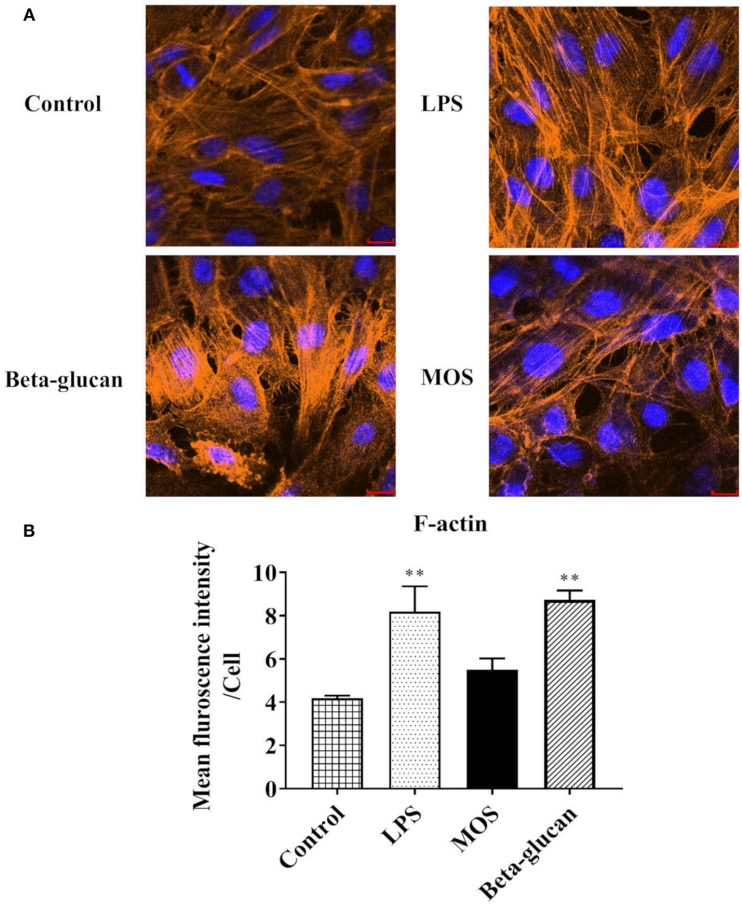

FIGURE 9 | RTgutGC cell morphology after treatments with LPS, MOS, and beta-glucan. **(A)** Staining of cell nuclei (blue) and skeleton (orange) after 6 h exposure to stimulants. **(B)** Mean Fluorescent Intensities (MFI) of RTgutGC skeleton (F-actin). Data represent mean + SEM of three independent experiments, with 3 technical replicates each. Asterisks denote treatment groups statistically significant different to the control (**$P < 0.01$). Scale bar = 100 μm.

to improve lysozyme concentration, classical pathway of complement (APCA and CPCA) (53), microvilli structure and absorptive surface area (12). Whether the immune-modulatory effects induced by MOS in the present study having any relation to the increase in epithelial permeability is a question that clearly warrants attention in future studies. Possibly, the increased permeability could lead to an increased antigen influx that would trigger mucosal immune responses, including modulation of cytokine expression.

Beta-glucan is one of the potent and promising immunostimulants in aquaculture which could be beneficial for growth, disease resistance and immune response of a range of fish species including rainbow trout (54–56). *In vitro*, beta-glucans were found to have positive effects on neutrophil degranulation of fathead minnows (57) and respiratory burst activity of Atlantic salmon (58). In the present study, beta-glucan treatment also produced increased mRNA levels of pro-inflammatory cytokine genes (*il1b* and *il8*). This observation is in agreement with previous studies demonstrating that beta-glucans up-regulated pro-inflammatory cytokine expression in head kidney cells of rainbow trout (9) and increased *il1b* expression in Atlantic cod after challenged with *Vibrio anguillarum* (10). A previous report also found that *il1b* production was induced by cathelicidin-2 variants and *il1b* expression upregulation was elicited by a synergic effect of zymosan and cathelicidin-2 variants in

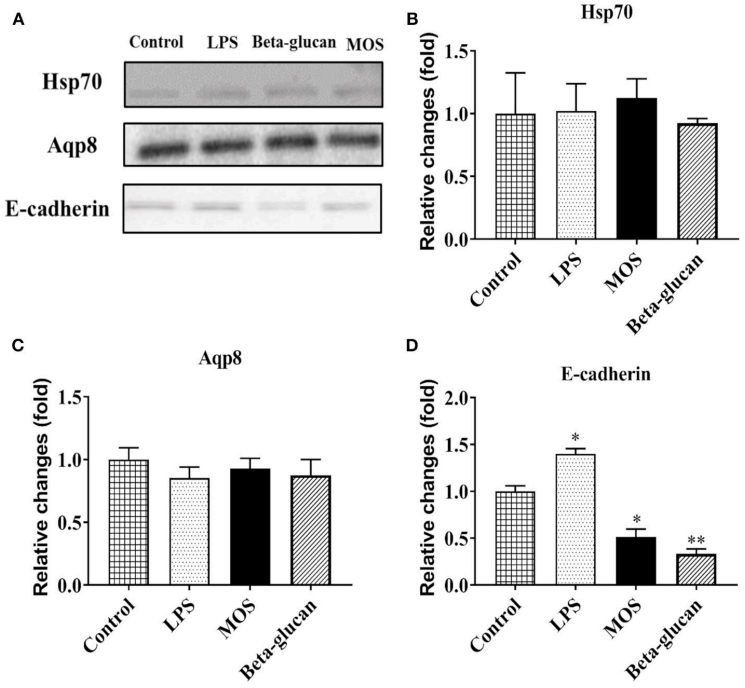

FIGURE 10 | (A) Representative Western blot pictures for target proteins. Statistical analysis of protein expression levels of heat shock protein 70 (hsp70, **B**), aquaporin 8 (Aqp8, **C**), and e-cadherin **(D)**. Data represent mean + SEM from two or three independent experiments with 3 technical replicates each. Asterisks denote treatment groups statistically significant different to the control (*$P < 0.05$, **$P < 0.01$). Full scans of the entire original blots are shown in **Supplementary Figure 2**.

RTgutGC cells (23). Beta-glucan lowered transactivation of NF-κB to stimulate immune response was also found in Caco-2 cells (15). Whether the expression of *il1b* and *il8* is affected by the cathelicidin-2 variants or the activation of NF-κB in RTgutGC still needs to be explored in future studies. *In vivo*, the expression of *il8* was not affected significantly in the distal intestine of Atlantic cod fed beta-glucans (10), which is different from our findings. Available literature suggests that beta-glucans may regulate inflammatory effects in an inconsistent pattern, possibly depending on the differences of composition, dosage, quality, route, and exposure time (11, 23, 54, 59). Nucleotides also produced elevated levels of *il1b* and *il8*, but the degree of response was minor compared to the other functional ingredients evaluated in the current study. Previous *in vivo* tests have found that dietary nucleotides might improve growth, disease resistance against *S. iniae* and pancreatic necrosis, serum alternative complement activity, serum lysozyme activity and crowding stress of rainbow trout (60–62) and influence macrophage activity, respiratory burst activity and expression of *il1b*, *il8,* and *tnfa* in turbot (63). However, the mechanism of growth and immune promotion by nucleotides still need to be identified *in vitro* or *in vivo* tests.

CONCLUSION

An increasing body of literature demonstrates that functional feed ingredients can support intestinal health and reduce disease susceptibility via multiple mechanisms, including direct effects on a variety of intestinal functions, e.g., barrier function, nutrient transport and immune responses (7, 23, 64–66). In fish, knowledge about basic mechanisms of functional ingredients and their interactions with the intestinal tissue is weak and fragmentary. The present study has provided new information on how functional ingredients commonly applied in aquafeeds can affect intestinal epithelial function in fish. Additionally, our study demonstrates the suitability of the RTgutGC transwell system as an alternative to fish feeding experiments for prediction of health effects of functional feeds.

AUTHOR CONTRIBUTIONS

JW, PL, AG, YY, ÅK, and TK: experiment design; JW, PL, AG, LL, YY, and TK: analyses; KS, LM, MØ, ÅK, and TK: supervision; JW and TK: writing, original draft; JW, PL, AG,

LL, YY, KS, LM, MØ, ÅK, and TK: writing, review, and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00152/full#supplementary-material

REFERENCES

1. Grosell M, Farrell AP, Brauner CJ. *Fish Physiology: The Multifunctional Gut of Fish*: London: Academic Press (2010).
2. Kiron V. Fish immune system and its nutritional modulation for preventive health care. *Anim Feed Sci Tech.* (2012) 173:111–33. doi: 10.1016/j.anifeedsci.2011.12.015
3. Ringø E, Zhou Z, Vecino JLG, Wadsworth S, Romero J, Krogdahl Å, et al. Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? *Aquacul Nutr*. (2016) 22:219–82. doi: 10.1111/anu.12346
4. Torrecillas S, Montero D, Izquierdo M. Improved health and growth of fish fed mannan oligosaccharides: potential mode of action. *Fish Shellfish Immunol*. (2014) 36:525–44. doi: 10.1016/j.fsi.2013.12.029
5. Carbone D, Faggio C. Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of *Sparus aurata* and *Dicentrarchus labrax*. *Fish Shellfish Immunol*. (2016) 54:172-8. doi: 10.1016/j.fsi.2016.04.011
6. Merrifield DL, Dimitroglou A, Foey A, Davies SJ, Baker RTM, Bøgwald J, et al. The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture* (2010) 302:1–18. doi: 10.1016/j.aquaculture.2010.02.007
7. Tacchi L, Bickerdike R, Douglas A, Secombes CJ, Martin SA. Transcriptomic responses to functional feeds in Atlantic salmon (*Salmo salar*). *Fish Shellfish Immunol*. (2011) 31:704–15. doi: 10.1016/j.fsi.2011.02.023
8. Gil A. Modulation of the immune response mediated by dietary nucleotides. *Eur J Clin Nutr*. (2002) 56:S1–4. doi: 10.1038/sj.ejcn.1601475
9. Chettri JK, Raida MK, Holten-Andersen L, Kania PW, Buchmann K. PAMP induced expression of immune relevant genes in head kidney leukocytes of rainbow trout (*Oncorhynchus mykiss*). *Dev Comp Immunol*. (2011) 35:476–82. doi: 10.1016/j.dci.2010.12.001
10. Lokesh J, Fernandes JM, Korsnes K, Bergh O, Brinchmann MF, Kiron V. Transcriptional regulation of cytokines in the intestine of Atlantic cod fed yeast derived mannan oligosaccharide or beta-glucan and challenged with *Vibrio anguillarum*. *Fish Shellfish Immunol*. (2012) 33:626–31. doi: 10.1016/j.fsi.2012.06.017
11. Skov J, Kania PW, Holten-Andersen L, Fouz B, Buchmann K. Immunomodulatory effects of dietary beta-1,3-glucan from Euglena gracilis in rainbow trout (*Oncorhynchus mykiss*) immersion vaccinated against *Yersinia ruckeri*. *Fish Shellfish Immunol*. (2012) 33:111–20. doi: 10.1016/j.fsi.2012.04.009
12. Dimitroglou A, Merrifield DL, Moate R, Davies SJ, Spring P, Sweetman J, et al. Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Anim Sci*. (2009) 87:3226–34. doi: 10.2527/jas.2008-1428
13. Hermes RG, Manzanilla EG, Martin-Orue SM, Perez JF, Klasing KC. Influence of dietary ingredients on *in vitro* inflammatory response of intestinal porcine epithelial cells challenged by an enterotoxigenic *Escherichia coli* (K88). *Comp Immunol Microbiol Infect Dis*. (2011) 34:479–88. doi: 10.1016/j.cimid.2011.08.006
14. He Y, Chu SH, Walker WA. Nucleotide supplements alter proliferation and differentiation of cultured human (Caco-2) and rat (IEC-6) intestinal epithelial cells. *J Nutr*. (1993) 123:1017–27. doi: 10.1093/jn/123.6.1017
15. Volman JJ, Helsper JP, Wei S, Baars JJ, van Griensven LJ, Sonnenberg AS, et al. Effects of mushroom-derived beta-glucan-rich polysaccharide extracts on nitric oxide production by bone marrow-derived macrophages and nuclear factor-kappaB transactivation in Caco-2 reporter cells: can effects be explained by structure? *Mol Nutr Food Res*. (2010) 54:268–76. doi:10.1002/mnfr.200900009
16. Amasheh M, Salah S, Amasheh S, Joachim M, Zeitz, M, Fromm M. Quercetin enhances epithelial barrier function and increases claudin-4 expression in Caco-2 cells. *J Nutr*. (2008) 138:1067–73. doi: 10.1093/jn/138.6.1067
17. Amasheh M, Andres S, Amasheh S, Fromm M, Schulzke JD. Barrier effects of nutritional factors. *Ann NY Acad Sci*. (2009) 1165:267–73. doi: 10.1111/j.1749-6632.2009.04063.x
18. Kawano A, Haiduk C, Schirmer K, Hanner R, Lee LEJ, Dixon B, et al. Development of a rainbow trout intestinal epithelial cell line and its response to lipopolysaccharide. *Aquacul Nutr*. (2011) 17:e241–e52. doi: 10.1111/j.1365-2095.2010.00757.x
19. Geppert M, Sigg L, Schirmer K. A novel two-compartment barrier model for investigating nanoparticle transport in fish intestinal epithelial cells. *Environ Sci Nano* (2016) 3:388–95. doi: 10.1039/c5en00226e
20. Minghetti M, Drieschner C, Bramaz N, Schug H, Schirmer K. A fish intestinal epithelial barrier model established from the rainbow trout (*Oncorhynchus mykiss*) cell line, RTgutGC. *Cell Biol Toxicol*. (2017) 33:539–55. doi: 10.1007/s10565-017-9385-x
21. Kawano A. (2009) *Developing and Characterizing a Salmonid Intestinal Epithelial Cell Line for Use in Studies of Inflammation in the Fish Gastrointestinal Tract*. Master's thesis. University of Waterloo.
22. Pumputis PG. (2017) *Development of RTgutGC as a Tool for Fish Feed Development*. Master´s thesis. University of Waterloo.
23. Schmitt P, Wacyk J, Morales-Lange B, Rojas V, Guzman F, Dixon B, et al. Immunomodulatory effect of cathelicidins in response to a beta-glucan in intestinal epithelial cells from rainbow trout. *Dev Comp Immunol*. (2015) 51:160–9. doi: 10.1016/j.dci.2015.03.007
24. Schirmer K, Chan A, Greenberg B, Dixon D, Bols N. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. *Toxi in vitro* (1997) 11:107–19.
25. Genz J, Esbaugh AJ, Grosell M. Intestinal transport following transfer to increased salinity in an anadromous fish (*Oncorhynchus mykiss*). *Comp Biochem Physiol A Mol Integr Physiol*. (2011) 159:150–8. doi: 10.1016/j.cbpa.2011.02.011
26. Schirmer K, Dixon D, Greenberg B, Bols N. Ability of 16 priority PAHs to be directly cytotoxic to a cell line from the rainbow trout gill. *Toxicology* (1998) 127:129–41.
27. Krogdahl A, Bakke-McKellep AM, Baeverfjord G. Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar L.*). *Aquaculture Nutr*. (2003) 9:361–71. doi: 10.1046/j.1365-2095.2003.00264.x
28. Dahlqvist A. Assay of intestinal disaccharidases. *Enzymol Biol Clin*. (1970) 11:52–66.
29. Venold FF, Penn MH, Krogdahl A, Overturf K. Severity of soybean meal induced distal intestinal inflammation, enterocyte proliferation rate, and fatty acid binding protein (Fabp2) level differ between strains of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* (2012) 364:281–92. doi: 10.1016/j.aquaculture.2012.08.035

30. Kortner TM, Valen EC, Kortner H, Marjara IS, Krogdahl A, Bakke AM. Candidate reference genes for quantitative real-time PCR (qPCR) assays during development of a diet-related enteropathy in Atlantic salmon (*Salmo salar L.*) and the potential pitfalls of uncritical use of normalization software tools. *Aquaculture* (2011) 318: 355–63. doi: 10.1016/j.aquaculture.2011.05.038
31. Muller PY, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques* (2002) 32:1372–4.
32. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* (2012) 9:671–5. doi: 10.1038/nmeth.2089
33. Drieschner C, Minghetti M, Wu SM, Renaud P, Schirmer K. Ultrathin alumina membranes as scaffold for epithelial cell culture from the intestine of rainbow trout. *ACS Appl Mater Inter*. (2017) 9: 9496–505. doi: 10.1021/acsami.7b00705
34. Krogdahl Å, Sundby A, Olli JJ. Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) digest and metabolize nutrients differently. Effects of water salinity and dietary starch level. *Aquaculture* (2004) 229:335–60. doi: 10.1016/s0044-8486(03)00396-x
35. Iliev DB, Roach JC, Mackenzie S, Planas JV, Goetz FW. Endotoxin recognition: in fish or not in fish? *FEBS Lett*. (2005) 579:6519–28. doi: 10.1016/j.febslet.2005.10.061
36. Chin AC, Flynn AN, Fedwick JP, Buret AG. The role of caspase-3 in lipopolysaccharide-mediated disruption of intestinal epithelial tight junctions. *Can J Physiol Pharmacol*. (2006) 84:1043–50. doi: 10.1139/y06-056
37. Baldwin DN, Vanchinathan V, Brown PO, Theriot JA. A gene-expression program reflecting the innate immune response of cultured intestinal epithelial cells to infection by *Listeria monocytogenes*. *Genome Biol*. (2002) 4:R2. doi: 10.1186/gb-2002-4-1-r2
38. Fawley J, Gourlay DM. Intestinal alkaline phosphatase: a summary of its role in clinical disease. *J Surg Res*. (2016) 202: 225–34. doi: 10.1016/j.jss.2015.12.008
39. McLaughlin J, Padfield PJ, Burt JPH, O'Neill CA. Ochratoxin A increases permeability through tight junctions by removal of specific claudin isoforms. *Am J Physiol-Cell Physiol*. (2004) 287:C1412–C7. doi: 10.1152/ajpcell.00007
40. Yilmaz E, Genc MA, Genc E. Effects of dietary mannan oligosaccharides on growth, body composition, and intestine and liver histology of rainbow trout, *Oncorhynchus mykiss*. *Isr J Aqua–Bam*. (2007) 59:182.e8.
41. Salze G, McLean E, Schwarz M, Craig S. Dietary mannan oligosaccharide enhances salinity tolerance and gut development of larval cobia. *Aquaculture* (2008) 274:148–52. doi: 10.1016/j.aquaculture.2007.11.008
42. Torrecillas S, Makol A, Benitez-Santana T, Caballero MJ, Montero D, Sweetman J, et al. Reduced gut bacterial translocation in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides (MOS). *Fish Shellfish Immunol*. (2011) 30:674–81. doi: 10.1016/j.fsi.2010.12.020
43. Torrecillas S, Makol A, Caballero MJ, Montero D, GinÉS R, Sweetman J, et al. Improved feed utilization, intestinal mucus production and immune parameters in sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides (MOS). *Aquaculture Nutr*. (2011) 17:223–33. doi: 10.1111/j.1365-2095.2009.00730.x
44. Schroers V, van der Marel M, Neuhaus H, Steinhagen D. Changes of intestinal mucus glycoproteins after peroral application of Aeromonas hydrophila to common carp (*Cyprinus carpio*). *Aquaculture* (2009) 288:184–9. doi: 10.1016/j.aquaculture.2008.12.013
45. Torrecillas S, Makol A, Betancor MB, Montero D, Caballero MJ, Sweetman J, et al. Enhanced intestinal epithelial barrier health status on European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides. *Fish Shellfish Immunol*. (2013) 34:1485–95. doi: 10.1016/j.fsi.2013.03.351
46. Torrecillas S, Montero D, Caballero MJ, Pittman KA, Custodio M, Campo A, et al. Dietary mannan oligosaccharides: counteracting the side effects of soybean meal oil inclusion on european sea bass (*Dicentrarchus labrax*) gut health and skin mucosa mucus production? *Front Immunol*. (2015) 6:397. doi: 10.3389/fimmu.2015.00397
47. Faggio C, Pagano M, Alampi R, Vazzana I, Felice MR. Cytotoxicity, haemolymphatic parameters, and oxidative stress following exposure to sub-lethal concentrations of quaternium-15 in *Mytilus galloprovincialis*. *Aquat Toxical*. (2016) 180:258–65. doi: 10.1016/j.aquatox.2016.10.010
48. Vallenius T. Actin stress fibre subtypes in mesenchymal-migrating cells. *Open Biol*. (2013) 3:130001. doi: 10.1098/rsob.130001
49. Zimmerman NP, Kumar SN, Turner JR, Dwinell MB. Cyclic AMP dysregulates intestinal epithelial cell restitution through PKA and RhoA. *Inflamm Bowel Dis*. (2012) 18:1081–91. doi: 10.1002/ibd.21898
50. Dezfuli BS, Giari L, Lui A, Squerzanti S, Castaldelli G, Shinn AP, et al. Proliferative cell nuclear antigen (PCNA) expression in the intestine of *Salmo trutta trutta* naturally infected with an acanthocephalan. *Parasit Vectors* (2012) 5:198. doi: 10.1186/1756-3305-5-198.
51. Iswarya A, Vaseeharan B, Anjugam M, Gobi N, Divya M, Faggio C. beta-1, 3 glucan binding protein based selenium nanowire enhances the immune status of *Cyprinus carpio* and protection against *Aeromonas hydrophila* infection. *Fish Shellfish Immunol*. (2018) 83:61–75. doi: 10.1016/j.fsi.2018.08.057
52. Torrecillas S, Makol A, Caballero MJ, Montero D, Dhanasiri AK, Sweetman J, et al. Effects on mortality and stress response in European sea bass, *Dicentrarchus labrax* (L.), fed mannan oligosaccharides (MOS) after *Vibrio anguillarum* exposure. *J Fish Dis*. (2012) 35:591–602. doi: 10.1111/j.1365-2761.2012.01384.x
53. Staykov Y, Spring P, Denev S, Sweetman J. Effect of a mannan oligosaccharide on the growth performance and immune status of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Inter*. (2007) 15:153–61. doi: 10.1007/s10499-007-9096-z
54. Meena DK, Das P, Kumar S, Mandal SC, Prusty AK, Singh SK, et al. Beta-glucan: an ideal immunostimulant in aquaculture (a review). *Fish Physiol Biochem*. (2013) 39:431–57. doi: 10.1007/s10695-012-9710-5
55. Verlhac V, Gabaudan J, Obach A, Schuep W, Hole R. Influence of dietary glucan and vitamin C on non-specific and specific immune responses of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* (1996) 143:123–33. doi: 10.1016/0044-8486(95)01238-9
56. Sealey WM, Barrows FT, Hang A, Johansen KA, Overturf K, LaPatra SE, et al. Evaluation of the ability of barley genotypes containing different amounts of beta-glucan to alter growth and disease resistance of rainbow trout *Oncorhynchus mykiss*. *Anim Feed Sci Tec*. (2008) 141:115–28. doi: 10.1016/j.anifeedsci.2007.05.022
57. Palic D, Andreasen CB, Herolt DM, Menzel BW, Roth JA. Immunomodulatory effects of beta-glucan on neutrophil function in fathead minnows (*Pimephales promelas Rafinesque*, 1820). *Dev Comp Immunol*. (2006) 30:817–30. doi: 10.1016/j.dci.2005.11.004
58. Bridle A, Carter C, Morrison R, Nowak B. The effect of β-glucan administration on macrophage respiratory burst activity and Atlantic salmon, *Salmo salar L.*, challenged with amoebic gill disease–evidence of inherent resistance. *J Fish Dis*. (2005) 28:347–56. doi: 10.1111/j.1365-2761.2005.00636.x
59. Ringø E, Erik Olsen R, Gonzalez Vecino JL, Wadsworth S. Use of Immunostimulants and nucleotides in aquaculture: a review. *J Marine Sci Res Dev*. (2012) 2:104. doi: 10.4172/2155-9910.1000104
60. Tahmasebi-Kohyani A, Keyvanshokooh S, Nematollahi A, Mahmoudi N, Pasha-Zanoosi H. Dietary administration of nucleotides to enhance growth, humoral immune responses, and disease resistance of the rainbow trout (*Oncorhynchus mykiss*) fingerlings. *Fish Shellfish Immunol*. (2011) 30:189–93. doi: 10.1016/j.fsi.2010.10.005
61. Leonardi M, Sandino AM, Klempau A. Effect of a nucleotide-enriched diet on the immune system, plasma cortisol levels and resistance to infectious pancreatic necrosis (IPN) in juvenile rainbow trout (*Oncorhynchus mykiss*). *Bull Eur Assoc Fish Pathol*. (2003) 23:52–9.
62. Tahmasebi-Kohyani A, Keyvanshokooh S, Nematollahi A, Mahmoudi N, Pasha-Zanoosi H. Effects of dietary nucleotides supplementation on rainbow trout (*Oncorhynchus mykiss*) performance and acute stress response. *Fish Physiol Biochem*. (2012) 38:431–40. doi: 10.1007/s10695-011-9524-x
63. Low C, Wadsworth S, Burrells C, Secombes CJ. Expression of immune genes in turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet. *Aquaculture* (2003) 221:23–40. doi: 10.1016/S0044-8486(03)00022-X

64. Wismar R, Brix S, Frokiaer H, Laerke HN. Dietary fibers as immunoregulatory compounds in health and disease. *Ann NY Acad Sci*. (2010) 1190:70–85. doi: 10.1111/j.1749-6632.2009.05256.x
65. Volman JJ, Ramakers JD, Plat J. Dietary modulation of immune function by beta-glucans. *Phys Behav*. (2008) 94:276–84. doi: 10.1016/j.physbeh.2007.11.045
66. Trischitta F, Faggio C. Effect of the flavonol quercetin on ion transport in the isolated intestine of the eel, *Anguilla anguilla*. *Comp Biochem Physiol C Toxicol Pharmacol*. (2006) 143:17–22. doi: 10.1016/j.cbpc.2005.11.012

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