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

Philosophiae Doctor (PhD)
Thesis 2021:75

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Mel av insektlarver som ingrediens i fôr til Atlantisk laks (*Salmo salar*): Effekter på tarmens funksjon, helse og mikrobiota

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“When you are studying any matter, or considering any philosophy, ask yourself only what are the facts and what is the truth that the facts bear out. Never let yourself be diverted, either by what you wish to believe, or by what you think would have beneficent social effects if it were believed. But look only, and solely, at what are the facts.”

—Bertrand Russell

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LIST OF ABBREVIATIONS

AMPs
antimicrobial peptides....17

ASVs
amplicon sequence variants....39

BBM
brush border membrane....17

BSA
bovine serum albumin....39

BSF
black soldier fly....9

DI
distal intestine....15

DMSO
dimethyl sulfoxide....39

MACs
microbiota-accessible carbohydrates
....25

MI
mid intestine....15

OTUs
operational taxonomic units....39

PI
proximal intestine....15

pIgR
polymeric Ig receptor....17

PRRs
pattern recognition receptors....19

SBMIE
soybean meal induced enteritis....13

SCFAs
short-chain fatty acids....24

sIgA
secretory IgA....17

UMIs
unique molecular identifiers....44

SUMMARY

To feed an increasing global population with finite resources, the food production sector must minimize resource input and maximize nutritional outputs for human consumptions. Human-edible plant feedstuffs are the main ingredients used in modern salmon feeds. To secure sustainable developments, the salmon aquaculture needs to decrease its dependency on human-edible feedstuffs and incorporate unexploited feed resources in its raw material portfolio. Being part of the salmonids' natural diet, insects can be part of the solution. Among insect species with potentials as alternative feed ingredients for aquaculture, black soldier fly is produced at an industrial scale for its good nutritional value. Feed conversion ratio, growth performance, and fish health are primary concerns when evaluating the performance of alternative feed ingredients. While the nutritional value of black soldier fly larvae meal for fish has been extensively evaluated in recent years, its influence on fish health remains to be fully explored.

The intestine is the first organ exposed to the diet and of pivotal importance for the growth, development, and protection against pathogens. A well-functioning, healthy intestine is the key to convert feeds into fish biomass efficiently. In this thesis, two feeding trials, one in freshwater and one in seawater, were conducted to generate new knowledge regarding the effects of insect meal diets on the intestinal function, health, and microbiota in Atlantic salmon. The feeding trials shared a similar experiment setup with major differences in the insect meal inclusion level and the life stage of experimental fish.

In the 8-week freshwater trial, pre-smolt Atlantic salmon were fed either a reference diet or an insect meal diet containing 60% black soldier fly larvae meal. The histological examination showed normal histological structures of intestinal segments except for steatosis in the proximal intestine in both diet groups. However, the steatosis was less severe in salmon fed the insect meal diet. These histological findings were supported by the expression profiles of genes related to lipid metabolism. The expression profiles of genes relevant to immune and barrier function were both generally unaffected by diets. However, salmon fed the insect meal diet showed increased expression of genes indicative of immune tolerance (*foxp3*), stress response (*hsp70*), and detoxification activity (*cyp1a1* and *mta*). The insect meal diet markedly modulated the intestinal microbiota. Overall, the microbial

diversity was lower in the digesta of salmon fed the insect meal diet but higher in the mucosa. A group of bacterial genera, dominated by members of the *Bacillaceae* family, was enriched in salmon fed the insect meal diet. We also found that microbiota in the intestine closely resembled that of the feeds but was distinct from the water microbiota. Notably, bacterial genera associated with the diet effect were present in the feed samples as well.

In the 16-week seawater trial, post-smolt salmon were fed either a reference diet or an insect meal diet containing 15% black soldier fly larvae meal. The histological examination showed steatosis in the proximal and mid intestine in both diet groups, albeit less severe in the proximal intestine of salmon fed the insect meal diet. Typical signs of soybean meal-induced enteritis were observed in all the intestinal segments in both diet groups. The only significant diet effect was a higher degree of submucosa cellularity in the proximal intestine of salmon fed the insect meal diet. Regarding gene expression analysis, few genes showed diet-induced differences in the expression level. The insect meal diet markedly modulated the intestinal microbiota, although the insect meal inclusion level was one-quarter of the freshwater feeding trial. In contrast to what we found in the freshwater feeding trial, the microbial diversity was higher in both digesta and mucosa of salmon fed the insect meal diet. Bacterial genera enriched in salmon fed the insect meal diet largely confirmed our results from the freshwater trial.

Collectively, these studies showed little evidence that insect meal diets containing 15% or 60% black soldier fly larvae meal impaired the intestinal function or health in Atlantic salmon. On the contrary, insect meal diets improved intestinal health status by reducing excessive lipid deposition in the proximal intestine. Despite a four-time difference in the insect meal inclusion level between these two feeding trials, the insect meal diets reproducibly modulated the salmon intestinal microbiota enriching a particular group of bacterial genera dominated by members of the *Bacillaceae* family. Additional work will be needed to elucidate the functional implications of these microbial changes in terms of host health and disease resistance.

Insect meal has become a realistic, sustainable feed ingredient for aquaculture. The present thesis work generated new knowledge regarding how diets containing insect meal may influence the intestinal function, health, and microbiota in Atlantic salmon. It strengthens the knowledge basis for applying insect meal in aquaculture at a full scale.

SUMMARY IN NORWEGIAN (SAMMENDRAG)

For å sikre matvareforsyningen til en stadig økende befolkning med begrensede råvareressurser, må matproduksjonssektoren minimere ressursinnsats og maksimere ernæringsverdi for humant konsum. Moderne laksefôr inneholder mye planteråvarer som også kan brukes til matproduksjon. For å bidra en bærekraftig utvikling av lakseoppdrett, må næringen redusere sin avhengighet av fôrråvarer som konkurrerer med humant konsum, og i større grad ta i bruk uutnyttede fôrressurser i råvareporteføljen. Insekter er en del av det naturlige kostholdet til laksefisk i vill tilstand, og kan være en del av løsningen. Svart soldatflue er blant de insektartene som har størst potensiale som en alternativ fôringrediens i akvakultur, og produseres i dag i industriell skala på grunn av sin gode næringsverdi. Fôrfaktor og fôrutnyttelse, vekst og fiskehelse er viktige faktorer som må evalueres når man studerer effekter av alternative fôringredienser. Ernæringsverdien av larver fra svart soldatflue har blitt grundig evaluert i fisk de siste årene. Det er imidlertid lite kunnskap om slike produkters mulige effekter på fiskehelse.

Tarmen er det første organsystemet i en organisme som eksponeres for fôret og dets ingredienser. Responsene i tarmen er av avgjørende betydning for et dyrs vekst, utvikling og beskyttelse mot patogener. En velfungerende, sunn tarm er nøkkelen til å konvertere fôr effektivt til fiskebiomasse. Denne studien omfattet to fôringsforsøk, et i ferskvann og et i sjøvann, gjennomført for å gi ny kunnskap om effekter av fôr med mel fra insektlarver på tarmfunksjon, helse og mikrobiota i atlantisk laks. Fôringsforsøkene hadde lignende forsøksdesign der fôr med store forskjeller i innhold av insektmel ble evaluert.

I ferskvannsforsøket ble atlantisk laks fôret to ulike fôr i åtte uker før smoltifisering, et referansefôr uten mel av insektlarver, eller et fôr som inneholdt 60% mel av larver fra svart soldatflue. Histologiske undersøkelser viste normal tarmstruktur, bortsett fra symptomer på steatose, dvs. opphopning av fett, i de fremre tarmavsnittene i begge fôrgruppene. Imidlertid var steatosen mindre alvorlig hos laks som fikk fôret med insektmel. De histologiske funnene ble bekreftet av ekspresjon av gener relatert til fettmetabolisme. Ekspresjonsprofilene til gener som er relevante for immun- og barrierefunksjon, viste ikke klare forskjeller mellom fôr. Imidlertid viste laks som ble gitt fôr med insektmel, økt ekspresjon av gener som indikerer immuntoleranse (*foxp3*), stressrespons (*hsp70*) og detoksifiseringsaktivitet (*cyp1a1* og *mta*). Fôret med insektmel ga tydelige endringer i tarmens mikrobiota. Tarminnholdet hos fisk som fikk fôr med insektmel viste et mindre

mangfold, mens mukosa viste ett større mangfold sammenlignet med fisk som fikk kontrollfôr. En gruppe bakterieslekter, dominert av medlemmer av *Bacillaceae*-familien, ble anriket hos laksen som fikk fôr med insektmel. Vi fant også at mikrobiota i tarmen hadde klare likhetstrekk med mikrobiota i fôret, mens den var klart forskjellig fra mikrobiota i vannet omkring.

I saltvannsforsøket ble laksen fôret med et referansefôr eller et fôr som inneholdt 15 % mel av larver fra svart soldatflue i 16 uker. De histologiske undersøkelsene viste steatose i den proksimale og midtre delen av tarmen i begge fôrgruppene, som var mindre alvorlig hos laksen som fikk fôr med insektmel. Inflammatoriske endringer, liknende de som induseres av standard soyabønnemel, ble observert i alle de undersøkte tarmsegmentene, med en høyere infiltrasjon av immunceller i mukosa i proksimal tarm hos fisk som fikk fôr med insektmel. Effekter av fôr ble observert i uttrykksnivå av noen gener som er involvert i fettmetabolisme. Innblanding av insektmel i fôret ga tydelige endringer i tarmens mikrobiota også hos fisken i saltvann, selv om inkluderingsnivået for insektmel kun var ¼ av nivået som ble studert i ferskvannsforsøket. Men, i motsetning til resultatene fra ferskvannsforsøket, var det mikrobielle mangfoldet hos fisken i saltvannsforsøket høyere både i tarminnholdet og i mukosa hos laksen som fikk fôr med insektmel. Det var i stor grad de samme bakterieslektene som ble anriket av innblanding av insektmel i fôret i saltvannsforsøket som i ferskvannsforsøket.

Totalt sett viser disse studiene at fôr som inneholder mel av larver fra svart soldatflue, ikke ser ut til å svekke tarmfunksjonen eller tarmhelsen hos atlantisk laks. Tvert imot ser fôr med insektmel ut til å kunne bedre tarmens helsetilstand ved å redusere fettopphopning i de fremre delene av tarmen. Til tross for en firegangers forskjell i innblandingsnivået av insektmel mellom de to fôringsforsøkene, viste resultatene liknende effekter på mikrobiota, dvs at fôret med insektmel ga en anrikning av særlig en gruppe bakterier dominert av medlemmer av familien *Bacillaceae*. Mer forskning er nødvendig for å forstå de funksjonelle implikasjonene av disse mikrobielle endringene.

Studiene viser at insektmel kan bli en realistisk, bærekraftig fôringrediens for oppdrettsindustrien. De har gitt ny kunnskap om hvordan fôr som inneholder insektmel kan påvirke tarmfunksjonen, helsen og mikrobiotaen i atlantisk laks. De styrker kunnskapsgrunnlaget for anvendelse av insektmel som næringskilde i lakseoppdrett i kommersiell skala.

LIST OF PAPERS

Paper I

Gut health and vaccination response in pre-smolt Atlantic salmon (*Salmo salar*) fed black soldier fly (*Hermetia illucens*) larvae meal

Yanxian Li, Trond M. Kortner, Elvis M. Chikwati, Hetron Mweemba Munang'andu, Erik-Jan Lock, Åshild Krogdahl

Fish and Shellfish Immunology 2019, 86, 1106-1113; DOI: 10.1016/j.fsi.2018.12.057

Paper II

Consistent changes in the intestinal microbiota of Atlantic salmon fed insect meal diets

Yanxian Li, Karina Gajardo, Alexander Jaramillo-Torres, Trond M. Kortner, Åshild Krogdahl

Manuscript

Paper III

Total replacement of fish meal with black soldier fly (*Hermetia illucens*) larvae meal does not compromise the gut health of Atlantic salmon (*Salmo salar*)

Yanxian Li, Trond M. Kortner, Elvis M. Chikwati, Ikram Belghit, Erik-Jan Lock, Åshild Krogdahl

Aquaculture 2020, 734967; DOI: 10.1016/j.aquaculture.2020.734967

Paper IV

Differential response of digesta- and mucosa-associated intestinal microbiota to dietary insect meal during the seawater phase of Atlantic salmon

Yanxian Li*, Leonardo Bruni*, Alexander Jaramillo-Torres, Karina Gajardo, Trond M. Kortner, Åshild Krogdahl

Animal Microbiome 2021, 3, 8; DOI: 10.1186/s42523-020-00071-3

*Equal contribution

INTRODUCTION

Despite an average annual increase rate of 8 percent from 1980 to 2018, a further increase in the world aquaculture fish production is foreseen in the next decade (FAO, 2020). However, the supply of fish meal and fish oil, in the past the two most crucial feed raw materials for the aquaculture industry, is not expected to increase. The competition for these excellent nutrient sources has been intense, resulting in an escalation of price and call for the search to find alternative sources. Finding alternative ingredients for Atlantic salmon (*Salmo salar*) diets has demanded great research efforts in the past 25-30 years, efforts which must be intensified to meet the future needs of the industry. So far, plant ingredients have been the main alternatives. The use of plant ingredients in the Norwegian salmon diet has increased from 10% in 1990 to 70% in 2016 (Aas *et al.*, 2019). Nonetheless, as the global human population is growing and projected to reach 9.7 billion in 2050 (UN, 2019), there is a need for the salmon industry to decrease its dependency on terrestrial plant materials that otherwise can be directly used as foods for humans. Future salmon production should incorporate unexploited feed resources in its raw material repertoire to secure sustainable developments. From a sustainability perspective, insects are promising alternative feed ingredients. They possess a remarkable capacity to upgrade low-quality organic material, require minimal water and cultivable land, and emit little greenhouse gases (Van Huis, 2013). One of the insect species which has gained the most attention as an alternative feed ingredient for salmon is the black soldier fly (BSF; *Hermetia illucens*). It is now produced at an industrial scale for its expected good nutritional value and suitability for intensive production. Feed conversion ratio, growth performance, and fish health are primary concerns when substituting fish meal with alternative protein sources. At the time this thesis work was initiated, the nutritional value of BSF larvae meal had been evaluated in several fish species, including Atlantic salmon (Bondari and Sheppard, 1987; Borgogno *et al.*, 2017; Devic *et al.*, 2017; Hu *et al.*, 2017; Kroeckel *et al.*, 2012; Li *et al.*, 2017; Li *et al.*, 2016; Lock *et al.*, 2016; Magalhães *et al.*, 2017; Renna *et al.*, 2017; Sealey *et al.*, 2011; St-Hilaire *et al.*, 2007). Yet there was a knowledge gap on how dietary BSF larvae meal may influence the fish health. A prerequisite for introduction of a new ingredient into fish feed is convincing evidence that it does not compromise fish health. The present work aims to reveal possible health effects of dietary BSF larvae meal for Atlantic salmon with focus on the intestinal function, health, and microbiota, as the intestine is the first organ exposed to the diet and of

pivotal importance for the growth, development, and protection against foreign compounds, such as antigens.

The following chapters present background information and knowledge of relevance for the present thesis.

General background

Plant feedstuffs as main ingredients used in Norwegian salmon feeds

The global human population is growing and projected to reach 9.7 billion in 2050 (UN, 2019), requiring an increase in the food supply of 25-70% (Hunter *et al.*, 2017). Fish are considered a nutritionally valuable part of the human diet and play an essential role in the global food supply (Khalili Tilami and Sampels, 2018; Tacon and Metian, 2013). Atlantic salmon is the most produced marine fish species and one of the most economically important farmed fish worldwide (FAO, 2020). Due to the limited supply of marine ingredients, plant feedstuffs in Norwegian salmon feeds have been steadily increasing since 1990. They are the main raw materials used in modern salmon feeds (Figure 1) (Aas *et al.*, 2019). Among the plant protein sources used in salmon feeds in 2016, soy protein concentrate accounted for 19.0% of the total diet ingredients, followed by wheat gluten (9.0%), corn gluten (3.6%), faba beans (3.4%), pea protein concentrate (1.3%), sunflower meal (1.1%), sunflower protein (0.5%) and other marginally used plant proteins (2.3%).

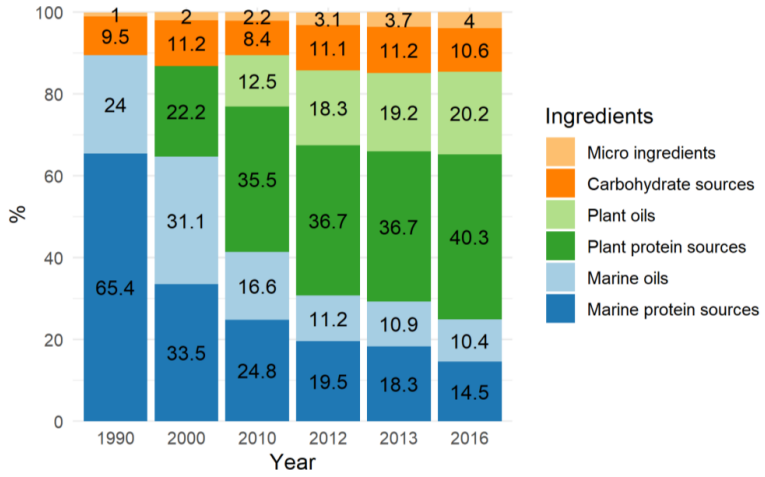

Figure 1. Ingredient sources (% of feed) in the Norwegian salmon feed in 2016 compared to previous years. Source: Aas *et al.* (2019).

Insect larvae meal as an alternative feed ingredient for salmon aquaculture

To feed an increasing global population, the food production sector must minimize the resource input and maximize nutritional outputs for human consumptions. To this end, salmon farming needs to decrease its dependency on human-edible terrestrial plant materials. Future salmon production should incorporate unexploited feed resources in the raw material repertoire to secure sustainable developments. At present, possible candidates include insects (Sánchez-Muros *et al.*, 2014), macroalgae (Wan *et al.*, 2019), and microorganisms (bacteria, yeasts, and microalgae) (Glencross *et al.*, 2020). Insects are part of the natural diets of many fish species, including Atlantic salmon. In terms of sustainability, insects are promising alternative protein sources. They possess a remarkable capacity to upgrade low-quality organic materials, require minimal water and cultivable land, and emit little greenhouse gases (Van Huis, 2013). So far, seven insect species have been approved by the EU for their use in aquafeeds, which include BSF, common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*) (EU, 2017). Due to the EU restrictions on feeds for farmed animals, materials of plant origin are the primary feed substrates used in the intensive insect rearing in Europe.

Black soldier fly is produced at an industrial scale in Europe for its good nutritional value and suitability for intensive production. On a dry matter basis, BSF larvae contain about 42% protein and 35% lipid (Newton *et al.*, 1977). In terms of protein quality, BSF larvae show a favorable essential amino acid profile closer to the fish meal than that of the standard soybean meal (Barroso *et al.*, 2014). On the other hand, the BSF larvae lipid is less preferable for salmon due to the very low poly-unsaturated fatty acids contents (PUFA) and absence of n-3 fatty acids. However, the lipid level and fatty acid profile are diet-dependent, allowing manipulation using different feed substrates (Makkar *et al.*, 2014). At the time this thesis work was initiated, the nutritional value of BSF larvae meal had been evaluated in several fish species, including Atlantic salmon (Bondari and Sheppard, 1987; Borgogno *et al.*, 2017; Devic *et al.*, 2017; Hu *et al.*, 2017; Kroeckel *et al.*, 2012; Li *et al.*, 2017; Li *et al.*, 2016; Lock *et al.*, 2016; Magalhães *et al.*, 2017; Renna *et al.*, 2017; Sealey *et al.*, 2011; St-Hilaire *et al.*, 2007). The optimal substitution level of fish meal in the diet by BSF larvae meal varied considerably among these studies (25-100%), possibly due to differences in the larvae meal quality, fish species, and variation in the other ingredients in the diets. The study in Atlantic salmon showed that the BSF larvae meal could replace up to 100% of the dietary fish meal without affecting the growth performance and sensory quality (Lock *et al.*, 2016). These promising

results led to the initiation of the AquaFly project, which investigated in-depth the growth, health, welfare, and fillet quality of Atlantic salmon fed diets containing BSF larvae meal.

A prerequisite for introducing a new ingredient into fish feed is convincing evidence that it does not compromise fish health. The BSF larvae meal had been indicated to be a valuable source of nutrients for Atlantic salmon. However, its influence on fish health remained unclear at the time this thesis work started. For instance, the chitin in the BSF larvae meal may interfere with the digestion of proteins and lipids, although positive effects of chitin such as growth-promoting and immunostimulatory effects have been reported in other fish species (Henry *et al.*, 2015; Ringo *et al.*, 2012). Depending on the source and quality, hazardous substances, such as heavy metals, may be transferred from feed substrates to BSF larvae causing potential feed and food safety issues (Diener *et al.*, 2015; Purschke *et al.*, 2017; Tschirner and Simon, 2015). As part of the AquaFly project, the present thesis work aimed to reveal the possible health effects of dietary BSF larvae meal for salmon. Most health endpoints of the present work focused on the intestinal function, health, and microbiota because the intestine is the first organ exposed to the diet and of pivotal importance for the growth, development, and protection against foreign compounds. As elaborated in the next chapter, alternative feed ingredients may challenge various aspects of intestinal function and health. Should bioactive compounds in the insect meal introduce any beneficial or detrimental biological effects, they are expected to occur in the intestine, if not only in this organ system.

Intestinal health problems associated with alternative feed ingredients

Imbalanced nutrient composition, antinutritional factors, and environmental pollutants in alternative feed ingredients may challenge farmed fish's intestinal function and health. Two main intestinal health problems associated with using alternative feed ingredients in Atlantic salmon are steatosis and inflammation.

Steatosis, often observed in the pyloric intestine and caeca, refers to excessive lipid accumulation in the intestinal mucosa, which is microscopically manifested as enterocyte hyper-vacuolization when stained by hematoxylin and eosin (Figure 2). Depending on the severity of steatosis, the pyloric intestine and caeca may macroscopically look swollen, pale, and coarse because of the excessive lipid accumulation. In severe cases, steatosis may be accompanied by accumulations of lipidic materials inside the intestinal lumen and referred

to as lipid malabsorption syndrome (LMS), eventually resulting in steatorrhea and so-called "floating feces" (Hanche-Olsen *et al.*, 2013; Penn, 2011). First reported by salmon farmers in 2002 and becoming prevalent in 2010, the lipid malabsorption syndrome seems to have largely disappeared in nowadays' salmon farming. However, an ongoing field survey of intestinal health status in farmed Norwegian salmon has reported a high incidence of steatosis occurring at different life stages (Chikwati *et al.*, 2018). Steatosis is thought to represent a lipid transport disorder within the enterocytes, possibly caused by deficiencies in major building blocks of lipoproteins, such as cholesterol and phosphatidylcholine. This is supported by recent findings showing that dietary supplementation of phosphatidylcholine or choline, both of which are low in plant ingredients, prevents steatosis in salmon fed low fish meal diets (Hansen *et al.*, 2020a; Hansen *et al.*, 2020b; Krogdahl *et al.*, 2020).

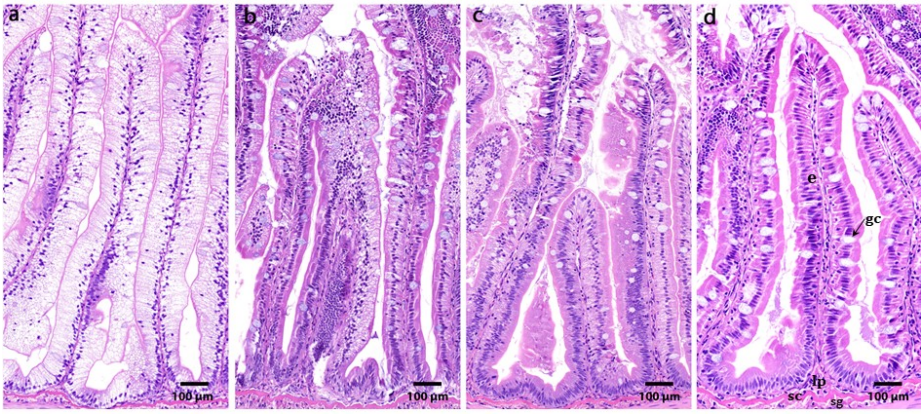

Figure 2. Severity of enterocyte hyper-vacuolization (steatosis) in pyloric caeca, representative for marked (a), moderate (b), mild (c), and normal (d). Abbreviations: e, epithelium; gc, goblet cells; lp, lamina propria; sc, stratum compactum; sm, sub-mucosa. Scale bar = 100 µm. Staining: H & E. Source: (Hansen *et al.*, 2020a)

Intestinal inflammation also represents a health and welfare issue in farmed salmon. The most well-known intestinal inflammation is the so-called soybean meal-induced enteritis (SBMIE) located in the distal intestine of salmon fed diets containing standard soybean meal. The SBMIE is characterized by widening and shortening of the intestinal folds; loss of the supranuclear vacuolization in epithelial cells; widening of the lamina propria within mucosal folds, with increased amounts of connective tissue and infiltration of mixed leukocytes in the lamina propria and submucosa (Figure 3) (Baeverfjord and Krogdahl, 1996). Concomitant with the histological changes, inferior growth performance is often

observed as well as altered intestinal functions, which include a reduction in brush border enzyme activities, reduced uptake of macromolecules, and increased intestinal permeability (Krogdahl *et al.*, 2010). Saponins, possibly along with other components present in the soybean meal, have been identified as the causative agents for the development of SBMIE in salmon (Knudsen *et al.*, 2008; Krogdahl *et al.*, 2015). Diet-induced enteritis in the distal intestine was also observed in salmon fed diets containing other legume ingredients such as pea protein concentrate, which induces enteritis at a 35% inclusion level in the diet (Penn *et al.*, 2011). As standard soybean meal causes enteritis in salmon, soybean protein concentrate, a more refined soybean protein product that does not induce enteritis, has been used as the primary source of plant protein in nowadays' Norwegian salmon diets. However, the ongoing intestinal health survey in farmed Norwegian salmon has also revealed a high incidence of inflammation in the distal intestine that closely resembles the SBMIE, though soybean meal is not used in commercial salmon diets (Chikwati *et al.*, 2018). The exact cause of intestinal inflammation in farmed salmon fed commercial diets is not clear. One possible explanation could be varying inclusion levels of less-refined plants ingredient in the diets, such as pea protein concentrate and fava bean, which are known to cause intestinal inflammation when the inclusion level is high (De Santis *et al.*, 2015; Penn *et al.*, 2011).

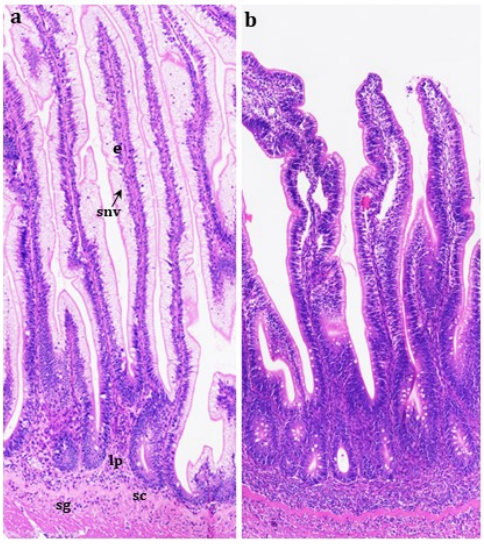

Figure 3. Representative histological sections showing normal (a) and inflamed (b) distal intestine in Atlantic salmon. Abbreviations: e, epithelium; lp, lamina propria; sc, stratum compactum; sg, stratum granulosum; snv, supranuclear vacuolization. Staining: H & E. Pictures: Elvis M. Chikwati.

The multifunctional intestine

In this chapter, the histomorphology and important functions of the Atlantic salmon intestine are introduced to serve as background knowledge for the results presented in this work. Background knowledge on the intestinal microbiota will be introduced in the next chapter.

Histomorphology

The alimentary tract of Atlantic salmon consists of the oral cavity, pharynx, esophagus, stomach, and intestine. The intestinal histomorphology of Atlantic salmon has been described in detail by Løkka *et al.* (2013). Macroscopically, the intestine can be divided into three distinguishable segments, i.e., the proximal intestine (PI), mid intestine (MI), and distal intestine (DI) (Figure 4). The PI has many blind-ended extensions termed pyloric caeca, which vastly increase the absorptive surface of the PI. As reflected in its morphology, the PI absorbs about 70% of the total nutrients (Bakke-McKellep *et al.*, 2000; Denstadli *et al.*, 2004). The MI is functionally analogous to the PI, responsible for the continued digestion and absorption of nutrients. The DI is immunologically more active than the other segments (Harstad *et al.*, 2008; Koppang *et al.*, 1998a; Koppang *et al.*, 1998b; Moldal *et al.*, 2014), though nutrient digestion and absorption also occur in this region. The SBMIE seems to occur only in the DI (Bjørgen *et al.*, 2019; Van den Ingh *et al.*, 1991). The structural organization of the intestinal mucosa is similar in all the intestinal segments, which consists of the epithelium (e), lamina propria (lp), stratum compactum (sc), and stratum granulosum (sg) (Figure 2-d; Figure 3-a). Enterocytes are the main building blocks of mucosal folds, segregated by goblet cells and intraepithelial lymphocytes. Simple mucosal folds are present in all the intestinal segments, whereas branched complex folds only occur in the DI. Under normal conditions, little to no vacuolization is present in the PI and MI enterocytes. In contrast, the DI enterocytes show varying degrees of supranuclear vacuolization which might be related to nutrient transport, digestive enzyme reabsorption, and antigen uptake. The supranuclear vacuoles diminish or disappear during starvation and inflammation (Baeverfjord and Krogdahl, 1996).

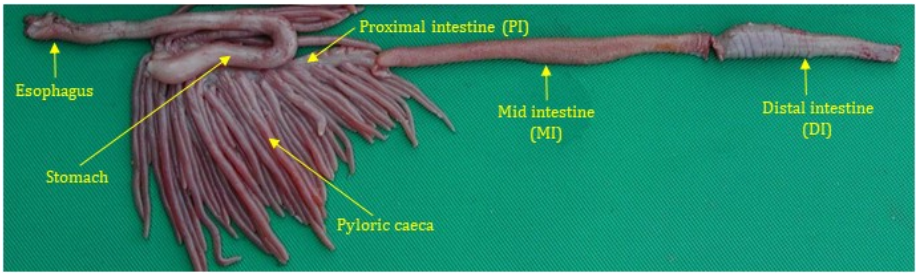

Figure 4. The alimentary tract of Atlantic salmon. Picture: Åshild Krogdahl.

Digestive function

The primary function of the intestine is to digest feeds and absorb nutrients. A well-functioning digestive system is the key to convert feeds into fish biomass efficiently. The digestive physiology of fish has been extensively reviewed elsewhere (Bakke *et al.*, 2011; Bjørgen *et al.*, 2020; Buddington *et al.*, 1997; Rust, 2002). A summary of the digestion process in fish is provided here, which to our knowledge, applies to salmon as well.

The digestion starts in the stomach, which is responsible for the mechanical and initial enzymatic digestion of foods. The hydrochloric acid, secreted by the oxynticopeptic cells in the gastric glands, secures a low pH in the stomach, denatures proteins in the feed, and activates pepsinogen, the pro-enzyme of the gastric protease pepsin. After the initial digestion in the stomach by pepsin, the acidic digesta enters the proximal intestine and stimulates secretions from the intestine and accessory digestive organs, i.e., pancreas, gall bladder, and mucosa. The bicarbonate in the secretions neutralizes the acidic digesta from the stomach so that digestive enzymes in the intestine can work at a pH close to their optimums. The primary digestive enzymes secreted from the pancreas are the proteolytic enzymes trypsin, chymotrypsin and elastase, lipase, phospholipases, α-amylase, as well as DNAase and RNAase. Notably, the proteolytic enzymes and co-lipase are secreted as inactive pro-enzymes, whereas the lipases and α-amylase are released in active forms (Bakke *et al.*, 2011). Enterokinase secreted from intestinal cells initiates the activation of pro-enzymes by activating trypsinogen (Ogiwara and Takahashi, 2007), the pro-enzyme of trypsin. After activation, trypsin activates the other pro-proteases. The bile salts released from the gall bladder emulsify lipids and fat-soluble components in the diet, facilitating their hydrolysis by lipases. The majority of primary bile acids in salmon are taurine-conjugated, with taurocholic acid being the predominant bile salt (Kortner *et al.*, 2014). The bile salt concentration is very high in the proximal intestine of salmon (up to 25% of dry matter). It

decreases gradually throughout the intestine (Gu *et al.*, 2014; Kortner *et al.*, 2013; Kortner *et al.*, 2016; Romarheim *et al.*, 2008; Romarheim *et al.*, 2006), which is indicative of efficient reabsorption and recycling of bile salts via mechanisms yet to be revealed. After the enzymatic digestion of feed particles in the intestinal lumen, intermediate products, such as small peptides and disaccharides, released during the hydrolysis are further broken down and absorbed by the brush border membrane (BBM) of the enterocytes. Some important BBM digestive enzymes are aminopeptidases (e.g., leucine-aminopeptidase), maltase, sucrases, alkaline phosphatases, and monoglyceride lipases. The absorption of nutrients across the BBM and subsequent transport into the circulatory system are not well characterized in fish compared to mammals. Interested readers are directed to the reviews given at the beginning of this section.

Immune function

Besides being a major digestive organ, the intestine is also the largest immune apparatus for fish. The intestinal mucosa is in contact with an enormous number of dietary antigens and commensal microbes that reside in the intestine. Yet, it is separated from the external environment only by a single layer of cells. To sustain the immune homeostasis at the intestine mucosal site, it is important that the intestinal immune system works properly such that it tolerates the benign antigens while fights against pathogens swiftly and potently. The intestinal immunity of fish has been extensively reviewed elsewhere (Gomez *et al.*, 2013; Rombout *et al.*, 2011; Salinas, 2015). A brief description of the fish intestinal immune system is provided here.

As illustrated in Figure 5, the apical side of the intestinal epithelium is covered by a layer of mucus that serves as the first line of defense. The mucus is secreted by the goblet cells and is mainly composed of heavily glycosylated proteins called mucin. Mucin-2, the dominant mucin in the mammalian intestine, has been identified in the genomes of several fish species, including Atlantic salmon (Lang *et al.*, 2007; Lang *et al.*, 2004; Sveen *et al.*, 2017). Apart from mucins, immune components including immunoglobulins, antimicrobial peptides (AMPs), complement proteins, and lysozyme are also present in the mucus. In mammals, secretory IgA (sIgA), a specialized antibody in mucosal immunity, is produced by plasma cells and transcytosed through the epithelial layer into the intestinal lumen by the polymeric Ig receptor (pIgR) expressed on the surface of epithelial cells.

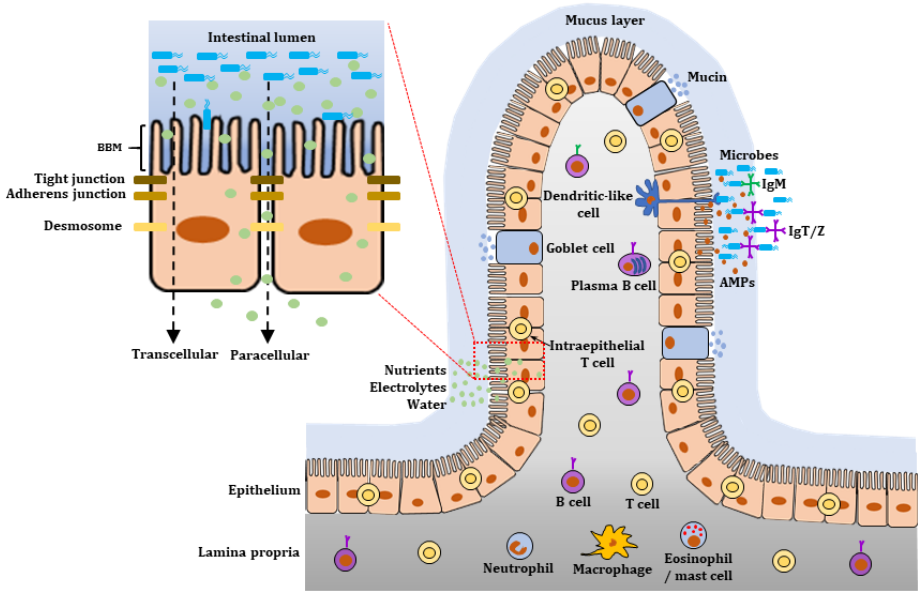

Figure 5. Schematic illustration of the intestinal mucosa in teleost fish. The mucus layer covering the epithelium is rich in humoral immune components such as immunoglobulins (IgT/Z mostly) and AMPs, which keep the commensal microbes and pathogens at bay. The epithelium forms a selectively permeable barrier between the intestinal lumen and internal milieu, controlling paracellular and transcellular transport of substances like nutrients, electrolytes, and water. Intestinal epithelial cells are connected by the so-called tight junctions, adherens junction, and desmosome; the former two regulate paracellular permeability. The gut-associated lymphoid tissue is diffused within the lamina propria, with a complex repertoire of immune cells including macrophages, granulocytes, dendritic-like cells, B cells and T cells. The schematic illustration was made based on reviews by Rombout *et al.* (2011), Groschwitz and Hogan (2009), and Gomez *et al.* (2013).

When the sIgA-pIgR complex is transported to the apical side of the enterocytes, it is cleaved off and released into the luminal space. The sIgA remains covalently bound to a portion of the pIgR designated as the SC (Salinas *et al.*, 2011). The dimeric sIgA can bind antigens and pathogenic microorganisms in the intestinal lumen, blocking their access to epithelial receptors, entrapping them in mucus and thus promoting their clearance (Mantis *et al.*, 2011). An IgA homolog has been identified in fish. The variant found in rainbow trout (*Oncorhynchus mykiss*) is named IgT (Zhang *et al.*, 2010), also known as IgZ in zebrafish (*Danio rerio*) (Danilova *et al.*, 2005). Different than its mammalian counterparts, salmonid IgT appears to be non-covalently bonded tetramers in mucus (Salinas *et al.*, 2011). A recent study in rainbow trout showed that the secretory IgT at the gill mucosal surface is functionally analogous to mammalian IgA in terms of pathogen clearance and microbiota

hemostasis (Xu *et al.*, 2020). It is unknown, but likely, that salmonid secretory IgT plays a similar role also in the intestinal mucosal immunity. The bactericidal AMPs play an important part in maintaining the integrity of epithelial barrier, limiting bacterial translocation, and promoting epithelium repair after injury by reducing the direct contact of bacteria with the epithelium. Fish possess a variety of AMPs with diverse functions including anti-bacterial, anti-viral and anti-fungal effects (Rajanbabu and Chen, 2011). Together, these elements in the mucus layer prevent most intestinal microbes from direct contact with epithelium and thus result in reduced inflammatory reactions and microbial translocation at the intestinal mucosal site.

Beneath the mucus layer is the intestinal epithelium, which consists of several different cell types. The absorptive enterocytes not only form a physical barrier but also function as signal sensors and transmitters. There are pattern recognition receptors (PRRs) localized at the different compartments of enterocytes that identify microorganisms through the recognition of microbe-associated molecular patterns (MAMPs) carried by the microbes. So far, four types of PRRs have been identified in teleosts, which include toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and peptidoglycan recognition proteins (PGRPs) (Boltaña *et al.*, 2011). Specifically, when PRRs bind the PAMPs of microbes that attach to or breach the intestinal epithelium, distinct intracellular signaling cascades will be triggered, releasing specific cytokines that induce anti-viral, pro-, or anti-inflammatory responses (Boltaña *et al.*, 2011). In addition, absorptive enterocytes constantly secret AMPs into the intestinal lumen, thereby contributing to the segregation of commensal bacteria from the epithelium barrier. In mammals, Paneth cells are specialized secretory cells located in small intestinal crypts, producing AMPs and proteins that regulate intestinal microbiota. Similar cells have not been discovered in fish. Cells sharing characteristics with mammalian M cells have been reported in Atlantic salmon (Fuglem *et al.*, 2010). However, there is no evidence that they can sample particles as large as bacteria or yeast cells. Rather, macrophage-like cells embedded within the epithelium have been shown to engulf yeast cells and bovine serum albumin in the intestinal lumen (Løkka *et al.*, 2014). It is suggested that antigen uptake by macrophage-like cells might be a more ancient uptake mechanism than specialized M cells.

The gut-associated lymphoid tissues (GALT) of teleosts are less organized than in mammals. Compartmented lymphoid tissues such as Peyer's patches, isolated lymphoid follicles and mesenteric lymph nodes are absent in fish. The immune cells, such as macrophages,

granulocytes, mast/eosinophilic granule cells, dendritic cells and lymphocytes, are diffusely located in the lamina propria, submucosa and epithelium. Macrophages and neutrophils are phagocytic cells that play major parts in the process and clearance of foreign antigens; eosinophils are granulocytic leucocytes involved in the defense against parasites (Balla *et al.*, 2010). Despite the heterogeneity of mast cells between fish species in terms of staining properties and tissue distribution, the functions of mast cells appear to be similar to those of mammals. Mast cells are abundant in the intestinal mucosa and react strongly to inflammation via migration, accumulation, and degranulation (Rombout *et al.*, 2011). Granules of fish mast/eosinophilic granule cells contain alkaline and acid phosphatases, 5'-nucleotidase, lysozyme, peroxidase, serotonin, histamine, and antimicrobial peptides such as piscidins, chrisophsin and pleurocidin (Sfacteria *et al.*, 2014). Mammalian dendritic cells, specialized antigen processing cells in the intestine, play a key role in shaping the intestinal immune response through their ability to orchestrate protective immunity and immune tolerance in the host (Coombes and Powrie, 2008). Dendritic cells have also been identified in zebrafish (Lugo-Villarino *et al.*, 2010) and rainbow trout (Bassity and Clark, 2012), however, their role in intestinal immunity needs clarification. Lymphocytes are the main players in the adaptive immunity at the intestinal mucosal site. Two types of B cells have been identified in the intestine of teleost fish to date, $IgM^{+,}$ and IgT^{+}/IgZ^{+}. IgM represents the main Ig in the plasma of teleosts, but it also plays a role in mucosal immunity (von Gersdorff Jørgensen *et al.*, 2011; Zhang *et al.*, 2010; Zhao *et al.*, 2008). In contrast, IgT is the only teleost Ig isotype characterized by a specialized mucosal function (Zhang *et al.*, 2010). Though it is recognized that T cells are abundant in the fish intestine, T cell subsets and their functions and distribution are largely not clear due to the lack of proper analytic tools. However, the expression of many T cell marker genes including CD3ε, CD4, CD8α and β, CD28, TCRα, TCRβ, TCRγ and TCRζ have been investigated in many fish species (Gomez *et al.*, 2013). Both lamina propria and the epithelium are populated with T cells, but intraepithelial T cells are the more studied ones. Intraepithelial T cells exist in large quantities in the fish intestine. They were found to account for 55% of the total intestinal leukocytes in seabass (*Dicentrarchus labrax*) as assessed by their reactivity to mAb DLT15, an anti-T cell monoclonal antibody specially produced for seabass (Romano *et al.*, 2011; Scapigliati *et al.*, 1995). The bulk of IELs are $CD8^{+}$ in seabass, rainbow trout, and Atlantic salmon (Bakke-McKellep *et al.*, 2007; Bernard *et al.*, 2006; Castro *et al.*, 2011; Picchietti *et al.*, 2011). Isolated DLT15-positive cells from seabass intestine express TCRβ, TCRγ, CD8α, CD4 and RAG-1 genes, with a much higher expression level of TCRγ than the others (Boschi *et al.*, 2011). These findings are indicative of a subset of T cells in the fish intestine analog to the

mammalian TCRγδ/CD8αα intraepithelial T cells, a predominant T cell subset in mammalian mucosal tissues that recognize unprocessed antigen in a manner similar to that of PRRs (Cheroutre *et al.*, 2011). There is evidence supporting this suggestion. Notably, intestinal lymphocytes purified from sea bass show significant cytotoxic activity against xenogeneic (K562) and allogeneic (DLEC) cell targets, suggesting the occurrence of non-specific, MHC-independent activity (Pichietti *et al.*, 2010).

Barrier function

A well-functioning barrier is pivotal for sustaining a healthy intestine. Impaired intestinal barrier function is associated with the pathogenesis of several human intestinal diseases such as inflammatory bowel disease, celiac disease, and irritable bowel syndrome (Camilleri *et al.*, 2012; Turner, 2009). As illustrated in Figure 5, the mucus layer is at the forefront of the intestinal epithelial barrier. It is rich in humoral immune components such as immunoglobulins (IgT/Z mostly) and AMPs, which keep the commensal microbes and pathogens at bay. Mucus layer defects allow for easier translocation of intestinal microbes and their toxins across the epithelium, causing increased mucosal immune activation and possibly development of the disease. For instance, mucin-2 knockout mice show augmented colonic *Citrobacter rodentium* infection (Bergstrom *et al.*, 2010) and develop spontaneous colitis (Van der Sluis *et al.*, 2006). The epithelial cells beneath the mucus layer form a selectively permeable barrier between the intestinal lumen and internal milieu, blocking the translocation of harmful entities such as dietary antigens, microbes, and their toxins while allowing the transport of water, electrolytes, and nutrients from the intestinal lumen into the circulation. The selective permeability of the intestinal epithelium is achieved by two major routes: the transcellular pathway through epithelial cells and the paracellular pathway between epithelial cells (Figure 5). Besides passive diffusion, transcellular permeability is mostly mediated by specialized transporters located in the epithelial cell membranes, which are selective for water, electrolytes, and nutrients. In mammals, uptake of larger peptides, proteins, and particles from the intestinal lumen can occur via endocytosis followed by transcytosis and exocytosis, whereas microbes can be taken up by antigen sampling cells like M cells (Menard *et al.*, 2010). In fish, endocytosis of intact proteins, bacteria, and yeasts has been reported to take place in the distal intestine (Løkka and Koppang, 2016). Intestinal epithelial cells are connected by the apical junctional complex, composed of tight and adherens junctions and the subjacent desmosomes (Figure 5). Paracellular permeability is regulated by the apical junctional complex, of which the tight

junctions are the rate-limiting factor for the paracellular passage of molecules (Adson *et al.*, 1994; Arrieta *et al.*, 2006). Tight junctions consist of four transmembrane protein families: claudins, occludin, junctional adhesion molecules (JAMs), and tricellulin (Groschwitz and Hogan, 2009). The intracellular domains of the transmembrane proteins interact with adaptor proteins such as zonula occludens (ZO), which mechanically link the tight junction complex to the actin cytoskeleton. The claudin protein family is pivotal for sustaining the tight junction function. It consists of 24 isoforms in mammals, which exhibit distinct expression patterns in different tissues and developmental stages (Lal-Nag and Morin, 2009). Differential expression of claudin isoforms affects tight junctions' size and charge selectivity (Groschwitz and Hogan, 2009). Studies in mammals suggest that claudin-1, -3, -4, -5, -8, -11, -14, and -19 are pore-tightening molecules, whereas claudin-2, -7, -10, -15, and -16 are pore-forming molecules (Amasheh *et al.*, 2011; Menard *et al.*, 2010). So far, 26 claudin isoforms have been identified in Atlantic salmon based on transcriptome data (Tipsmark *et al.*, 2008). Of the 26 isoforms, claudin-15 and claudin-25b are suggested as intestine-specific isoforms (Tipsmark *et al.*, 2010; Tipsmark *et al.*, 2008). The claudin-15 was similar to zebrafish and mammalian claudin-15, whereas claudin-25b is similar to mammalian claudin-4 (Tipsmark *et al.*, 2010). Hence, claudin-15 and claudin-25b in Atlantic salmon were suggested to be pore-forming and pore-tightening, respectively, supported by gene expression data from other studies (Sundell and Sundh, 2012). Adherens junctions are required for the assembly of the tight junctions. Similar to tight junctions, they are formed by interactions between transmembrane proteins (e.g., E-cadherin), intracellular adaptor proteins (e.g., catenins) and the actin cytoskeleton (Groschwitz and Hogan, 2009). Disruption of adherens junctions results in weakened cell-cell adhesion, perturbed epithelial cell polarization and differentiation, and premature apoptosis (Hermiston and Gordon, 1995).

Xenobiotic metabolism

The liver is the primary organ where xenobiotic metabolism takes place. However, one can also expect to observe activation of xenobiotic metabolism in the intestine, given that this organ is in direct and constant contact with the components of the diet. Xenobiotic metabolism consists of three phases. In phase I, enzymes such as cytochrome P450 oxidases add reactive or polar groups to xenobiotics via oxidation, reduction, or hydrolysis. These modified compounds are then conjugated to large and often polar compounds in phase II reactions, catalyzed by transferase enzymes such as glutathione S-transferases. Finally, the

conjugated xenobiotics are catabolized and excreted in phase III. In fish, various types of biomarkers for monitoring exposure to xenobiotics have been evaluated and validated, which include, but are not limited to, biotransformation enzymes, antioxidant enzymes, stress proteins and metallothioneins (van der Oost *et al.*, 2003). Of note, phase I biotransformation enzyme, cytochrome P450 1A (CYP1A), is the best-studied biomarker for environmental pollution in aquatic ecosystems (Bucheli and Fent, 1995; Uno *et al.*, 2012). It is one of the most sensitive biomarkers and has been validated in numerous studies in fish, including Atlantic salmon (Mortensen and Arukwe, 2007a; b; Sanden and Olsvik, 2009). Many environmental contaminants exert toxic effects via inducing oxidative stress (Winston and Di Giulio, 1991). Hence, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione-dependent peroxidase (GPOX), which are critical for the clearance of reactive oxygen species (ROS), can also serve as valuable biomarkers (Regoli and Giuliani, 2014; Valavanidis *et al.*, 2006). Stress proteins, also known as heat-shock proteins (HSPs), are involved in the protection and repair of cells. They are induced by stress and harmful conditions, including high/low temperature, ultraviolet light, anoxia, oxidants, toxins, and xenobiotics (Basu *et al.*, 2002; Gupta *et al.*, 2010). Metallothioneins (MTs) are low-molecular-weight, cysteine-rich proteins playing a central role in regulating the essential metals zinc and copper and detoxifying these and other nonessential metals such as cadmium and mercury (Roesijadi, 1992). The metallothionein induction is strongest in organs active in uptake, detoxification, and excretion, such as the liver, kidney, gills, and intestine of fish.

Intestinal microbiota

Aside from acting as a chemical barrier to block the envision of pathogens, the intestinal mucus layer also harbors diverse populations of microorganisms, including viruses, bacteria, archaea, and fungi collectively termed the microbiota. The advances in sequencing technologies and the establishment of germ-free animal models in recent years have transformed our understanding of the importance of intestinal microbiota in host development, health, and disease. It is now well established that intestinal microbiota is involved in normal host development (Bates *et al.*, 2006b; Rawls *et al.*, 2004) and many physiological activities such as food digestion and absorption (Ray *et al.*, 2012), lipid metabolism and energy balance (Falcinelli *et al.*, 2015; Semova *et al.*, 2012), social behavior (Archie and Tung, 2015) and aging (Smith *et al.*, 2017). In particular, the intestinal microbiota is, in various aspects, closely connected to intestinal function and health (Round

and Mazmanian, 2009; Shreiner *et al.*, 2015). Therefore, it is crucial to consider the intestinal microbiota when evaluating fish intestinal function and health.

Microbiota and intestinal health

Studies in germ-free mice and zebrafish have demonstrated that microbiota is essential for developing normal intestinal structure and functions (Bates *et al.*, 2007; Bates *et al.*, 2006a; Falk *et al.*, 1998; Rawls *et al.*, 2004). Besides this, intestinal microbiota interacts directly and indirectly with the intestinal immune system to regulate the differentiation of both pro- and anti-inflammatory T cell populations. It plays a fundamental role in maintaining the homeostasis of the intestinal immune response. The interaction may be mediated by direct contact between microbes and intestinal epithelial cells (Ivanov *et al.*, 2009) or immune cells (Niess *et al.*, 2005), or by microbial-derived products such as lipopolysaccharide (LPS) (Bates *et al.*, 2007), polysaccharide A (PSA) (Mazmanian *et al.*, 2008) and short-chain fatty acids (SCFAs) (Liu *et al.*, 2014; Smith *et al.*, 2013b). Given the immunomodulatory effects of intestinal microbiota, intestinal microbiota has become a therapeutic target for human intestinal diseases like recurrent *Clostridium difficile* infection (Hryckowian *et al.*, 2018) and inflammatory bowel disease (Jeon *et al.*, 2018; Narula *et al.*, 2017). In salmon aquaculture, dietary supplementation of probiotics and microbial-derived products has been applied to mitigate intestinal inflammation. For instance, dietary supplementation of a lactic acid bacteria cocktail (*Lactococcus lactis* and *Carnobacterium maltaromaticum*) was found to diminish enteritis induced by diets containing 38% soybean meal (Navarrete *et al.*, 2013), whereas the inclusion of a commercial probiotic product containing *Pediococcus acidilactici* abated intestinal inflammation chemically induced by oxazolone (Vasanth *et al.*, 2015). Bacterial meal and cell wall fractions derived from *Methylococcus capsulatus* have also been shown to prevent enteritis induced by 20% soybean meal (Romarheim *et al.*, 2011; Romarheim *et al.*, 2013a; Romarheim *et al.*, 2013b). Besides bacteria, dietary inclusion of yeast (*Candida utilis*) has also been reported to counteract enteritis induced by 20% soybean meal (Grammes *et al.*, 2013). However, later studies showed that the same dose of *Candida utilis* was unable to counteract enteritis induced by 20% (Reveco-Urzua *et al.*, 2019) or 40% (Hansen *et al.*, 2019) soybean meal. Taken together, these results provide evidence that microbiota is a promising target that can be selectively manipulated to improve the fish intestinal health status.

Interplays between intestinal microbiota and diet

One of the evolutionary advantages provided by the intestinal microbiota is the vastly increased gene catalog and metabolomic capacity, which enable the synthesis and catabolism of compounds that cannot be metabolized by the host alone (Fontaine and Kohl, 2020). The intestinal microbiota is known to modify dietary components, producing metabolites often beneficial to the host. For instance, intestinal microbes can ferment non-digestible carbohydrates producing SCFAs (Liu *et al.*, 2014; Smith *et al.*, 2013a) and synthesize B-group vitamins (LeBlanc *et al.*, 2013). However, the intestinal microbiota is also known to affect the drug efficacy and toxicity (Haiser *et al.*, 2013; Kaddurah-Daouk *et al.*, 2011) and convert food components into metabolites that are harmful to the host health. For example, intestinal microbiota can produce trimethylamine (TMA) from dietary choline, which after absorption is converted to trimethylamine N-oxide (TMAO), a metabolite associated with increased risk for cardiovascular disease (Wang *et al.*, 2011; Wang *et al.*, 2015).

Diet is one of the most critical environmental factors in shaping the host intestinal microbiota. It is an attractive target for modifying intestinal microbiota as it can be easily manipulated. Long-term dietary intake patterns have been shown to have a considerable effect on the structure of host intestinal microbiota (De Filippo *et al.*, 2010; Muegge *et al.*, 2011; Wu *et al.*, 2011), whereas short-term dietary changes also alter the host intestinal microbiota in a rapid and reproducible way (David *et al.*, 2014). Different dietary components may selectively induce compositional and functional alterations of the intestinal microbiota, which in turn could inflict important effects on the host health and disease resistance (Devkota *et al.*, 2012; Hryckowian *et al.*, 2018; Lukens *et al.*, 2014; Suez *et al.*, 2014). For instance, consuming a diet high in saturated fat promotes the expansion of a sulfite-reducing pathobiont, *Bilophila wadsworthia*, which was associated with a pro-inflammatory Th1 immune response and increased incidence of colitis in IL-10$^{-/-}$ mice (Devkota *et al.*, 2012). In contrast, dietary supplementation of microbiota-accessible carbohydrates (MACs) suppressed antibiotic-induced *Clostridium difficile* infection in mice, which was associated with the outgrowth of MAC-utilizing taxa and end products of MAC metabolism, i.e., the SCFAs acetate, propionate, and butyrate (Hryckowian *et al.*, 2018). In salmonid aquaculture, the use of alternative feed ingredients for fishmeal and fish oil in fish feeds can result in altered intestinal microbiota composition (Gajardo *et al.*, 2016; Jin *et al.*, 2019; Reveco *et al.*, 2014; Schmidt *et al.*, 2016), though the functional implications of such changes have not yet been revealed. For instance, less-refined plant-based ingredients such

as soybean meal seemed to selectively increase the abundance of lactic acid bacteria in the salmon intestine (Gajardo *et al.*, 2016; Reveco *et al.*, 2014; Schmidt *et al.*, 2016), whereas dietary inclusion of BSF larvae meal was found to increase the abundance of specific microbial clades including *Actinomyces*, *Bacillus*, *Brevibacterium*, *Corynebacterium,* and *Enterococcus* in the rainbow trout intestine (Huyben *et al.*, 2019; Terova *et al.*, 2019).

Temporal and spatial variation of Atlantic salmon intestinal microbiota

Sequencing-based studies of salmon intestinal microbiota have increased dramatically in recent years. We recently summarized important findings from these studies (Bjørgen *et al.*, 2020). Of note, the salmon intestinal microbiota shows temporal and spatial variations.

In the early developmental stages in freshwater, the salmon intestinal microbiota seems to be dominated mainly by *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes,* and *Tenericutes*. As the salmon enters the seawater and mature, the abundance of *Bacteroidetes* and *Firmicutes* declines while the abundance of *Tenericutes* and *Spirochaetes* increases (Llewellyn *et al.*, 2016; Lokesh *et al.*, 2019). The intestinal microbiota of salmon in later life stages is often dominated by a few phylotypes, including *Aliivibrio* (*Proteobacteria*), *Photobacterium* (*Proteobacteria*), *Mycoplasma* (*Tenericutes*), and *Brevinema* (*Spirochaetes*) (Gupta *et al.*, 2019a; Gupta *et al.*, 2019b; Karlsen *et al.*, 2017; Llewellyn *et al.*, 2016; Lokesh *et al.*, 2019), resulting in much lower microbial richness. The lower microbial richness in the later life stages is suggested to result from the increased filtering of microbial communities from the host (Heys *et al.*, 2020). However, the observed temporal variations in the salmon intestinal microbiota are confounded by many factors such as changes in the diet composition, environmental conditions (e.g., water salinity, temperature, and microbiota composition), and fish behavior (e.g., seawater drinking).

Like in mammals (Yasuda *et al.*, 2015; Zhang *et al.*, 2014), the salmon intestinal microbiota also shows spatial heterogeneity in its composition (Gajardo *et al.*, 2016). Microbial communities are different along the intestinal tract and between digesta and mucosa within the same intestinal segment. The microbial richness and diversity are usually lower in the intestinal mucosa than digesta (Gajardo *et al.*, 2016; Gajardo *et al.*, 2017). Notably, the digesta- and mucosa-associated microbiota also differ in their resilience to dietary changes. Existing studies suggest that the mucosa-associated microbiota is more resilient to dietary

changes than the digesta-associated microbiota (Gajardo *et al.*, 2017; Gupta *et al.*, 2019a; Jaramillo-Torres *et al.*, 2019).

AIMS OF THE STUDY

The overall goal of the present work was to secure that the intestinal function and health of Atlantic salmon are not compromised by the inclusion of insect larvae meal in the diet.

To achieve the main goal of this project, the following sub-goals were formulated:

- To document how dietary inclusion of BSF larvae meal may affect Atlantic salmon's intestinal function and health (**Paper I** and **III**).

- To characterize the intestinal microbiota of Atlantic salmon fed diets containing BSF larvae meal while accounting for its spatial heterogeneity (**Paper II** and **IV**).

HYPOTHESES

The following hypotheses were formulated based on the information presented in the introductory chapter. These hypotheses were the basis for the aims of the current thesis work:

- The inclusion of BSF larvae meal in the Atlantic salmon diet does not cause intestinal health problems.

- Inclusion of BSF larvae meal in the Atlantic salmon diet modulates the intestinal microbiota, possibly dependent on the intestinal compartments investigated.

SUMMARY OF RESULTS

Two feeding trials, one in freshwater and one in seawater, were carried out to evaluate how insect meal diets may affect the intestinal function, health, and microbiota in Atlantic salmon. The feeding trials shared a similar experiment setup but with major differences in the insect meal inclusion level and the life stage of experimental fish. In the 8-week freshwater trial, pre-smolt Atlantic salmon were fed either a reference diet containing fish meal, soy protein concentrate, and wheat gluten as protein sources, or an insect meal diet wherein BSF larvae meal (60%) replaced the bulk of fish meal and soy protein concentrate. Four tanks of fish were used per diet. In the 16-week seawater trial, post-smolt salmon were fed either a reference diet containing fish meal, soy protein concentrate, pea protein concentrate, corn gluten, and wheat gluten as protein sources, or an insect meal diet wherein BSF larvae meal (15%) replaced all the fish meal and most of the pea protein concentrate. Three tanks of fish were used per diet. In both experiments, the intestinal function and health were evaluated by histological examination and gene expression analysis, whereas the intestinal microbiota was profiled by sequencing V1-2 regions of the 16S rRNA gene. In total, four panels of genes relevant to lipid metabolism, immune modulation, barrier function, and detoxification response were profiled by qPCR.

Paper I presents results from the freshwater feeding trial regarding intestinal function and health. The histological examination showed normal histological structures of intestinal segments except for steatosis in the proximal intestine in both diet groups. However, the steatosis was less severe in salmon fed the insect meal diet.

In the proximal intestine, the expression level of *plin2*, a surface marker of lipid droplets, showed a 50% reduction in salmon fed the insect meal diet, supporting the histological findings. Additionally, genes responsible for the absorption (*cd36*) and intracellular transportation (*fabp2b*) of fatty acids showed increased expression in salmon fed the insect meal diet, so did those involved in the cholesterol uptake (*npc1l1*) and *de novo* synthesis (*srebp2* and *cyp51*). The expression profiles of genes relevant to immune and barrier function were generally not affected by diets. However, the expression levels of *cd3γδ* (T-cell marker) and *foxp3* (master transcription factor of regulatory T-cells) were higher in salmon fed the insect meal diet and showed a positive correlation. Most of the genes

indicative of detoxification response showed higher expression in salmon fed the insect meal diet. These genes included *cyp1a1* (a marker for xenobiotic metabolism), *mta* (a marker for heavy metal detoxification), *hsp70* (a marker for stress response), *sod1* (an indicator of oxidative stress), and *cat* (an indicator of oxidative stress).

In the distal intestine, a much lower number of differentially expressed genes were identified than in the proximal intestine. Contrary to what we found in the proximal intestine, the expression of *plin2* increased in salmon fed the insect meal diet. The *cd3γδ* and *foxp3* expression levels were also higher in salmon fed the insect meal diet and showed a strong positive correlation. The expression level of *cyp1a1* also showed a more than 2-fold increase in salmon fed the insect meal diet.

Paper II presents microbiota profiling results from the freshwater feeding trial. Microbiota of digesta and mucosal origin from the proximal and distal intestine were collected and sequenced, together with feed and water samples. The data analysis was done for digesta and mucosa samples independently due to batch effects between sequencing runs. The results showed that microbiota in the intestine closely resembled that of the feed but was distinct from the water microbiota. Five core OTUs were shared between the diets, classified as *Bacillus*, *Globicatella*, *Kurthia*, *Lactobacillus*, and *Ureibacillus*. Overall, the microbiota variation between intestinal segments was slight, but was significant in the digesta of salmon fed the reference diet. In contrast, diet markedly modulated the salmon intestinal microbiota in both digesta and mucosa, regardless of intestinal segments. Overall, the microbial diversity was lower in the digesta of salmon fed the insect meal diet but higher in the mucosa compared to fish fed the reference diet. In total, 93 and 36 taxa were associated with the diet effect in the digesta and mucosa, respectively. Collectively, 32 taxa were associated with the diet effect in both digesta and mucosa. Salmon fed the insect meal diet showed lower relative abundances of unclassified *Peptostreptococcaceae*, *Peptostreptococcus*, *Photobacterium,* and lactic acid bacteria including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus,* and *Streptococcus*; but higher relative abundances of *Actinomyces*, unclassified *Bacillales*, unclassified *Bacillaceae*, *Bacillus*, unclassified *Beutenbergiaceae*, *Brevibacterium*, *Cellulosimicrobium*, *Clostridium sensu stricto 1*, *Corynebacterium 1*, unclassified *Enterococcaceae*, *Enterococcus*, *Exiguobacterium*, *Globicatella*, *Gracilibacillus*, unclassified *Lactobacillales*, *Lysinibacillus*, *Macrococcus*, *Microbacterium*, *Nosocomiicoccus*, *Oceanobacillus*, *Ornithinibacillus*, *Paenibacillus*, unclassified *Planococcaceae*, and unclassified *RsaHF231*. Some of these taxa, such as

Paenibacillus and *Streptococcus*, were positively associated with the expression of *foxp3* in the intestine. Notably, bacterial taxa associated with the diet effect were present in the feed samples as well.

Paper III presents results from the seawater feeding trial regarding intestinal function and health. The histological examination showed steatosis in the proximal and mid intestine in both diet groups. It was, however, less severe in the proximal intestine of salmon fed the insect meal diet. Typical signs of soybean meal-induced enteritis were observed in all the intestinal segments in both diet groups. The only significant diet effect was a higher degree of submucosa cellularity in the proximal intestine of salmon fed the insect meal diet. Regarding gene expression analysis, few differentially expressed genes were identified. In the proximal intestine, *mmp13*, a marker gene involved in tissue reorganization, was the only differentially expressed gene showing lower expression in salmon fed the insect meal diet. In the distal intestine, *chk*, a marker gene involved in *de novo* synthesis of phosphatidylcholine, was the only differentially expressed gene showing lower expression in salmon fed the insect meal diet.

Paper IV presents microbiota profiling results from the seawater feeding trial. Microbiota of digesta and mucosa origin from the distal intestine were collected and sequenced. The results showed that three core ASVs were shared between the diets, classified as *Aliivibrio*, *Brevinema andersonii*, and *Mycoplasma*. The intestinal digesta and mucosa harbored microbial communities with clear differences. Regardless of diet, microbial diversity was much higher in the digesta than in the mucosa. Forty-seven taxa were identified as differentially abundant between the digesta and mucosa, but only two of these taxa showed higher relative abundance in the mucosa. These two mucosa-enriched taxa, *Brevinema andersonii,* and unclassified *Spirochaetaceae*, belong to the Spirochaetes phylum and were associated with gene expression in the intestine related to immune responses and barrier function, respectively. The insect meal diet markedly modulated the intestinal microbiota, resulting in higher microbial diversity in both digesta and mucosa. The magnitude of diet effect, however, was different between the digesta and mucosa. A much stronger diet effect was observed in the digesta than that in the mucosa. Thirty-six taxa were associated with the diet effect. Corroborating with our findings in the freshwater feeding trial, salmon fed the insect meal die showed lower relative abundances of *Geobacillus*, *Peptostreptococcus*, *Photobacterium, Streptococcus*, *Tepidimicrobium*, *Vagococcus,* and *Weissella*; but higher relative abundances of *Actinomyces*, unclassified *Bacillaceae*, *Bacillus*, unclassified

Beutenbergiaceae, *Brevibacterium*, *Cellulosimicrobium*, *Corynebacterium 1*, unclassified *Enterococcaceae*, *Enterococcus*, *Erysipelatoclostridium*, *Gracilibacillus*, unclassified *Lachnospiraceae*, unclassified *Lactobacillales*, *Lysinibacillus*, *Microbacterium*, *Oceanobacillus*, *Ornithinibacillus*, and unclassified *RsaHF231*.

DISCUSSION: MATERIALS AND METHODS

Materials

In the freshwater feeding trial, feed, water, intestinal digesta, and mucosa samples were collected for microbiota profiling. In the seawater feeding trial, only intestinal digesta and mucosa samples were collected for microbiota profiling due to resource limitations. Results from the freshwater feeding trial showed that water microbiota was distinct from the intestinal microbiota. In contrast, many taxa found in the intestine were also present in the feed samples (**Paper II**). The observed diet effects could be partly attributed to the differences in the feed-associated microbiota. Given the hydrothermal treatments during the extrusion step in the feed production, the feed-associated microbiota could have primarily originated from dead/dormant bacteria rather than living bacteria. As such, the feed-associated microbiota could be a major confounding factor when digesta is used for evaluating diet effects on the intestinal microbiota. Feed samples should be collected in sequencing-based microbiota studies, which do not distinguish between living and dead bacteria. Unfortunately, feed samples were not included in the microbiota analysis in the seawater feeding trial.

Methodological considerations for microbiota profiling

Since the histological examination (H&E staining) and gene expression profiling (qPCR) were carried out using conventional and well-established methods in our group, the discussion herein on the methodology will focus on microbiota profiling, which is still under rapid development. Best practices for conducting a microbiota study have been reviewed elsewhere (Goodrich *et al.*, 2014; Knight *et al.*, 2018). Methodological considerations for microbiota analysis will be discussed below.

Experimental design

A well-designed experiment is pivotal for obtaining meaningful data. There are many aspects to consider when designing a microbiota study, such as experimental controls, sample size, batch effect, confounding factors, sampling scheme, and sequencing strategy. Here we discuss the experimental controls, sample size, and batch effects.

Positive controls. Setting up proper experimental controls is vital for obtaining valid results. A mock community is a defined mixture of known microbial strains created in vitro to simulate the composition of a microbiota sample (Bokulich *et al.*, 2016). It can be processed like a biological sample, starting from DNA extraction to sequencing. The real sequence data generated from the mock can then be processed, and the results are compared to true values in the mock. As such, the use of mock as a positive control allows researchers to evaluate their results critically and optimize the data generation process. The mock community ((ZymoBIOMICS™, Zymo Research, USA; catalog no., D6300) used in the present work is widely used in microbiota studies. It contains three easy-to-lyse Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica*), five tough-to-lyse Gram-positive bacteria (*Lactobacillus fermentum*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis*), and two tough-to-lyse yeasts (*Cryptococcus neoformans*, *Saccharomyces cerevisiae*). The use of mock has allowed us to optimize our wet-lab protocol and compare the performance of different bioinformatic pipelines/tools. For instance, we processed the sequence data from the same mock community using the QIIME1 and QIIME2 pipeline, respectively. Compared to true values in the mock, the QIIME2 pipeline produced a much more accurate taxonomic profile than the QIIME1 pipeline. Based on this result, we chose QIIME2 over QIIME1 for amplicon sequence data processing. It should also be noted that a mock community is overly simplified compared to a real biological sample. One should use caution to avoid overfitting of methods to unrealistic conditions and use expert knowledge to judge the quality of results.

Negative controls. As the universal primers amplify the 16S rRNA gene of most bacteria, amplicon sequencing is inherently subjective to contamination, given that bacteria are ubiquitously distributed. Contamination may come from the environment, reagents, lab personnel, and lab equipment. While good lab hygiene is helpful, it is impractical to eliminate all the living or dead microbes in the lab. Contamination can be a severe problem, especially when dealing with samples with low microbial biomass (Eisenhofer *et al.*, 2019; Glassing *et al.*, 2016; Karstens *et al.*, 2019; Weyrich *et al.*, 2019). Failing to account for contamination issues in molecular-based microbiota studies can lead to flawed or false conclusions (de Goffau *et al.*, 2019; Lauder *et al.*, 2016; Salter *et al.*, 2014). The inclusion of negative controls in the molecular-based microbiota studies helps to monitor and mitigate the contamination issues. Negative controls should be included from the early stage of a microbiota study, starting from the sampling (low microbial biomass samples) or DNA

extraction stage (Kim *et al.*, 2017). Also, they should be subjected to the same treatments that the biological samples receive and always sequenced even when they show weak or no PCR amplification. If the samples are processed in batches, a negative control should also be included in each batch. Including negative controls in the present work has enabled us to quickly discern contaminating sequences from true biological sequences (**Paper II** and **IV**). For instance, sequences classified as *Pseudomonas* are frequently identified as the most abundant reagent contaminants in our lab. Notably, *Pseudomonas* has also been isolated from intestinal digesta and mucosa of Atlantic salmon by traditional culturing approaches (Askarian *et al.*, 2012; Cantas *et al.*, 2011; Hatje *et al.*, 2014; Navarrete *et al.*, 2013). Without negative controls, one cannot distinguish contaminating *Pseudomonas* from the indigenous *Pseudomonas,* and conclusions based on such results are likely flawed or false.

Sample size. A study with a small sample size has low statistical power, reducing the chance of detecting an actual effect. Underpowered studies may produce false-negative and unreproducible results. However, the effect size is usually unknown in a microbiota study, and effect size calculation remains challenging (Debelius *et al.*, 2016). As such, one should maximize biological replication to reduce the chance of conducting an underpowered study. Based on the available resources, we sampled 12 and 18 fish per treatment group in the freshwater and seawater feeding trial, respectively. A recent study suggests that a minimum of 9 fish is needed to draw valid conclusions in dietary metabarcoding studies (Panteli *et al.*, 2020). Future studies will benefit from statistical power and effect size calculation.

Batch effect. High-throughput sequencing data are often generated in batches due to logistical or practical constraints. Technical sources of variation across batches, referred to as batch effects, can distort biological findings resulting in false conclusions (Leek *et al.*, 2010). For instance, microbiota samples may cluster together based on non-biological factors such as sample preparation date, handling personnel, reagent lot, and sequencing batch, obscuring biological variations of interest. To reduce confounding between biological and batch effects, it is crucial to design the study such that sample classes are distributed evenly across batches. However, in the freshwater feeding trial, digesta samples were first amplified and sequenced due to technical challenges in obtaining high-quality PCR products from mucosa samples. The technical replicates of digesta samples showed a clear batch effect between the sequencing runs (**Paper II**). The performance of batch effect correction methods, initially developed for gene expression data, on microbiota data was recently reviewed by Wang and LeCao (2019). Commonly used methods such as ComBat (Johnson *et*

al., 2007) and removeBatchEffect (Ritchie *et al.*, 2015) require data transformation to meet the methods' parametric assumptions. As many downstream data analyses in microbiota studies require a count table as input, the resulting batch effect corrected data are limited to analyses like dimension reduction (PCA) and differential abundance testing. At present, batch effect correction methods that produce count data as output include RUVSeq (Risso *et al.*, 2014) and ComBat-seq (Zhang *et al.*, 2020). However, the latter method is not suited for data with severely or completely confounded study designs. RUVSeq removes batch effects by performing factor analysis on sample replicates and negative control variables. It can be used to correct batch effects in experiments where biological effects are severely or completely confounded by batch effects. Based on the beta-diversity analysis, RUVSeq seemed to effectively correct the PCR batch effects. However, it removed a large proportion of sequences from samples and the resulting sample taxonomic composition was very different from that before the batch effects correction. Therefore, we did not perform batch effects corrections for our data. The microbiota data generated from the freshwater feeding trial were analyzed separately based on the sequencing runs (**Paper II**).

Sample collection

Sampling methods. Compared to the collection of digesta samples, collecting mucosa samples is more complicated. The mucosa-associated microbiota is usually collected by flushing, scraping, or dissecting the intestinal mucosa. We chose to dissect a small piece of the intestinal segment as it is probably more representative of the mucosal microbial community. While this sampling method is fine for amplicon sequencing, it is not suitable for shotgun metagenomics and metatranscriptomics analysis as the sample is heavily contaminated by host DNA. Alternative sampling methods such as swabbing should be considered if samples are used for multi-omics analysis.

Sample preservation. Different sample preservation methods are known to cause systematic bias in microbiota profiling results (Song *et al.*, 2016; Vogtmann *et al.*, 2017b). Immediate freezing at -20 ℃ or below is considered as the "gold standard" method for preserving samples for microbiota profiling. However, this approach is not always feasible for field studies. Alternative methods such as 95% ethanol and RNAlater are effective in sample preservation as well (Song *et al.*, 2016; Vogtmann *et al.*, 2017a; Vogtmann *et al.*, 2017b). Samples kept in these fixatives are more stable in ambient temperature compared to those preserved by snap freezing. However, negative controls should be included during

the sampling stage when samples are kept in these fixatives as reagent contamination may be introduced. We chose the "gold standard" method (snap-frozen in liquid N_2 and stored at -80 °C) for sample preservation as we were able to keep samples frozen from the sampling and underway to the freezers at the lab.

DNA extraction

DNA extraction is one of the main factors causing variations in microbiota analysis results between studies (Sinha *et al.*, 2017). The use of commercial DNA extraction kits is advised so that results between studies are more comparable. The QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany; catalog no., 51504) was a widely used DNA extraction kit at the time this thesis work was initiated. DNA extraction efficiency is one of the major concerns in sequencing-based studies of intestinal microbiota. Mechanical cell lysis, which is not included in the QIAamp® kit, increases the DNA extraction efficiency of tough-to-lyse microbes from complex microbial ecosystems (de Boer *et al.*, 2010; Nylund *et al.*, 2010; Salonen *et al.*, 2010; Walker *et al.*, 2015). We, therefore, added a bead-beating step to the cell lysis step in addition to the chemical and enzymatic treatments. In later experiments, we found that the size of beads also affected the DNA extraction efficiency. A mixture of beads with different sizes produced a more accurate taxonomic composition in the mock than those with the same size. Moreover, the relative abundance of the tough-to-lyse Gram-positive bacteria in the mock was higher and closer to the ground truth.

Amplicon PCR

Primers. The choice of primers is known to critically influence amplicon sequencing results (Huse *et al.*, 2008; Kim *et al.*, 2011; Klindworth *et al.*, 2013). Different primers have their own biases, resulting in over-representation or selection against certain microbial clades in the samples (Cai *et al.*, 2013; Klindworth *et al.*, 2013; Walters *et al.*, 2016; Wear *et al.*, 2018). Previously, we tested the performance of commonly used universal primers, including V1-2 (Roeselers *et al.*, 2011), V3 (Muyzer *et al.*, 1993), V4 (Rodrigues *et al.*, 2013), and V4-5 (Schwieger and Tebbe, 2000), for our samples (Gajardo, 2016). The V1-2 primers showed a single bright band in the agarose gel, whereas other primers showed no band (V4), a single band with smearing (V3), or multiple bands (V4-5). Therefore, we chose the V1-2 primers over other more commonly used primers (V4, V4-5) for the taxonomic profiling of our samples. Results so far show that the performance of V1-2 primers is broadly comparable to that of the more commonly used primer sets such as V3-4 and V4. Another practical

reason to keep using the V1-2 primers in the present work is to keep experimental conditions consistent in different studies conducted in our lab so that we can integrate data for a meta-analysis.

PCR enhancers. Compared to other samples collected in the present work, mucosa samples can be very challenging to amplify. We found that diluting DNA templates often increased PCR efficiency, probably due to reduced PCR inhibitory effects. The reason why mucosa samples showing more potent PCR inhibitory effects than digesta samples, which are known to contain PCR inhibitors, remains to be explored. When DNA template dilution did not work, we tested the performance of several PCR enhancers, including bovine serum albumin (BSA), dimethyl sulfoxide (DMSO) and formamide. Our results showed that 0.4% BSA improved the PCR performance, whereas formamide and DMSO did not (Figure 6).

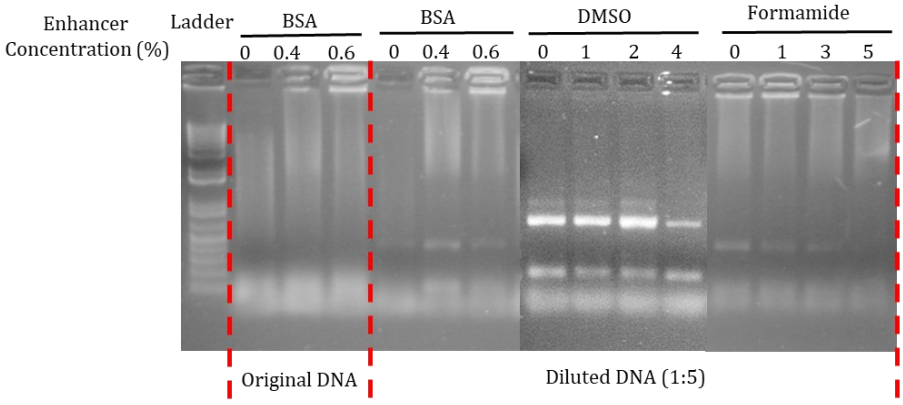

Figure 6. Gel images showing the effects of DNA template dilution and PCR enhancers on the PCR performance of mucosa samples. The DNA was extracted from the colon mucosa of mink and diluted 1:5 in ATE buffer. The concentration of the PCR enhancer in the PCR reaction mix is shown on the top. The results apply to salmon intestinal mucosa as well.

Bioinformatics

To cluster or not: OTUs versus ASVs. Due to errors in sequences generated by Illumina platforms, 16S amplicon sequences are conventionally clustered into operational taxonomic units (OTUs) at a specific sequence similarity threshold, often 97%, to avoid interpreting sequencing artifacts as real biological sequence variants. In recent years, "denoising" algorithms such as DADA2 (Callahan *et al.*, 2016), UNOISE2 (Edgar, 2016), and Deblur (Amir *et al.*, 2017) have been developed to address the issue by correcting sequencing errors, generating amplicon sequence variants (ASVs) with a single-nucleotide resolution, which is also known as sub-OTUs or zero-radius OTUs. Compared to OTU-based approaches, ASV

methods provide improved taxonomic resolution. Moreover, ASVs are consistent labels making them more reproducible and comparable among different studies. As such, ASV methods have gained increasing popularity over the traditional OTU methods. However, intragenomic heterogeneity of 16S rRNA genes is common in many bacteria species (Sun *et al.*, 2013), which means ASV methods may split a single taxon into multiple ASVs resulting in inflated alpha and beta diversity of microbial communities. In addition, ASV methods can lead to false discoveries in taxon co-occurrence analysis as a result of splitting a single taxon into multiple ASVs: ASVs from the same taxon are inherently strongly correlated. Apparently, ASV methods are not necessarily better than the traditional OTU methods. Studies indicate that conclusions based on the ASV or OTU methods often show high concordance (Capunitan *et al.*, 2020; Glassman and Martiny, 2018; Moossavi *et al.*, 2020). In agreement, our exploratory data analysis in **Paper II** showed that most results were similar between the ASV and OTU approach. We decided to cluster the AVSs into OTUs in **Paper II** because it ameliorated the batch effects and reduced the data sparsity. Ultimately, the choice of sequence processing method is based on the research question being asked. If one is not concerned about the intragenomic heterogeneity of 16S rRNA genes, ASV methods can be advantageous. Otherwise, the traditional OTU methods also deliver reliable results.

Phylogeny: de novo versus reference-based fragment insertion. After constructing the OTU/ASV table, a typical step is to build a phylogenetic tree for downstream phylogeny-related analyses, such as calculating Faith's phylogenetic diversity and UniFrac distance. Conventionally, the phylogenetic tree is built using *de novo* approaches. To build a *de novo* tree, the representative sequences are first aligned by a multiple sequence aligner like MAFFT (Katoh and Standley, 2013). The resulting alignment is masked to reduce alignment ambiguity. A phylogenetic tree is then inferred from the masked alignment by algorithms like FastTree (Price *et al.*, 2010), RAxML (Stamatakis, 2014), and IQ-TREE (Nguyen *et al.*, 2015). Finally, the inferred phylogenetic tree is rooted. The *de novo* approaches use all the representative sequences to build the tree and are suitable for samples that contain many novel clades not represented in the reference database. However, the *de novo* tree built from short amplicon sequences may not be reliable as they do not contain much phylogenetic information. Analyses based on *de novo* tree may yield incorrect biological conclusions (Janssen *et al.*, 2018). The reference-based fragment insertion approach like SEPP, which inserts the representative sequences into a reference tree based on the sequence similarity, is probably a better choice (Janssen *et al.*, 2018; Knight *et al.*, 2018). Unlike the *de novo* approaches, representative sequences that are not similar enough to any record in the

reference tree are discarded. Therefore, this approach is not suitable for samples that contain many novel clades not represented in the reference tree. Eventually, the choice of the approach to build a phylogenetic tree is dependent on the sample characteristics. In our experiences, most of, if not all, the representative sequences could be reliably inserted into the reference tree. Therefore, the reference-based fragment insertion was considered the better approach for our data.

Statistics

A major recent development in the statistical analysis of microbiota data is the adoption of compositional data analysis. In sequencing-based microbiota studies, each sample consists of proportions of different taxa with a sum constrained to a constant. This characteristic makes microbiota sequence data compositional (Gloor *et al.*, 2017; Tsilimigras and Fodor, 2016). Compositional data reside in the simplex rather than Euclidean space, making standard statistical methods such as the Pearson correlation, *t*-test, *ANOVA,* and linear regression analysis not directly applicable for analyzing microbiota sequence data (Aitchison, 1982; Mandal *et al.*, 2015). Standard statistical methods have been reported to have high false positive rates when directly applied for correlation analysis and differential abundance testing in microbiota studies (Friedman and Alm, 2012; Hawinkel *et al.*, 2017; Kurtz *et al.*, 2015; Lovell *et al.*, 2015; Thorsen *et al.*, 2016). As such, compositional data analysis was introduced into microbiota research to deal with the compositionality in sequence data. The first step in compositional data analysis is to perform a log-ratio transformation of data such as centered log-ratio (CLR) transformation and isometric log-ratio (ILR) transformation. The log-ratio transformation moves compositional data from simplex space to real space, allowing the application of standard statistical methods in microbiota sequence data analysis (Aitchison, 1986). Alternative compositional analysis approaches for standard statistical methods are summarized in Table 1 below.

In recent years, many bespoke compositional data analysis methods, such as ANCOM (Mandal *et al.*, 2015), ALDEx2 (Fernandes *et al.*, 2014), mixMC (Lê Cao *et al.*, 2016), Corncob (Martin *et al.*, 2020), gneiss (Morton *et al.*, 2017), selbal (Rivera-Pinto *et al.*, 2018) and Songbird (Morton *et al.*, 2019), have been developed for differential abundance testing of microbiota data.

Table 1. Standard statistical methods for microbiota data analysis and their compositional substitutes*.

	Standard methods	Compositional methods
Normalization	Rarefaction TSS (total sum scaling) CSS (cumulative sum scaling) DESeq's variance stabilization edgeR's TMM (trimmed mean of M-values)	CLR (centered log-ratio transformation) ILR (isometric log-ratio transformation)
Distance	Jaccard distance Bary-Curtis distance UniFrac distance	Aitchison distance (Aitchison *et al.*, 2000; Gloor *et al.*, 2017) PHILR transformed Euclidean distance (Silverman *et al.*, 2017)
Ordination	PCoA (principal coordinate analysis)	PCA (principal component analysis)
Correlation	Pearson Spearman	SparCC (Friedman and Alm, 2012) SpiecEasi (Kurtz *et al.*, 2015) Φ (Lovell *et al.*, 2015) P (Erb and Notredame, 2016)
Differential abundance	LEfSe (Segata *et al.*, 2011) metagenomeSeq (Paulson *et al.*, 2013) DESeq2 (Love *et al.*, 2014) edgeR (Robinson *et al.*, 2010) Voom (Law *et al.*, 2014)	ANCOM (Mandal *et al.*, 2015) ALDEx2 (Fernandes *et al.*, 2014) mixMC (Lê Cao *et al.*, 2016) Corncob (Martin *et al.*, 2020) gneiss (Morton *et al.*, 2017) selbal (Rivera-Pinto *et al.*, 2018) Songbird (Morton *et al.*, 2019)

*Adapted from Gloor *et al.* (2017) and Weiss *et al.* (2017).

While compositional data analysis has become popular among researchers, it should be noted that the interpretation of compositional data analysis results can be challenging (Quinn *et al.*, 2018). The results need to be interpreted based on the selected log-transformation to avoid erroneous interpretations (Quinn *et al.*, 2019). Also, the performance of these new methods has not been extensively benchmarked. A recent study suggested that compositional methods devised for differential abundance testing of microbiota data did not outperform count-based methods initially developed for differential expression analysis of RNA-seq data, such as limma-voom and DESeq2 (Calgaro *et al.*, 2020). It was argued that the best method for differential abundance testing is data-dependent and that researchers should choose methods based on a careful exploratory data analysis. However, two later studies suggested otherwise. These count-based methods failed to control the false discovery rates (Mallick *et al.*, 2021; Nearing *et al.*, 2021). Comparing the performance of 14 commonly used differential abundance tools on 38 16S datasets, Nearing *et al.* (2021) reported that conservative methods such as ALDEx2 and ANCOM produced the most consistent results across methods and studies. However, these methods suffer from low statistical power. If higher statistical power is needed, MaAsLin2 is recommended as a reasonable choice but at the potential cost of higher false discoveries. In agreement, Mallick *et al.* (2021) reported that MaAsLin2 preserved statistical power in the presence of multiple covariates and repeated measures while showing a reasonable control of false discoveries. Based on these results, we chose MaAsLin2 for the association analysis of microbiota data for its higher statistical power, good control of false discoveries and flexibility in handling complex experimental designs.

Methodological limitations

Limitations of short-read amplicon sequencing. High-throughput, accurate, short-read Illumina sequencing has been the standard way for 16S rRNA gene profiling. However, the short sequence size (100-500 bps) limits the taxonomic resolution of amplicon sequences, most of which can only be reliably annotated at the genus or higher taxonomic rank level. Moreover, the short sequence does not contain enough information to build well-resolved phylogeny. The error rate has been high in long-read sequencing platforms. The Pacific Biosciences (PacBio) has an error rate of 13% (Wagner *et al.*, 2016), and the Oxford Nanopore Technologies has an error rate of 5-25% (Volden *et al.*, 2018). However, recent advancements in sequencing technologies have enabled the application of highly accurate long-read sequencing in 16S rRNA gene profiling. The PacBio CCS (circular consensus

sequences) was reported to measure the full-length 16S rRNA gene with single-nucleotide resolution and a per-base error rate of ~0.04% (Callahan *et al.*, 2019). The combination of unique molecular identifiers (UMIs) with Oxford Nanopore Technologies was reported to yield full-length 16S rRNA sequences with a per-base error rate of ~0.004% (Karst *et al.*, 2021). Synthetic long reads assembled from UMI-tagged Illumina short reads were also reported to have a per-base error rate of ~0.005% when applied to sequence the full-length 16S rRNA gene (Callahan *et al.*, 2021). Highly accurate long-read sequencing provides higher taxonomic resolution (often species or even strain level) and well-supported phylogeny. As cost-efficiency improves, long-read sequencing should be considered a better alternative to the current standard Illumina short-read sequencing for the 16S marker gene survey.

Limitations of amplicon sequencing. Targeted amplicon sequencing is well suited for microbial community taxonomic profiling, especially when samples are heavily contaminated by host DNA. Although tools like PICRUSt/PICRUSt2 (Douglas *et al.*, 2020; Langille *et al.*, 2013) and Tax4Fun/Tax4Fun2 (Aßhauer *et al.*, 2015; Wemheuer *et al.*, 2020) have been developed for functional prediction from 16S amplicon sequence data, their performance is generally poor for non-human samples, and the prediction accuracy varies between gene function categories (Sun *et al.*, 2020). In addition, there could be a huge discrepancy between differentially abundant functions computed from predicted and actual metagenomics data (Douglas *et al.*, 2020). To gain functional insights into the microbiome data, one should resort to other omics technologies such as shotgun metagenomics (functional potential), metatranscriptomics (active functions), metaproteomics, and metabolomics.

Limitations of sequencing-based methods. Advancements in sequencing technologies have enabled rapid, in-depth taxonomic and functional profiling of microbial communities from diverse environments at an unprecedented scale. While sequencing-based analysis has become the mainstream method for studying microbiota, its limitations exist. First and foremost, the sequencing-based methods do not differentiate between living and dormant/dead cells. DNA molecules can persist in the environment for a long time (Levy-Booth *et al.*, 2007; Pietramellara *et al.*, 2009; Torti *et al.*, 2015). The sequenced DNA molecules may originate from relic DNA, dormant/dead cells, and viable cells, resulting in misinterpretation of results. A recent study in Atlantic salmon indicated that ~45% of OTUs identified in intestinal digesta originated from dead bacteria (Dvergedal *et al.*, 2020). This

result is particularly alarming given that we assume most of the DNA extracted from intestinal digesta originates from viable microbes. As DNA from non-living microbes confounds results, future studies should explore methods that distinguish living cells from dead/dormant cells in microbial ecosystems, such as viability PCR, and RNA sequencing (Emerson *et al.*, 2017).

Measurement bias is another major limitation of sequencing-based methods. Bias is introduced at different steps of the sample processing pipeline due to preferential measurement of some taxa over others, resulting in distorted measurements of true values (Brooks *et al.*, 2015; McLaren *et al.*, 2019). For instance, bacteria species differ in their resistance to lysing during DNA extraction (de Boer *et al.*, 2010; Nylund *et al.*, 2010; Salonen *et al.*, 2010; Walker *et al.*, 2015), which results in differential DNA extraction efficiency. Different primers are known to amplify or select against some groups of taxa over others, causing differences in the PCR amplification efficiency (Cai *et al.*, 2013; Klindworth *et al.*, 2013; Walters *et al.*, 2016; Wear *et al.*, 2018). Variations in the 16*S* rRNA gene copy numbers also lead to unequal representation of taxa in the samples (Kembel *et al.*, 2012; Louca *et al.*, 2018). As a result, the measured values may markedly deviate from the true values. This is exemplified by the low concordance between the expected and observed taxonomic composition of the mock community sequenced in the present work (**Paper II** and **IV**). The discrepancies between measured and actual values in sequencing-based experiments highlight the necessity of validating such results by absolute quantification techniques such as cultivation (if possible), qPCR, flow cytometry, and fluorescence in situ hybridization.

DISCUSSIONS: MAIN FINDINGS

Insect meal diets do not impair the intestinal health in Atlantic salmon

As indicated by its essential amino acid profile, BSF larvae meal is a good protein source for fish (Henry *et al.*, 2015; Hua, 2021). In accordance, the feed utilization and growth performance were similar in salmon fed the reference or insect meal diets in both feeding trials as published elsewhere (Belghit *et al.*, 2018; Belghit *et al.*, 2019b). Regarding the health aspects of salmon fed the insect meal diets, the histological examination of the intestine showed no clear evidence of adverse effects when the insect meal was included in the salmon diets (**Paper I** and **III**). Concordant with our results, most fish species have shown normal histological structures in the intestine when fed diets containing BSF larvae meal, although some studies reported shortening or atrophy of intestinal mucosal folds (Zarantoniello *et al.*, 2021). Nonetheless, salmon fed the insect meal diet containing 60% BSF larvae meal showed increased expression of genes indicative of stress (*hsp70*) and detoxification responses (*cpy1a1* and *mta*) (**Paper I**). These responses may be associated with the higher concentration of certain heavy metals in the insect meal diets such as cadmium and lead (Belghit *et al.*, 2018; Biancarosa *et al.*, 2019), which BSF larvae selectively enriched from the growing substrate (Diener *et al.*, 2015; Purschke *et al.*, 2017; Tschirner and Simon, 2015). The consequences of increased exposure to these heavy metals seemed benign on the intestinal health as no significant diet effects were observed for the expression levels of toxicity indicators such as *casp6* (cell apoptosis) and *mmp13* (tissue regeneration). The insect meal diet containing 60% BSF larvae meal also seemed to stimulate regulatory T-cells activity in the proximal and distal intestine, as indicated by the increased expression of *cd3γδ* and *foxp3* in these intestinal segments (**Paper I**). These results may suggest an oral tolerance to the insect meal diet (Rombout *et al.*, 2014) or immune modulation by bacteria taxa, such as *Paenibacillus* (**Paper II**), in salmon fed the insect meal diet (Furusawa *et al.*, 2013; Qiao *et al.*, 2020; Round and Mazmanian, 2010).

Insect meal diets ameliorate the intestinal steatosis in Atlantic salmon

A recent field survey revealed a high incidence of steatosis in the intestine of farmed salmon throughout the production cycle, especially during warm periods when the feed intake is high (Chikwati, Midtlyng, and Krogdahl 2018). In accordance, we found steatosis in both feeding trials. However, the steatosis in both feeding trials was less severe in salmon fed the insect meal diets, either containing 15% or 60% BSF larvae meal (**Paper I** and **III**). It is now known that steatosis represents a lipid transport disorder within the enterocytes, caused by deficiencies in major building blocks of lipoproteins such as phosphatidylcholine. Dietary supplementation of phosphatidylcholine or choline, an essential nutrient for *de novo* synthesis of phosphatidylcholine, effectively prevents steatosis in salmon fed low fish meal diets (Hansen *et al.*, 2020a; Hansen *et al.*, 2020b; Krogdahl *et al.*, 2020). Given that in both feeding trials feed intake and lipid digestibility were similar between the reference and insect meal diet group (Belghit *et al.*, 2018; Belghit *et al.*, 2019b), one possible explanation for the reduction in the steatosis is increased supplement of nutrients in the insect meal diets that are critical for the lipid transport across the enterocytes such as choline or phosphatidylcholine. Unfortunately, we did not measure the contents of these nutrients in the experimental diets. An alternative explanation is increased lipid peroxidation within enterocytes in salmon fed the insect meal diets due to the higher levels of medium-chained fatty acids, especially lauric acid (12:0), in the insect meal (Belghit *et al.*, 2019b). Medium-chained fatty acids are readily oxidized for energy production. They have been reported to reduce lipid storage in mammals and fish (Belghit *et al.*, 2019a; Nordrum *et al.*, 2003; Smith *et al.*, 2005; St-Onge and Jones, 2002; St-Onge *et al.*, 2008; Williams *et al.*, 2006). As steatosis represents a sub-optimal intestinal health status, with possible negative effects on dietary lipid uptake, the inclusion of BSF larvae meal in salmon diets may have functional benefits to the intestinal health besides its nutritional value.

Temporal and spatial variations of the Atlantic salmon intestinal microbiota

During the freshwater and early life stages in the seawater, salmon is commonly populated by bacterial taxa such as *Peptostreptococcus*, *Photobacterium*, and lactic acid bacteria, including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Vagococcus*, and *Weissella* in the intestine (Dehler *et al.*, 2017; Rudi *et al.*, 2018). As salmon continue to grow and mature in the seawater, only a few of these taxa persist, whereas taxa like *Aliivibrio*,

Brevinema, and *Mycoplasma* start to emerge and sporadically dominate the intestinal microbial community (Gupta *et al.*, 2019a; Gupta *et al.*, 2019b; Karlsen *et al.*, 2017; Llewellyn *et al.*, 2016; Lokesh *et al.*, 2019). In accordance with these observations, we identified *Aliivibrio*, *Brevinema andersonii*, and *Mycoplasma* as the core microbes of adult salmon in the seawater feeding trial (**Paper IV**). Our association analysis (**Paper IV**) and results from other studies indicate that these taxa may be associated with salmon growth and health (Cheaib *et al.*, 2021; Klakegg *et al.*, 2020a; Rasmussen *et al.*, 2021). For instance, Klakegg *et al.* (2020a) reported that probiotic bath with *Aliivibrio* strains improved the growth, reduced the mortality, and lowered the prevalence of ulcers in post-smolt salmon (Klakegg *et al.*, 2020a). Similar results were also observed in cleaner fish, lumpfish (*Cyclopterus lumpus*), after receiving a bath with two probiotic strains of *Aliivibrio* (Klakegg *et al.*, 2020b). Advancements in genome-resolved metagenomics will facilitate the functional analysis of these taxa in host nutrition and health (Cheaib *et al.*, 2021; Rasmussen *et al.*, 2021).

Besides the temporal variations, the salmon intestinal microbiota is also spatially heterogeneous in its composition. In line with previous findings (Gajardo *et al.*, 2016), we discovered minor microbiota variations between intestinal segments (**Paper II**), but clear differences between digesta- and mucosa-associated microbiota within the same intestinal segment (**Paper IV**). The substantial differences between the digesta- and mucosa-associated microbiota in seawater salmon may result from an increasing host selection pressure in the later life stages that determines which microbial clades colonize and flourish in the intestinal mucus layer (Heys *et al.*, 2020; Van den Abbeele *et al.*, 2011). This explanation is supported by our results showing that the microbial diversity was much lower in the mucosa, and that only a few taxa, i.e., *Brevinema andersonii* and unclassified *Spirochaetaceae*, were enriched in the mucosa (**Paper IV**). Notably, the digesta- and mucosa-associated microbiota differed in their response to insect meal diets (**Paper II** and **IV**); the mucosa-associated microbiota appeared more resilient to dietary perturbations than the digesta-associated microbiota (**Paper IV**), as reported in other studies in salmon (Gajardo *et al.*, 2017; Gupta *et al.*, 2019a; Jaramillo-Torres *et al.*, 2019). Taken together, these results have important implications for the experiment design when conducting a diet-microbiota study in fish. Firstly, collecting only digesta or mucosa samples or a homogenate of both can lead to biased or incorrect conclusions on the diet effects. To fully unveil the response of intestinal microbiota to dietary interventions, one should profile digesta- and mucosa-associated intestinal microbiota in parallel whenever feasible. Secondly, to increase the statistical power, resources may be better allocated by collecting more digesta and mucosa

samples from one intestinal segment (e.g., the distal intestine) than collecting less digesta and mucosa samples from multiple intestinal segments.

Insect meal diets modulate the Atlantic salmon intestinal microbiota

Despite a four-time difference in the BSF larvae meal inclusion level, the insect meal diets markedly modulated the salmon intestinal microbiota in both feeding trials resulting in consistent changes across different experiments (**Paper II** and **IV**). In both feeding trials, salmon showed higher microbial diversity in the intestinal mucosa when fed the insect meal diets (**Paper II** and **IV**). This finding corroborates results in rainbow trout fed diets containing 10-30% BSF larvae meal (Bruni *et al.*, 2018; Huyben *et al.*, 2019; Terova *et al.*, 2019). Our findings, showing in both feeding trials that a particular group of bacterial genera was enriched in salmon fed the insect meal diets (**Paper II** and **IV**), partly confirm results in other fish species. For instance, rainbow trout also have shown increased relative abundances of *Actinomyces*, *Bacillus*, *Brevibacterium*, *Corynebacterium 1*, *Enterococcus*, *Oceanobacillus*, and *Paenibacillus* in the intestine when fed diets containing 15% or 30% BSF larvae meal (Huyben *et al.*, 2019; Rimoldi *et al.*, 2021; Terova *et al.*, 2019). Similar observations have been made in Siberian sturgeon (*Acipenser baerii*) fed a diet containing 15% BSF larvae meal, inducing higher absolute abundances of *Bacillus* and *Enterococcus* (Jozefiak *et al.*, 2019). In this latter study, fluorescence in situ hybridization (*FISH*) was used for the bacteria quantification.

The observed diet effects can be explained by feed microbiota and perhaps dietary nutrients. We found evidence for the former because bacterial genera associated with the diet effects were present in the feed samples. Some of these taxa, such as *Actinomyces*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Enterococcus*, *Oceanobacillus,* and *RsaHF231*, may have originated from BSF larvae (Bruno *et al.*, 2019; Jiang *et al.*, 2019; Wynants *et al.*, 2019; Zheng *et al.*, 2013). Given the high temperature and pressure during the extrusion step in the feed production, most feed-associated microbes are expected to be inactive, i.e., dormant or dead. As DNA sequencing methods cannot differentiate between active and inactive microbes, it is unclear the extent to which the observed diet effects are attributable to the carry-over of inactive microbes and colonization of active microbes from the feeds. Methods such as viability PCR and RNA sequencing can reveal the answers (Emerson *et al.*, 2017). On the other hand, dietary nutrients in the insect meal diet, such as chitin, may have promoted the

growth of certain bacterial taxa. *Actinomyces* species are often identified as active chitin degraders, showing enhanced growth and activity upon chitin addition (Beier and Bertilsson, 2013). Many *Bacillus* species are well-known as chitin degraders (Cody, 1989). For instance, *Bacillus* was one of the predominant taxa in the intestinal mucosa of salmon fed a chitin-supplemented diet (5%), displaying the highest in vitro chitinase activity (Askarian *et al.*, 2012). This hypothesis can be tested by supplementing insect meal-specific nutrients to the same basal diet and sequencing the intestinal microbiota of salmon fed such diets.

MAIN CONCLUSIONS

Conclusions on methodology

- Positive and negative controls should be included in sequencing-based microbiota studies to obtain valid results. Sample collection and processing should be carefully planned to avoid confounding biological effects with batch effects.

- Given that feed-associated microbiota can be a confounding factor of diet effects, feed samples should be collected and analyzed when running a diet-microbiota study in fish.

- Stronger mechanical cell lysis, such as bead-beating with mixed-size beads, improves DNA extraction efficiency.

- Regarding samples for which it is difficult to obtain high-quality PCR products, DNA template dilution and PCR enhancers like BSA effectively improve the amplicon PCR performance.

- The ASV approach is not inherently better than the OTU clustering, nor is the reference-based fragment insertion always better than the *de novo* approach for constructing phylogeny. One should choose the method that is best suited for answering the research question.

- There is no consensus on which methods are the best, if any, for differential abundance testing of microbiota data. A possible way to obtain robust results is to report consensus results from several differential abundance testing tools that have shown good performance in benchmarking studies.

- Amplicon sequencing has limitations such as low taxonomic resolution, lack of functional insights, measurement biases, and inability to distinguish microbial cells that are active or not. Researchers should be aware of these methodological limitations and vigorously validate such results in new studies.

Conclusions on main findings

- The present study showed no clear evidence that insect meal diets containing 15% or 60% BSF larvae meal impaired the intestinal function or health in Atlantic salmon.

- The insect meal diets tended to improve the intestinal health status in Atlantic salmon by reducing excessive lipid deposition in the proximal intestine.

- The insect meal diet containing 60% BSF larvae meal modulated the expression of genes in the intestine, indicating changes in immune tolerance, stress response, and detoxification activity.

- The Atlantic salmon intestinal microbiota showed spatial heterogeneity in its composition. Microbiota variations between intestinal segments were minor but substantial between digesta and mucosa. Notably, the digesta- and mucosa-associated microbiota differed in their response to insect meal diets.

- In the pre-smolt Atlantic salmon, the microbial overlap was high between the intestine and feeds but low between the intestine and water.

- The insect meal diets modulated the Atlantic salmon intestinal microbiota, resulting in higher mucosal microbiota diversity and enrichment of a particular group of bacteria taxa dominated by members of the *Bacillaceae* family.

FUTURE PERSPECTIVES

The present thesis has generated new knowledge regarding the effects of insect meal diets on the intestinal function, health, and microbiota in Atlantic salmon. Nonetheless, some questions remain unresolved due to resource and methodological limitations. Additional studies will be needed to address the following research questions:

- What is the mechanism behind the reduced intestinal steatosis in salmon fed the insect meal diets?
- What are the functional implications of the intestinal microbiota changes in salmon fed the insect meal diets? Does it affect host health and disease resistance?
- Is the feed microbiota a confounding or contributing factor to the observed diet effects? Or both?
- Why are members of the *Bacillaceae* family enriched in salmon fed the insect meal diets? Is it related to chitin degradation?

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APPENDIX: PAPERS I-IV

I

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Full length article

Gut health and vaccination response in pre-smolt Atlantic salmon (*Salmo salar*) fed black soldier fly (*Hermetia illucens*) larvae meal

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ABSTRACT

Limited availability of sustainable feed ingredients is a serious concern in salmon aquaculture. Insects may become an important, sustainable resource for expanding the raw material repertoire. Herein, we present data from an 8-week feeding trial with pre-smolt Atlantic salmon (initial body weight 49 ± 1.5 g) fed either a reference diet containing fish meal, soy protein concentrate and wheat gluten as protein sources, or a test diet wherein 85% of the protein was supplied by black soldier fly larvae meal. Possible diet effect on the systemic immune response was evaluated by measuring plasma antibody titers after vaccination against infectious pancreatic necrosis virus (IPNV). The gut health of fish was evaluated using endpoints including organ and tissue indices, histopathological parameters and gene expression. Both diets induced the same level of antibody responses against IPNV. In fish fed the reference diet, the histological examination of the pyloric caeca mucosa showed clear hyper-vacuolization suggestive of lipid accumulation in enterocytes, whereas this was less pronounced in the insect meal fed fish. Expression of genes relevant to lipid metabolism confirmed these histological findings. Immune and barrier-function gene expression profiles were both generally not affected by diet. However, the fish fed insect meal showed increased expression of genes indicative of stress response, immune tolerance and increased detoxification activity. In summary, our results showed no indications that dietary inclusion of insect meal affected the gut health of Atlantic salmon negatively. The insect meal based diet seemed to reduce excessive lipid deposition in the pyloric caeca and stimulate xenobiotic metabolism.

1. Introduction

The world's wild fish catches seem to have reached their limit, whereas fishmeal and fish oil demands continue to grow, mostly due to the rapid growth in aquaculture. Therefore, marine ingredients in salmon diets have been gradually replaced by plant ingredients, decreasing from ~90% in 1990 to ~35% in 2012 [1]. Of the plant-based protein sources, 58% was from soy protein concentrate (SPC) followed by sunflower expeller (16%), wheat gluten (16%), fava beans (5%) and other marginally used plant proteins. However, some of these plant-based ingredients cause proliferative or inflammatory conditions in the intestinal mucosa as well as changes in host immune responses and gut enzymatic profiles, while others have been reported to increase fish susceptibility to various diseases [2–5]. Although the future availability of SPC is guaranteed in the short-term [6], there is a need for new nutrient sources in salmon aquaculture to cope with the increasing demand. Moreover, as the world population is projected to reach 9 billion in 2050, global food production must maximize nutritional output for human consumption while minimizing the input of resources [1]. Salmon feed producers need to reduce their dependency on terrestrial plant products that may be used directly for human consumption. Hence, several novel feed ingredients have emerged as promising candidates such as bacterial meal (*Methylococcus capsulatus*) [6], yeasts [7] and insects [8].

Insects are promising alternative protein sources as they possess an outstanding capacity to upgrade low-quality organic material, require minimal water and cultivable land, and emit little greenhouse gases [9]. Among species with the greatest potential for large-scale feed production are black soldier fly (BSF; *Hermetia illucens*), yellow mealworm (*Tenebrio molitor*), silkworm (*Bombyx mori*) and common housefly (*Musca domestica*) [10]. Black soldier fly has been produced at industrial scale in Europe due to its exceptionally good nutritional value

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and suitability for massive production [11–14]. On a dry matter basis, BSF larvae contain about 42% protein and 35% lipid [12]. In a survey of the nutritional composition of various insects belonging to the order Diptera, Orthoptera and Coleoptera, Barroso et al. found that BSF prepupae possessed an essential amino acid profile closest to fishmeal [14]. While the amino acid profile of insects is genetically determined and stable, the fat level and fatty acid profile are diet-dependent [13]. The potential of BSF larvae as a feed ingredient has been evaluated in several carnivorous and omnivorous fish species [13,15–25]. The optimal inclusion level of BSF larvae meal in the diet seems to vary considerably among fish species. For Atlantic salmon, results by Lock et al. have indicated that all fish meal could be replaced by BSF larvae meal without affecting the salmon growth performance or sensory quality [25]. Together, these results suggest that BSF is a realistic protein source for the salmon aquaculture.

A prerequisite for introducing a new ingredient into fish feed is convincing evidence that it will not compromise fish health. Intestinal inflammation and lipid malabsorption are two frequently observed gut health problems associated with increased use of plant-based salmon feed [4,26–29]. How feed ingredients of insect-origin may affect gut health and function is one of the main research questions that must be answered before commencing on full-scale commercial production. One of the immunological reactive compounds in insect meals is chitin, an essential component of the insect cuticle also found in bacterial and fungal cell walls. Chitin polymers (40–70 μm) act as pathogen associated molecular patterns (PAMPs) that bind pattern recognition receptors (PRRs) on various antigen presenting cells stimulating the production of various cytokines and immune mediators [30]. It may also interfere with protein and lipid digestion. However, positive effects such as growth promotion and immune-stimulation in fish have been reported [31,32]. Although impacts of BSF dietary inclusion on growth performance, sensory, feed conversion ratio and several other factors have been investigated in different fish species [13,15,20,33], its effect on gut health, function and host immune response has not been elucidated. Hence, the objectives of this study with Atlantic salmon are to: (i) evaluate the morphological and organosomatic changes induced by BSF in the gut, (ii) profile host immune gene expression evoked by BSF, (iii) and to evaluate the impact of BSF on antibody responses induced by parenteral vaccination.

2. Materials and methods

2.1. Diets and fish husbandry

An 8-week feeding trial was conducted at the Cargill AquaNutrition (former EWOS Innovation) experimental facility at Dirdal, Norway, in accordance with laws regulating the experimentation with live animals in Norway. A total of 800 mixed-gender pre-smolt Atlantic salmon with a mean initial body weight of 49 g (1.5 g SEM) were randomly assigned into 8 fiberglass tanks (450 L, 100 fish per tank) supplied with running freshwater. Quadruplicate tanks of fish were fed either a reference diet (REF) containing fish meal, soy protein concentrate and wheat gluten as protein sources, or an insect meal based diet (IM) wherein 85% of the protein was supplied by black soldier fly larvae meal, replacing the bulk of fish meal and soy protein (Table 1). The fish were fed continuously by automatic disk feeders under a photoperiod regimen of 24 h daylight. During the feeding trial, water temperature stabilized around 13.7 °C. Further details on insect meal and diet composition (amino acids, fatty acids and minerals) were reported elsewhere [34].

Table 1
Formulation of the experimental diets*.

Ingredients (g/100 g)	REF	IM
Fishmeal LT94	35.0	6.0
Insect meal	0	60.0
Soy protein concentrate	29.6	5.0
Wheat gluten	14.3	14.4
Fish oil	4.6	6.9
Rapeseed Oil	12.0	4.8
Vitamin & mineral premix	0.3	0.3
Yttrium	0.2	0.2
Miscellaneous	4.0	2.4
Chemical composition		
DM (%)	94	96
Crude lipid (%)	18	22
Crude protein (%)	47	44
Carbohydrates (%)	11	12
Ash (%)	8	7
Gross energy (MJ Kg^{-1} DM)	22	23
TBARS (nmol g^{-1})	7	17

*REF, reference diet; IM, insect meal diet; DM, dry matter; TBARS, Thiobarbituric acid reactive substances.

2.2. Sample collection

At the termination of the feeding trial, fish were randomly taken from the tanks, anesthetized with tricaine methanesulfonate (MS222) (80 mg/L; Pharmaq AS, Oslo, Norway) and euthanized by a sharp blow to the head before tissue sampling. The body weight was recorded for all the fish sampled. Subsequently, the whole digestive tract was dissected from three fish per tank, cleaned of attached adipose tissue and opened longitudinally. Only fish with chyme present along the whole intestine were sampled to ensure exposure to the diets until the point of sampling. The chyme was removed using a spatula. The emptied intestine was divided into proximal (PI), mid (MI) and distal (DI) segments and weighed respectively. The gut tissue was rinsed in phosphate buffered saline (PBS) three times to remove traces of remaining chyme and cut into pieces for RNA extraction (preserved in RNAlater solution, incubated at 4 °C for 24 h and stored at −20 °C) and histological evaluation (fixed in 4% phosphate-buffered formaldehyde solution for 24 h and transferred to 70% ethanol for storage).

2.3. Vaccination and ELISA

To investigate possible diet effect on the systemic immune response, another 20 fish were randomly selected from each tank and intraperitoneally injected with 0.1 mL of inactivated whole viral vaccine based on the highly immunogenic strain of infectious pancreatic necrosis virus (IPNV) [35]. Due to constraints on the availability of experimental facilities, the quadruplicate tanks per diet group were reduced to duplicate tanks per diet with 40 fish each. The fish were fed the same diets for another six hundred degree days. Thereafter, blood samples were taken from the caudal vein of 20 fish per tank using heparinized syringes equipped with 22 G needles (35 IU heparin per mL; S-Monovette®, SARSTEDT, Germany) and placed on ice before plasma collection. Plasma was collected after centrifugation at 2000 g for 10 min (4 °C) and snap-frozen in liquid N_2 for the determination of antibody level against IPNV.

The vaccine was prepared after culturing IPNV in Asian Grouper-strain K cells [36] at a concentration of 1×10^9 $TCID_{50}$/mL as previously standardized [37,38]. The virus suspension was inactivated using 0.5% formaldehyde using a magnetic stirrer for 48 h followed by dialysis for 48 h. The vaccine was prepared as a water-in-oil (w/o) formulation using the ISA 763 VG (SEPPIC, France) adjuvant following manufacturer's recommendation. Emulsification of the viral antigen with adjuvant was done as previously described [35].

The enzyme linked immunosorbent assay (ELISA) used to evaluate antibody responses was carried out as previously described [39]. Briefly, ELISA plates (Immunoplates, Nunc Maxisorb, Denmark) were coated with rabbit anti-IPNV designated as K95 [40] in coating buffer and were incubated overnight at 4 °C. After washing using PBS containing 20% Tween (PBST) thrice, plates were blocked using 5% dry milk at room temperature (RT) for 2 h. Thereafter, 0.5% formalin

inactivated IPNV antigen at a concentration of 1×10^5 $TCID_{50}$/mL was added to each well and incubated for 2 h at room temperature after washing thrice using PBST. Test plasma diluted in a twofold dilution starting from 1:10 to 1:320 was added to each well together with high and low positive controls as well as plasma and blank (1% dry milk PBST) controls. The plates were incubated overnight at 4 °C after adding diluted plasma samples. A mouse monoclonal anti-salmon antibody designated as 4C10 [41] targeting IgM heavy chain was diluted at 1:5000 and added to each well after washing three times using PBST. After incubation for 1 h, all plates were washed and goat-anti-mouse antibody conjugated with horse-radish peroxidase (DAKO; Glostrup, Denmark) was added to each well and incubated for 1 h at RT. After washing, substrate-containing OPD (O-phenylenediamine dihydrochloride, DAKO) constituted at manufacturer's recommendation was added to each well. Results were read using spectrophotometer (TECAN, Genios, Boston, USA) at 490 optic density wave length.

2.4. Organosomatic indices

Organosomatic indices (OSI) of the PI, MI and DI were calculated as the percentages of the weight of intestinal segments relative to the fish body weight; OSI = 100 * TW/BW, where TW is the tissue weight and BW is the fish body weight.

2.5. Histology

After fixation, PI, MI, and DI samples were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin according to standard histological techniques. The paraffin blocks were placed on a cooling block before cut by an automatic microtome (HM 355S, Thermo Scientific™, US) to produce sections of 3 μm thickness. The slides were then stained by hematoxylin and eosin, and examined blindly with a light microscope (Axio Scope.A1, Zeiss, Germany) equipped with a camera (AxioCam ICc 3, Zeiss) paying attention to typical inflammatory morphological changes observed in salmonid intestine fed soybean meal diets: that is, shortening and fusion of mucosal folds, cellular infiltration within the lamina propria and submucosa, enterocyte vacuolization and nucleus position disparity. Normally, little to no vacuolization is present in the enterocyte of the PI and MI whereas enterocytes of the DI show various degrees of supranuclear vacuolization that diminishes or disappears during the inflammation. For each histological characteristic evaluated, a value of normal, mild, moderate, marked or severe was assigned.

2.6. Quantitative real-time PCR

Real-time qPCR assays were performed following the MIQE guidelines [42]. Total RNA was extracted from PI and DI samples on a Biomek® 4000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA, USA) using a Maxwell® HT simplyRNA Kit (Custom) (Premage, Madison, WI, USA) according to the manufacturer's protocol. RNA purity and concentration were measured using Epoch Microplate Spectrophotometer (BioTeK Instruments, Winooski, VT, USA) and the integrity was verified by the 2100 Bioanalyzer with the 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA, USA). The mean A_{260}/A_{280} ratio was 2.3 (S.D. = 0.01) and the average RIN (RNA integrity number) value was 8.4 (S.D. = 0.76). First-strand complementary DNA (cDNA) synthesis was performed using 1.0 μg total RNA from all samples using a Superscript™ IV VILO™ cDNA synthesis kit (catalog no., 11756050) (Invitrogen, Carlsbad, CA, USA). A negative control was set up by omitting RNA and the obtained cDNA was diluted 1:10 before use. The qPCR primers were obtained from the literature or designed using the Primer 3 (http://frodo.wi.mit.edu/primer3/). Primer efficiency was determined using 2-fold serial dilutions of randomly pooled cDNA. The qPCR assays were performed using the LightCycler 96 (Roche Applied Science, Basel, Switzerland) and a 10-μL reaction volume was used, which contained 2 μL of PCR-grade water, 2 μL diluted cDNA template, 5 μL LightCycler 480 SYBR Green I Master (Roche Applied Science) and 0.5 μL (10 μM) of each forward and reverse primer. Samples were run in duplicates in addition to a no-template control for each gene. A three-step qPCR programme was applied incorporating an enzyme activation step at 95 °C (5 min) and 45 cycles of 95 °C (10 s), 55–62 °C (10 s) and 72 °C (15 s). The plate pipetting was done using the Biomek® 4000 automation workstation. Quantification cycle (Cq) values were determined using the second derivative method. The specificity of qPCR amplification was confirmed by evaluating the melting curve of qPCR products and the band pattern on the agarose gel after electrophoresis. Beta-actin (*actb*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), RNA polymerase 2 (*rnapo2*) and hypoxanthine phosphoribosyltransferase 1 (*hprt1*) were evaluated for use as reference genes according to their stability across and within the treatments [43]. The expression of target genes in the PI was normalized to the geometric mean of *actb*, *rnapo2* and *hprt1*. For DI samples, the geometric mean of *gapdh* and *rnapo2* was used for the normalization. The mean normalized expression of the target genes was calculated from raw Cq values [44]. The genes profiled and the primers used for the qPCR assays are given in Table S1.

2.7. Statistics

Unless specified, statistical analyses were performed in JMP Pro 13.0.0 (SAS Institute, United States). For continuous variables, data were fitted to linear mixed model treating diet as fixed effect and tank as random effect. The homoscedasticity and normality of residuals was assessed visually by checking the "residual by predicted" plot and histogram, respectively. When necessary, data were subjected to box-cox power transformation to meet the model assumptions and refitted again. For ordinal variables, data were initially fitted to cumulative link mixed model implemented by the R package *ordinal* [45], treating diet as fixed effect and tank as random effect. As the full model resulted in high Hessian number and the random effect was not significant, the tank effect was dropped from the full model. The proportional odds assumption was checked by the nominal test. Spearman's rank correlation analysis was carried out to discover associations between variables of interests. Multiple comparisons were adjusted by the Bonferroni correction or adaptive two-stage Benjamini-Hochberg procedure where applicable. Data were presented as mean ± SEM. Statistically significant results were indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

3. Result

To aid readers in interpreting data reported here, results on general fish performance and nutrients utilization, which have been published elsewhere [34], are summarized below.

In brief, no differences between the diet groups were recorded for feed intake, feed conversion ratio, body weight gain or protein productive value, despite the test group showing a lower protein digestibility. Higher condition factor, hepatosomatic and viscerosomatic indices were observed in fish fed the IM based diet. Regarding gut function, no diet effect on chyme trypsin activity or bile salt level was noted, but the brush border enzyme, leucine aminopeptidase, showed lower activity in the IM diet group.

3.1. Antibody responses

No significant difference was observed between the two groups for the plasma antibody level against IPNV ($p > 0.05$) (Fig. 1).

3.2. Somatic indices of intestinal sections

No significant diet effect was observed for PI-somatic index (PISI).

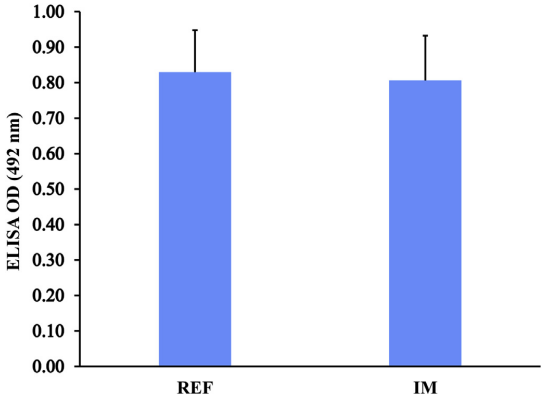

Fig. 1. Plasma antibody level against IPNV of salmon presmolts fed the experimental diets. REF, reference diet; IM, insect meal test diet. The results were obtained from plasma diluted 1:40.

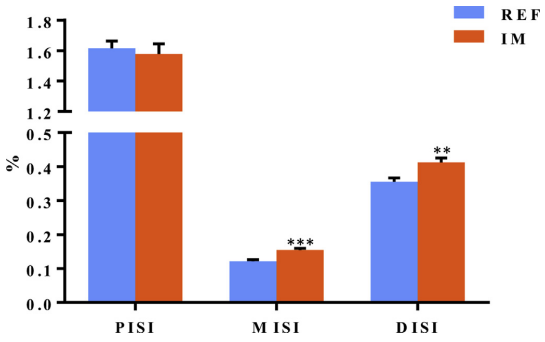

Fig. 2. Intestine-somatic indices of salmon presmolts fed the experimental diets. PISI, proximal intestine-somatic index; MISI, mid intestine-somatic index; DISI, distal intestine-somatic index. REF, reference diet; IM, insect meal test diet. Asterisks denote statistically significant differences between the diets (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

However, both MI-somatic index (MISI) and DI-somatic index (DISI) were significantly higher in fish fed the IM diet (Fig. 2) compared to those fed the reference diet.

3.3. Histological appearance

In the PI, increased vacuolization or hyper-vacuolization of the enterocytes, suggestive of lipid accumulation (steatosis), was observed in fish from both diet groups, but was significantly less prevalent in fish fed the IM diet (Fig. 3). The MI and DI showed normal and healthy histological structure for all sampled fish.

3.4. Gene expression

The results are presented as fold change of gene expression level in fish fed the IM diet relative to those fed the REF diet (Fig. 4). Correlations between fold changes of functionally-related genes are shown in Table 2.

In the PI, IM inclusion had minor effects on the expression profile of immune genes. The exception was the increase in expression of *cd3*$_{\gamma\delta}$ and *foxp3*. Similarly, no effect of diet was observed on the expression of tight junction genes *cldn15* or *cldn25b*. In support of the histological observation, there was a 2-fold decrease in the expression of *plin2*, a

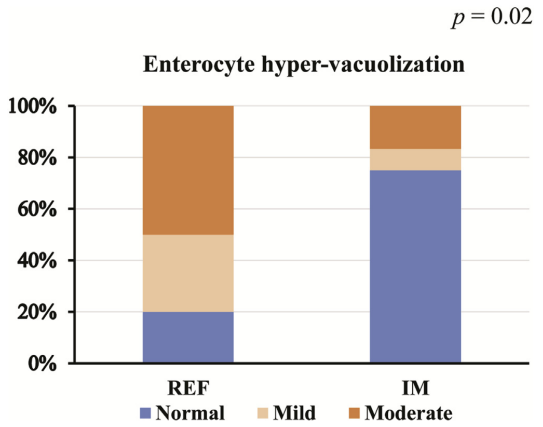

Fig. 3. Contingency chart showing proportions of sampled individuals scored normal, mild, or moderate (none scored above moderate) regarding hyper-vacuolization of enterocytes in the PI. REF, reference diet; IM, insect meal test diet. The *p* value of the diet effect was given. For the illustration of enterocyte hyper-vacuolization at various degrees, one can refer to Fig. S1.

surface marker of lipid droplets. Genes involved in cholesterol uptake (*npc1l1*) and de novo synthesis (*srebp2* and *cyp51*) showed increased expression, and so were those responsible for the absorption (*cd36*) and intracellular transportation (*fabp2b*) of fatty acids. Most of the detoxification response relevant genes were up-regulated, which included *cyp1a1* (a marker for xenobiotic metabolism), *mta* (a marker for heavy metal detoxification), *hsp70* (a marker for stress response), *sod1* (an indicator of oxidative stress) and *cat* (an indicator of oxidative stress). However, no correlations were found between *cyp1a1* and other genes profiled under the same category.

In the DI, less differences in the gene expression were observed, most of which showed similar responses to those observed in the PI. These genes included *cd3*$_{\gamma\delta}$, *foxp3*, *pcna*, *cyp1a1* and *cat*. Notably, the increased expression of *cd3*$_{\gamma\delta}$ showed a strong positive correlation with that of *foxp3*. *Cyp1a1* also showed an increase in the mRNA level of more than 2 folds in fish fed the IM diet. In contrast to what we found in the PI, the expression of *plin2* was increased, coinciding with a marked induction of *apoa4* expression.

4. Discussion

In summary, the main findings of the present study showed that dietary inclusion of IM as high as 60% did not cause appreciable negative effects on the gut health of Atlantic salmon. Its inclusion, however, reduced enterocyte hyper-vacuolization in the pyloric caeca, and seemed to stimulate regulatory T cell activity as well as xenobiotic metabolism both in the proximal and distal intestine.

The inclusion of certain legume-based alternative feed ingredients in salmon feed is known to cause proliferative or inflammatory conditions in the intestinal mucosa, resulting in compromised fish health and welfare [4,27]. In the present study, no signs of local inflammatory response to the IM inclusion was noted. Insect meal inclusion seemed however to stimulate regulatory T cell activity both in the proximal and distal intestine, as indicated by the increased expression of both *cd3*$_{\gamma\delta}$ and *foxp3* in these gut segments. It has been shown that the expression pattern of some cytokines that enhance T-cell responses are prone to induce oral tolerance to vaccination while others are not [46]. As a transcription factor for the differentiation of naïve CD4 T-cells into T-regulatory genes (T-regs), *foxp3* is the main marker of oral tolerance induced by prolonged exposure to the same antigenic proteins through

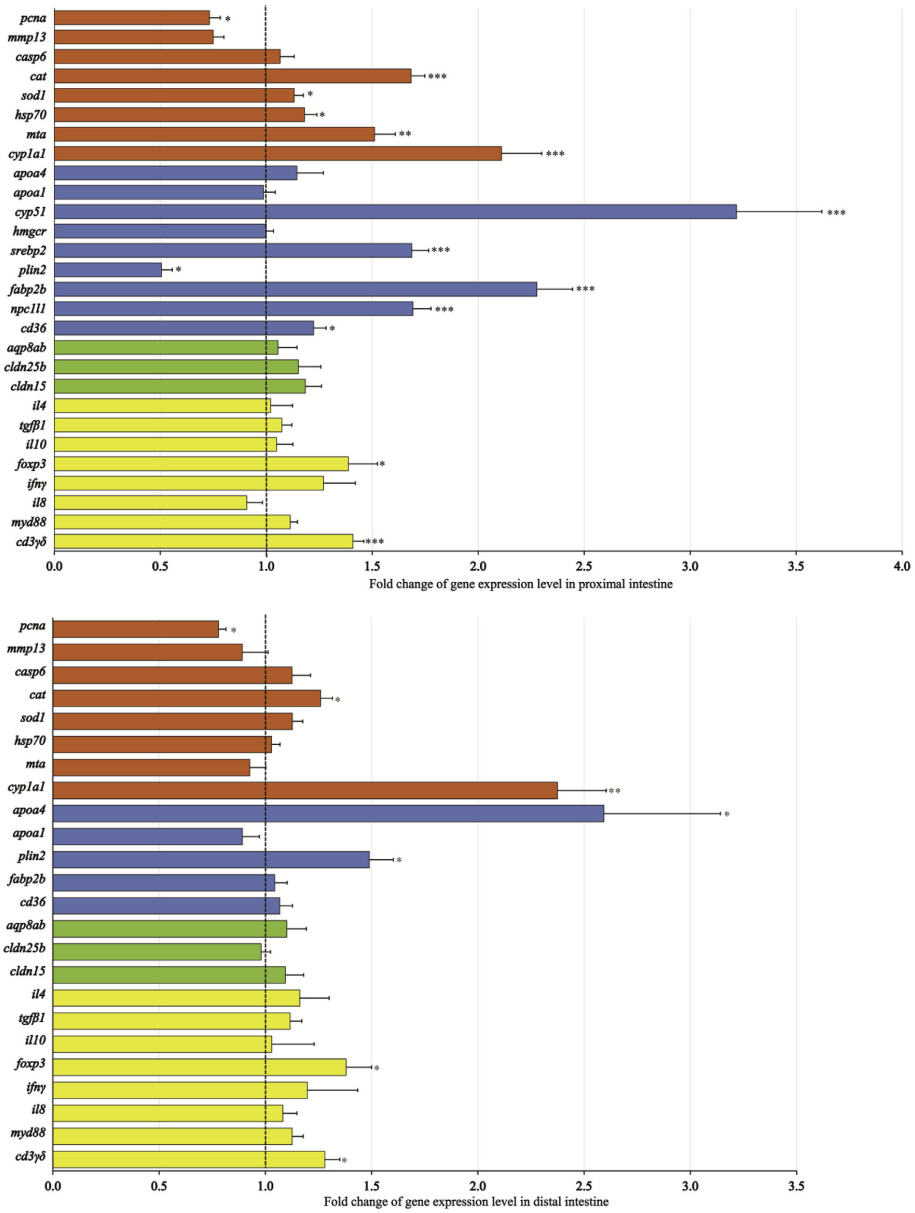

Fig. 4. The fold change of gene expression level in proximal and distal intestine of fish fed the IM diet relative to those fed the REF diet. The fold change was calculated by dividing the gene expression level of individual fish fed the IM diet by the mean expression level of fish fed the REF diet. Hence, the error bar indicates the variation in gene expression level in fish fed the IM diet. Values > 1 indicate an increased gene expression level in the IM fed fish, and vice versa. Genes relevant to immune response, barrier function, lipid metabolism and xenobiotic metabolism are shaded in yellow, green, blue and orange, respectively. For the explanation of gene abbreviations, see Table S1. *P* values of test statistics were adjusted by the Benjamini-Hochberg procedure for the proximal and distal intestine, respectively. Asterisks denote statistically significant differences between the diets (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the feed. Its overexpression has been linked to significant loss of systemic immune responses to vaccination [47,48]. As pointed out by other scientists, oral tolerance through feed can be initiated by tolerogenic protein molecules [49–51]. Oral tolerance following vaccination has been reported in different fish species and it can reduce immune protection [52–56]. Hence, there is a need for follow-up studies using oral vaccines to determine whether the larvae meal from black soldier fly produces tolerogenic proteins that evoke increased expression levels

Table 2
Correlations between fold changes of functionally related genes*.

Variables of interest	Spearman ρ	*p* value	Adjusted *p* value
PI			
cd3$_{\gamma\delta}$ & foxp3	0.63	0.0280	0.0583
cd36 & fabp2b	0.15	0.6482	0.5834
cyp1a1 & mta	0.11	0.7452	0.5868
cyp1a1 & hsp70	0.28	0.3715	0.4393
cyp1a1 & sod1	−0.61	0.0370	0.0583
cyp1a1 & cat	−0.26	0.4184	0.4393
sod1 & cat	0.87	0.0002	0.0013
srebp2 & cyp51	0.80	0.0019	0.0060
DI			
cd3$_{\gamma\delta}$ & foxp3	0.95	0.0001	0.0003
cyp1a1 & cat	0.12	0.7129	1.0000
apoa4 & plin2	0.34	0.2861	0.8583

*p values of test statistics for the PI were adjusted by the Benjamini-Hochberg procedure whereas those for the DI were adjusted by the Bonferroni correction. PI, proximal intestine; DI, distal intestine.

of *foxp3*, which could culminate in tolerance. On the other hand, previous studies have shown divergent and size-dependent effects of chitin on immune functions in mice cell models [57]. Small chitin molecules (< 40 μm), in particular, have been observed to induce production of anti-inflammatory cytokine *il10* [58]. In most cases, *il10* upregulation corroborates with increased expression of *tgf-β*, which is a regulatory cytokine for *foxp3* in T-regs. Whether the increased expression of *cd3$_{\gamma\delta}$* and *foxp3* may be associated with exposure to chitin, given that chitin has tolerogenic properties, deserves further investigation.

Dietary composition has been shown to influence the outcome of antibody responses in vaccinated fish. For example, Burrells et al. [59] showed elevated antibody responses against *Aeromonas salmonicida* in a diet containing novel protein supplements added to a standard commercial diet (EWOS VEXTRA Alpha®). On the contrary, Erdal et al. [60] observed decreased antibody levels against *Yersinia ruckeri* in fish fed higher levels of dietary ω-3 fatty acids. In the present study, there was no significant difference observed between the groups fed the REF and IM diets in antibody response against IPNV. These findings suggest that the two feeds have a similar impact on the inactivated whole vaccine against IPNV and that replacing the REF diet with the IM diet would not alter the protective ability of the vaccines. Given the increasing demand for oral vaccines in salmon aquaculture, future studies should investigate the effect of the BSF diet on enhancing mucosal immunity using oral vaccines.

The proximal intestine of IM-fed fish displayed increased expression of genes involved in uptake of fatty acids (*cd36*, *fabp2*) and cholesterol uptake and de novo synthesis (*npc1l1*, *srebp2*, *cyp51*). Associations between differential expression of fatty acid uptake genes and reductions of enterocyte hyper-vacuolization have been reported previously [61]. Our observations in the current work could reflect a generally increased uptake, turnover and transport of fatty acids in IM-fed fish. Several previous studies on salmon have also demonstrated the close relationship between dietary sterol levels and expression of genes related to cholesterol uptake and synthesis [61–64]. In the present study, the increased expression of genes related to cholesterol metabolism was likely caused by the different sterol composition of the two feeds, in which the IM diet contained about 50% less cholesterol but much higher phytosterol levels than the REF diet (unpublished data).

The use of plant ingredients in Atlantic salmon diets has been linked to increased hyper-vacuolization of enterocytes in the proximal intestine [61]. This hyper-vacuolization is apparently reflecting an abnormal lipid droplet accumulation within the enterocytes, i.e. steatosis [65–67]. The mechanism underlying the intracellular lipid accumulation is currently unknown, but has previously been proposed to involve a disrupted assembly of lipoproteins, possibly caused by lack of major building blocks, such as cholesterol, phosphatidylcholine and/or apolipoproteins [61]. In severe cases, the hyper-vacuolization may be accompanied by large accumulations of lipidic material in the intestinal chyme and is referred to as lipid malabsorption, eventually resulting in steatorrhea and the so-called “floating feces” on the surface of sea cages [28,29]. In the current study, the replacement of fishmeal and plant-based protein sources with IM resulted in less lipid accumulation within proximal intestinal enterocytes, observed as less prevalent enterocyte hyper-vacuolization. This was reflected in the marked suppression of *plin2* (also known as adipophilin) mRNA levels, a surface marker of lipid droplets [68], in the proximal intestine of fish fed the IM based diet. The reason for the reduction in enterocyte hyper-vacuolization in IM-fed fish is not clear but could be related to lower levels of plant ingredients (i.e. SPC and rapeseed oil) and/or actions of certain bioactive components present in the IM. It should be noted that there were no macroscopic appearances of lipid malabsorption in any fish at the time of sampling, and no apparent indications of reduced fish health as a result of the hyper-vacuolization.

To assess the exposure to toxic substances in fish, some useful biomarkers have been suggested which include, but are not limited to, biotransformation enzymes (Cyp1a1), anti-oxidant enzymes (Sod, Cat), stress proteins (Hsp70) and metallothioneins (Mta; a specific marker for exposure to metals) [69]. Of note, the phase I biotransformation enzyme, Cyp1a, is among the most sensitive biomarkers and has been validated in numerous studies in fish including Atlantic salmon [70–72]. In the present study, a more than 2-fold increase in the expression level of *cyp1a1* was found in the distal intestine of IM fed fish, while other marker genes showed no expression differences. Despite a lack of significant correlations in the expression levels between *cyp1a1* and other marker genes, their increased mRNA levels in the proximal intestine of fish fed the IM diet is indicative of elevated detoxification response to the diet. The responses may partially associate with the higher concentration of certain heavy metals in the IM diet such as nickel, cadmium, and lead [34], the latter two of which were found to be selectively enriched in the BSF larvae from the growing substrate [73–75]. However, the consequences on gut health of increased exposure to heavy metals seemed mild, as no significant diet effects were observed for toxicity indicators such as *casp6* (cell apoptosis) and *mmp13* (tissue regeneration), a finding in agreement with the absence of abnormalities on histological appearance and organ indices of the gut tissue.

In conclusion, our results showed no indications that the insect meal-based diet negatively affected the gut health of Atlantic salmon. On the contrary, the diet tended to improve gut function by reducing excessive lipid deposition within the enterocytes of the pyloric caeca. Stimulation of xenobiotic metabolism in the proximal intestine could possibly be a response to increased exposure to heavy metals selectively enriched by BSF larvae, such as cadmium and lead. Upregulation of Foxp3, a molecular marker for immune tolerance, calls for further investigations using oral vaccines to determine whether BSF has tolerogenic properties. The similarity in plasma IgM levels against IPNV indicated that BSF can safely replace fishmeal without inducing adverse effects that would reduce antibody responses for parenteral vaccines.

Conflicts of interest

The authors declare no competing financial interest.

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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.2018.12.057.

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II

Consistent changes in the intestinal microbiota of Atlantic salmon fed insect meal diets

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Background: Being part of fish's natural diets, insects have become a realistic, sustainable feed ingredient for aquaculture. While nutritional values of insects have been extensively studied in various fish species, their impact on the fish microbiota remains to be fully explored. In an 8-week freshwater feeding trial, Atlantic salmon (*Salmo salar*) were fed either a commercially relevant reference diet or an insect meal diet wherein black soldier fly (*Hermetia illucens*) larvae meal comprised 60% of total ingredients. Microbiota of digesta and mucosa origin from the proximal and distal intestine were collected and profiled along with feed and water samples.

Results: The insect meal diet markedly modulated the salmon intestinal microbiota. Overall, the microbial diversity was lower in the digesta of salmon fed the insect meal diet but higher in the mucosa. A group of bacterial genera, dominated by members of the *Bacillaceae* family, was enriched in salmon fed the insect meal diet, which confirms our previous findings in a seawater feeding trial. We also found that microbiota in the intestine closely resembled that of the feeds but was distinct from the water microbiota. Notably, bacterial genera associated with the diet effects were present in the feeds as well.

Conclusions: We conclude that salmon fed the insect meal diets show consistent changes in the intestinal microbiota. The next challenge is to evaluate the extent to which these alterations are attributable to feed microbiota and dietary nutrients and what these changes mean for fish physiology and health.

Atlantic salmon | Insect meal | Black soldier fly | Intestinal microbiota | Feed microbiota | Water microbiota

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Background

The global population is projected to reach 9.7 billion in 2050 (1), requiring an increase in the food supply by 25-70% (2). To fulfil this demand, the food production sector must minimize resource input and maximize nutritional outputs for human consumptions. Atlantic salmon, *Salmo salar*, is the most produced marine fish species and one of the most economically important farmed fish worldwide (3). Human-edible plant feedstuffs are the main ingredients used in modern salmon feeds (~70%) (4). To secure sustainable developments, salmon farming needs to decrease its dependency on human-edible feedstuffs and incorporate unexploited feed resources in its raw material repertoire. So far, possible candidates include insects (5), macroalgae (6), and single-cell organisms such as bacteria, yeasts, and microalgae (7). In terms of sustainability, insects are a promising candidate. They possess a remarkable capacity to upgrade low-quality organic materials, require minimal water and cultivable land, and emit little greenhouse gases (8). One insect species with the potential as an alternative protein source for salmon aquaculture is the black soldier fly (*Hermetia illucens*), produced at an industrial scale for its good nutritional value (9). Feed conversion ratio, growth performance, fish health, sustainability and price/availability are primary concerns when evaluating the performance of alternative feed ingredients. While the nutritional value of black soldier fly larvae meal has been extensively evaluated in various fish species, including Atlantic salmon (10–16), its influence on fish health remains largely unexplored.

The intestine is the first organ exposed to the diet and of pivotal importance for the growth, development, and protection against pathogens. A well-functioning, healthy intestine is the key to convert feed into fish biomass efficiently. It is now well established that the intestinal microbiota is, in various ways, closely connected to intestinal function and health (17–21). Diet is arguably one of the most important environmental factors shaping intestinal microbiota (22–24). Different dietary components may selectively induce compositional and functional alterations of the intestinal microbiota, which in turn could inflict important implications on the host health and disease resistance (19, 24–26).

Characterizing the response of intestinal microbiota to dietary shifts and its associations with host responses is a critical step towards identifying key microbial clades for promoting fish health and welfare. The main aims of the work presented herein were (i) to compare intestinal microbiota of Atlantic salmon fed a commercially relevant reference diet and an insect meal-based test diet, and (ii) to identify potential associations between intestinal microbial clades and host responses. This work was part of a larger study consisting of a freshwater and a seawater feeding trial. The present work reports the intestinal microbiota in freshwater Atlantic salmon fed an insect meal diet containing 60% black soldier fly larvae meal for 8 weeks.

Results

To aid readers in interpreting the data from this feeding trial, results on feed utilization, growth performance, intestinal histomorphology, and gene expression, which have been reported elsewhere (27, 28), are summarized as the following. In brief, there was little evidence that the insect meal diet

negatively affected salmon's feed utilization or growth performance. Histopathological examination showed excessive accumulation of lipid (steatosis) in the proximal intestine in both diet groups, but it was less severe in salmon fed the insect meal diet. The expression of the lipid droplet marker gene, *plin2*, supported these histological findings. Immune and barrier-function gene expression profiles were generally not affected by diet. However, salmon fed the insect meal diet showed increased expression of genes indicative of immune tolerance (*foxp3*), stress response (*hsp70*), and detoxification activity (*cpy1a1*).

Taxonomic analysis. All the bacterial species in the mock were correctly identified at the genus level with *E. faecalis*, *L. fermentum*, *L. monocytogenes*, and *S. aureus* further being assigned at the species level (Figure S1). At the genus level, the average Pearson's *r* was 0.58 for the correlation between expected and observed mock composition. The exact sequence, relative abundance, and taxonomy of contaminating features identified in the negative control samples are available in Table S1. The primary contaminating features, in descending order according to the mean relative abundance, were classified as *Pseudomonas*, *Halomonas*, *Shewanella algae*, *Undibacterium*, *Bradyrhizobium*, *Chitinophagaceae*, *Ralstonia*, *Sediminibacterium*, *Curvibacter*, *Afipia,* and *Cutibacterium*.

The top 10 most abundant bacterial genera across all the samples are shown in Figure 1. At visual observation, the microbiota in the digesta collected from the two intestinal segments of the salmon fed the reference diet appeared homogenous, but more heterogeneous in the sampled mucosa. Dominant genera in the reference diet group included *Lactobacillus*, unclassified *Peptostreptococcaceae,* and *Peptostreptococcus*. The microbiota in salmon fed the insect meal diet differed greatly from that of the reference diet fed fish, but the difference between the results of the digesta and mucosa appeared less than for fish fed the reference diet. Dominant genera in the insect meal diet group included unclassified *Bacillaceae*, *Bacillus*, *Corynebacterium 1*, *Enterococcus*, *Oceanobacillus*, and *Ornithinibacillus*. The microbiota in the intestine closely resembled that of the feed but was distinct from the water microbiota. In agreement with this, we found that the OTU overlap between the intestine and feed was much higher than that between the intestine and water (Figure 2).

Core microbiota. In total, 45 and 60 OTUs were identified as core microbiota (present in at least 50% of the samples) in salmon fed the reference and insect meal diet, respectively (Figure 3). Five core OTUs were shared between the diets, classified as *Bacillus*, *Globicatella*, *Kurthia*, *Lactobacillus*, and *Ureibacillus*. Primary core OTUs in salmon fed the reference diet comprised *Peptostreptococcaceae* (1 OTU), *Peptostreptococcus* (1 OTU), and lactic acid bacteria including *Lactobacillus* (13 OTUs), *Weissella* (3 OTUs), *Vagococcus* (3 OTUs), *Lactococcus* (1 OTU), *Leuconostoc* (1 OTU), *Pediococcus* (1 OTU) and *Streptococcus* (1 OTU). In contrast, primary core OTUs in salmon fed the insect meal diet comprised *Bacillus* (12 OTUs), *Enterococcus* (7 OTUs), *Corynebacterium 1* (4 OTUs), *Lysinibacillus* (3 OTUs), *Lactobacillus* (3 OTUs), *Actinomyces* (3 OTUs), *Oceanobacillus* (2 OTUs), *Bacillaceae* (2 OTUs), *Brevibacterium* (2 OTUs), *Microbacterium* (2 OTUs), *Ornithinibacillus* (1 OTU) and *RsaHF231* (1 OTU).

Alpha-diversity. Overall, the diet effects on the alpha-diversity showed opposite results for digesta and mucosa samples when evaluated independently (Figure 4). In the digesta, the insect meal diet reduced microbial diversity compared to the reference diet, whereas in the mucosa the insect meal diet increased diversity.

In the digesta, Faith's phylogenetic diversity (Faith's PD) showed a significant diet and intestinal segment effect, and the interaction between these terms was not significant. Faith's PD was lower in the insect meal diet group than in the reference diet group. Also, it was lower in the distal intestine than in the proximal intestine. Similar results were found for the Shannon index, but the interaction was significant. In both intestinal segments, Shannon's index was lower in salmon fed the insect meal diet, but the diet effect was stronger in the distal intestine than in the proximal intestine. In contrast, a significant intestinal segment effect was only found in salmon fed the insect meal diet, with the distal intestine showing a lower Shannon's index.

In the mucosa, Faith's PD showed a significant diet effect, with no significant difference between the intestinal segments. The salmon fed the insect meal diet showed a higher Faith's PD in both intestinal segments. A similar effect was indicated by Shannon's index, but only for the proximal intestine, i.e., the interaction between diet and segment was significant.

The differences between the alpha diversity of water and intestinal mucosal microbiota were not significant (Figure S2).

Beta-diversity. In the digesta, the PERMANOVA showed a significant diet and intestinal segment effect on the beta-diversity, and the interaction between these terms was significant (Figure 5a; Table 1). The diet effect on the beta-diversity was significant in both intestinal segments, whereas a significant intestinal segment effect was only found in salmon fed the reference diet. The PERMDISP showed that, in both intestinal segments, the multivariate dispersion was higher in the reference diet group than in the insect meal diet group. Differences in the multivariate dispersion between intestinal segments were not significant in both diets (Figure S3a).

In the mucosa, the PERMANOVA showed a significant diet but not a significant intestinal segment effect on the beta-diversity, and the interaction between these terms was significant (Figure 5b; Table 1). The diet effect on the beta-diversity was significant in both intestinal segments, but it was marginally stronger in the proximal intestine than in the distal intestine. The PERMDISP showed that differences in the multivariate dispersion between the diet groups were not significant at the tank or diet level (Figure S3b).

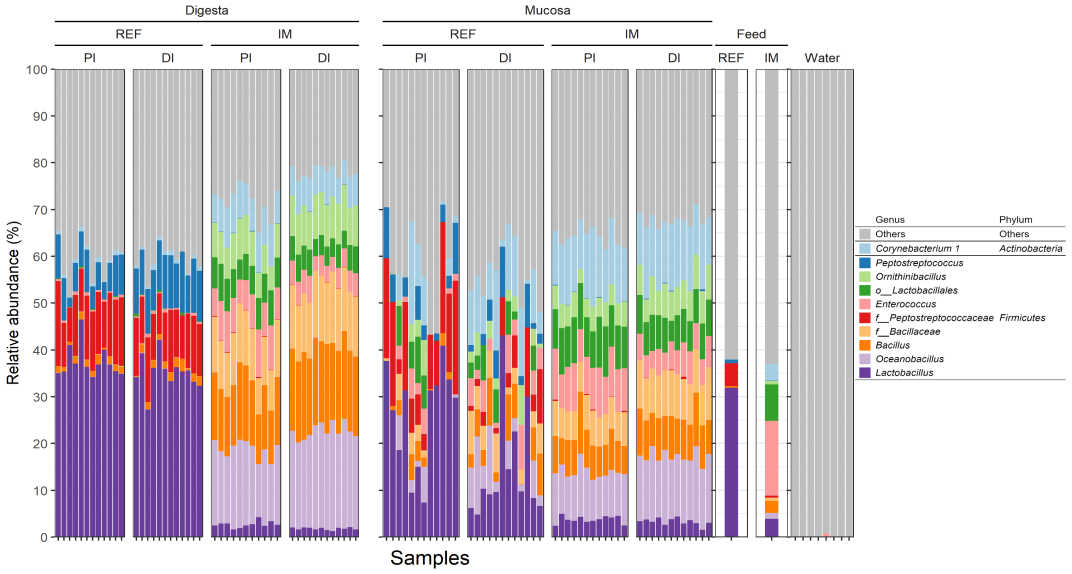

Fig. 1. Consistent changes in the taxonomic composition of intestinal microbiota from salmon fed the insect meal diet. Note that feed microbiota shows close resemblance to that observed in the intestine whereas water microbiota is very distinct from the intestinal microbiota. Only the top 10 most abundant bacterial genera are displayed in the plot whereas the other taxa are shown as "Others". Taxa not assigned at the genus level are prepended with letters indicating whether the taxonomic assignment was made at the order (o_) or family (f_) level. Abbreviations: REF, reference diet; IM, insect meal diet; PI, proximal intestine; DI, distal intestine.

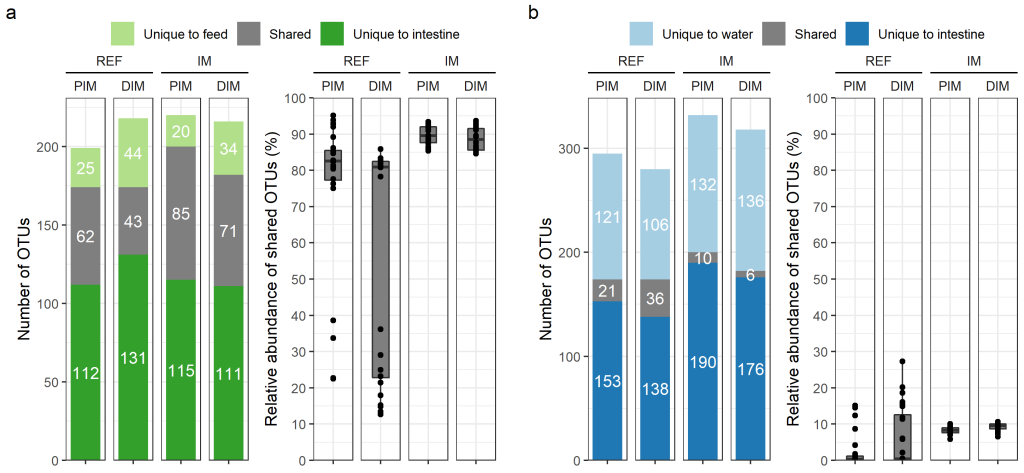

Fig. 2. Higher microbial overlap between the intestine and feeds (a) than that between the intestine and water (b). In each panel, the number of shared OTUs is shown on the left whereas the relative abundance of shared OTUs in the intestinal mucosa is shown on the right. To reduce the influence of rare OTUs and differences in sequencing depth, only OTUs with a minimum relative abundance of 0.05% were considered as present in a sample. Abbreviations: REF, reference diet; IM, insect meal diet; PIM, proximal intestine mucosa; DIM, distal intestine mucosa.

The water microbiota was significantly different from the intestinal mucosal microbiota (p = 0.001). The PERMDISP showed that differences in the multivariate dispersion between water and intestinal mucosal samples were not significant (p = 0.391).

Association analysis. Significant associations between sample metadata and bacterial genera in the digesta and mu-

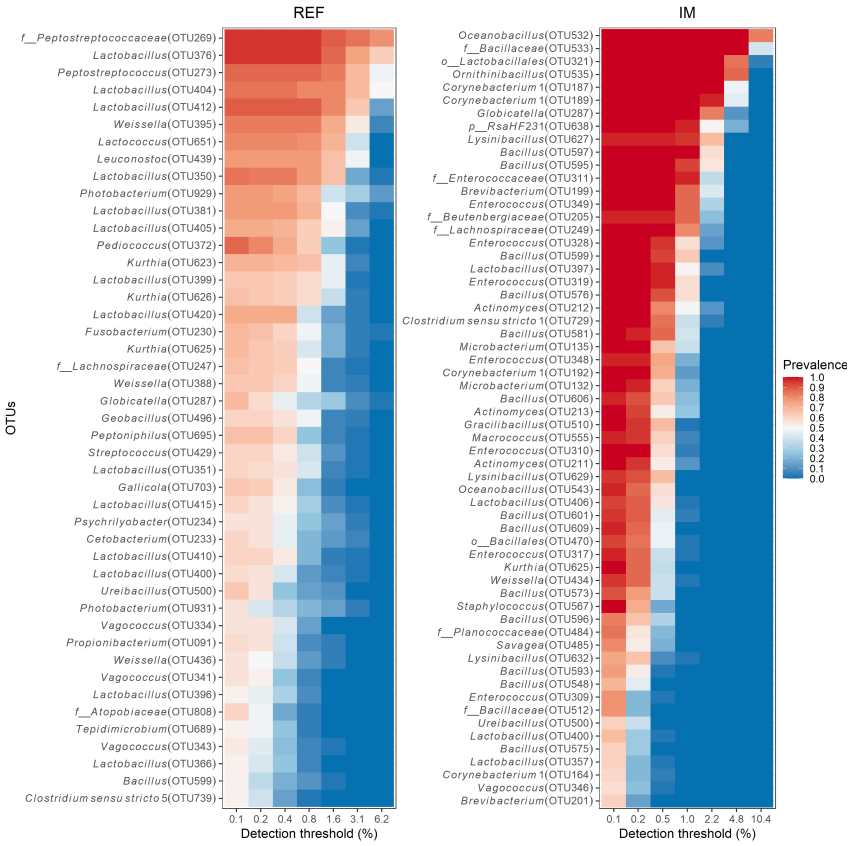

Fig. 3. Heatmaps showing the prevalence of core OTUs at different detection thresholds in salmon fed the reference (REF) or insect meal (IM) diet. The core OTUs were computed and visualized by the R package microbiome (29), with a minimum detection threshold of 0.1% and a minimal prevalence threshold of 50%. The taxonomy of core OTUs at the genus level is displayed on the y axis. OTUs not assigned at the genus level are prepended with letters indicating whether the taxonomic assignment was made at the phylum (p_), order (o_), or family (f_) level.

cosa are shown in Figure 6 and Figure 7, respectively. In total, 93 and 36 taxa were associated with the diet effect in the digesta and mucosa, respectively. Collectively, 32 taxa were associated with the diet effect in both digesta and mucosa. Among these taxa, bacterial genera enriched in salmon fed the reference diet consisted of unclassified *Peptostreptococcaceae*, *Peptostreptococcus*, *Photobacterium*, and lactic acid bacteria including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* (partially illustrated in Figure 6b and Figure 7b). In contrast, bacterial genera enriched in salmon fed the insect meal diet comprised *Actinomyces*, unclassified *Bacillales*, unclassified *Bacillaceae*, *Bacillus*, unclassified *Beutenbergiaceae*, *Brevibacterium*, *Cellulosimicrobium*, *Clostridium sensu stricto 1*, *Corynebacterium 1*, unclassified *Enterococcaceae*, *Enterococcus*, *Exiguobacterium*, *Globicatella*, *Gracilibacillus*, unclassified *Lactobacillales*, *Lysinibacillus*, *Macrococcus*, *Microbacterium*, *Nosocomiicoccus*, *Oceanobacillus*, *Ornithinibacillus*, *Paenibacillus*, unclassified *Planococcaceae*, and unclassified *RsaHF231* (partially illustrated in Figure 6c and Figure 7c). Regarding associations between bacterial genera and host gene expressions, the relative abundance of *Paenibacillus* and *Streptococcus* in the mucosa showed positive correlations with the expression level of *foxp3* , the master transcription factor of regulatory T-cells, in the intestine (partially illustrated in Figure 7d). Additionally, the relative abundance of unclassified *RsaHF231* in the digesta, and the relative abundance of unclassified *Bacillaceae*, *Corynebacterium 1*, *Enterococcus*, and *Oceanobacillus* in the mucosa, showed negative correlations with the expression level of *plin2* , a surface marker of lipid droplets, in the intestine (partially illustrated in Figure 7e).

Discussion

We found that the insect meal diet markedly modulated the Atlantic salmon intestinal microbiota. A group of bacterial genera, dominated by members of the *Bacillaceae* family,

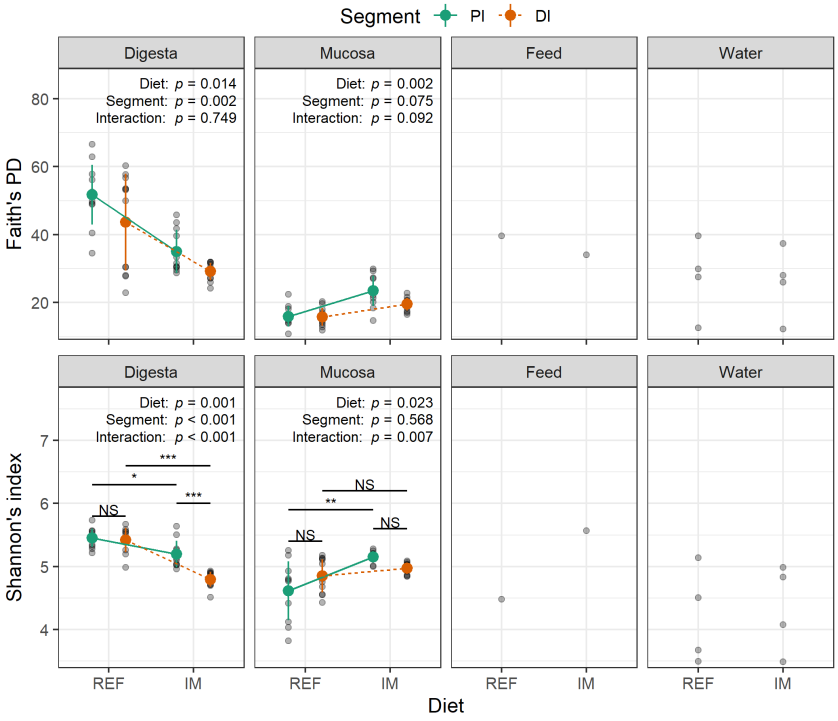

Fig. 4. Diet effects on the alpha-diversity are opposite when independently evaluated using digesta or mucosa samples. The error bars denote standard deviations of the means. The *p* values of the main effects and their interaction are displayed on the top of each subplot. The asterisks denote statistically significant differences in the post-hoc conditional contrasts (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Abbreviations: REF, reference diet; IM, insect meal diet; PI, proximal intestine; DI, distal intestine; NS, not significant; PD, phylogenetic diversity.

was enriched in salmon fed the insect meal diet. These results confirm our previous findings in a seawater feeding trial (32). We also found that microbiota in the intestine closely resembled that of the feeds. Notably, bacterial genera associated with the diet effects were present in the feeds as well. We conclude that salmon fed the insect meal diets show consistent changes in the intestinal microbiota. The next challenge is to evaluate the extent to which these alterations are attributable to feed microbiota and dietary nutrients.

Insect meal diet markedly modulated the intestinal microbiota. Higher microbial diversity has been reported in intestinal digesta, and mucosa of salmonids fed diets containing black soldier fly larvae meal (32–35). In the present study, however, this was the case for the mucosa, but the opposite was the result for the digesta. Our observation that a particular group of bacterial genera, dominated by members of the *Bacillaceae* family, was enriched in salmon fed the insect meal diet is in line with findings in our previous seawater trial, wherein salmon were fed an insect meal diet containing 15% black soldier fly larvae meal for 16 weeks (32). Among these bacterial genera, *Actinomyces*, *Bacillus*, *Brevibacterium*, *Corynebacterium 1*, *Enterococcus*, *Oceanobacillus*, and *Paenibacillus* were also reported to be enriched in rainbow trout fed diets containing 15% or 30% black soldier fly larvae meal (34–36). Similar observations have been made in Siberian sturgeon (*Acipenser baerii*) fed a diet containing 15% BSF larvae meal, inducing higher absolute abundances of *Bacillus* and *Enterococcus* (37). In this latter study, fluorescence in situ hybridization (*FISH*) technique was used for the bacteria quantification.

Feed microbiota and dietary nutrients may explain the observed diet effects. We found evidence for the former, because bacterial genera associated with the diet effects were present in the feed samples. Given the hydrothermal treatments during the extrusion step in the feed production, the viability of feed-associated microbes is expected to be low. As sequencing-based methods cannot differentiate between active (living) and inactive (dormant/dead) microbes, additional work will be needed to elucidate the extent to which the observed diet effects are attributable to the carry-over of inactive microbes and colonization of active microbes from feeds. Methods like viability PCR and RNA sequencing can be applied for such experiments (38). Changes in the feed components may have also contributed to the observed diet effects. For instance, dietary inclusion of soy proteins was suggested to associate with increased relative abundance of lactic acid bacteria in the salmon intestine (39). Thus, the replace-

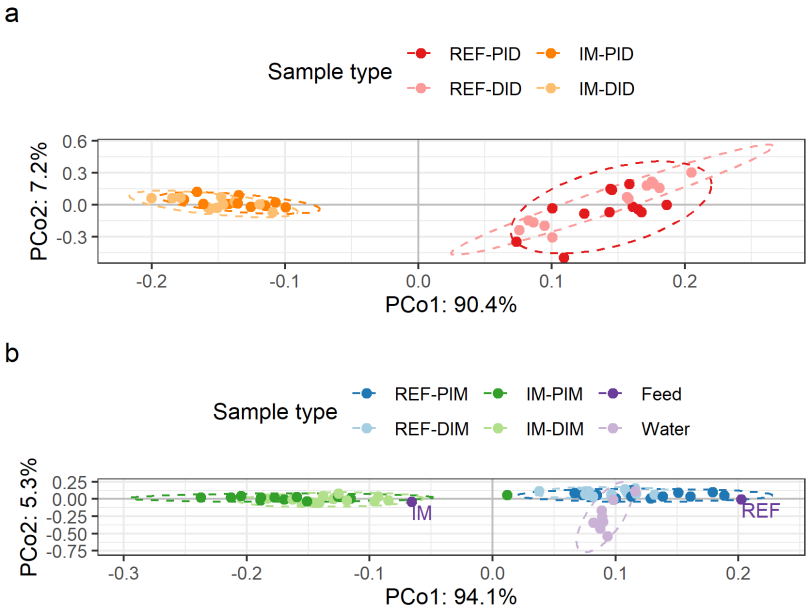

Fig. 5. The insect meal diet markedly modulated the salmon intestinal microbiota in both digesta (a) and mucosa (b), irrespective of intestinal segments. The dimensionality reduction was performed using a compositional beta-diversity metric called robust Aitchison PCA and visualized by EMPeror (30). The height-to-width ratio of the PCoA plot was set to reflect the ratio between the corresponding eigenvalues as recommended (31). Abbreviations: REF, reference diet; IM, insect meal diet; PID, proximal intestine digesta; DID, distal intestine digesta; PIM, proximal intestine mucosa; DIM, distal intestine mucosa; PCoA, principal coordinate analysis.

Table 1. PERMANOVA and subsequent conditional contrasts.

Source	Main effects		Interaction	Conditional contrasts			
	Diet	Segment		REF-PI VS. IM-PI	REF-DI VS. IM-DI	REF-PI VS. REF-DI	IM-PI VS. IM-DI
Digesta	0.001	0.041	0.041	0.002	0.002	0.04	0.59
Mucosa	0.001[1]	0.633	0.010	0.001[1]	0.001[1]	NA	NA

Abbreviations: REF, reference diet; IM, insect meal diet; PI, proximal intestine; DI, distal intestine; NA, not applicable.
[1]Monte Carlo *p* value

ment of soy protein concentrate with insect meal may explain the reduction in lactic acid bacteria in salmon fed the insect meal diet. On the other hand, nutrients from the insect meal, such as chitin, may have also promoted the growth of certain bacterial taxa including *Actinomyces* and *Bacillus*. *Actinomyces* species are often identified as active chitin degraders, showing enhanced growth and activity upon chitin addition (40). Many *Bacillus* species are well-known as chitin degraders (41). *Bacillus* was one of the predominant taxa in the intestinal mucosa of salmon fed a chitin-supplemented diet, displaying the highest *in vitro* chitinase activity (42). The latter hypothesis can be tested by supplementing insect meal-specific nutrients to the same basal diet and sequencing the intestinal microbiota of salmon fed these diets.

Microbiota was similar between intestinal segments. Like its mammalian counterparts (43, 44), the salmon intestinal microbiota is also spatially heterogeneous in its composition (45). Specifically, microbial communities differ along the intestinal tract and vary substantially between digesta and mucosa within the same intestinal segment. Due to the batch effects between sequencing runs, we could not directly compare microbial communities in the digesta and mucosa. Nonetheless, our study suggests that conclusions on the diet effect can be different when evaluated using digesta or mucosa samples alone. This is supported by our results showing that diet effects on the alpha-diversity and differential abundance testing were quite different when evaluated independently using digesta or mucosa samples. In contrast, our comparative analysis showed that microbiota variations be-

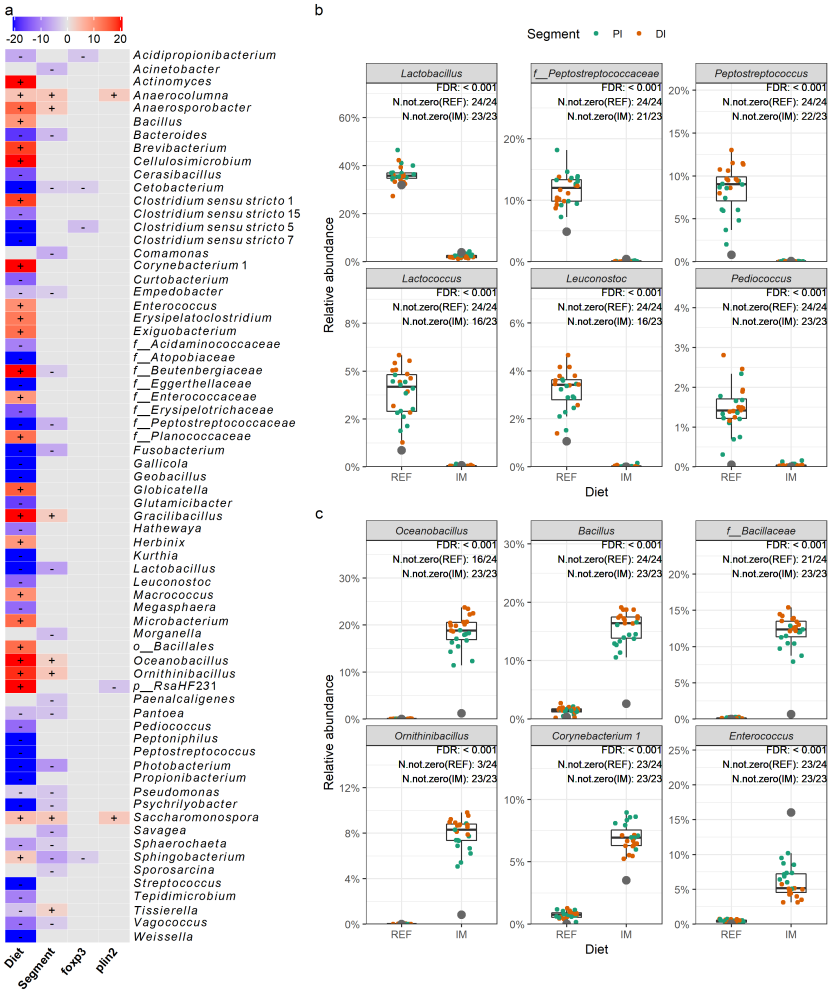

Fig. 6. Significant associations between sample metadata and microbial clades in the digesta. (a) Heatmap summarizing significant associations between sample metadata and microbial clades in the digesta. Color key: -log(*q*-value) * sign(coefficient). Cells that denote significant associations are colored in red or blue and overlaid with a plus (+) or minus (-) sign that indicates the direction of association: Diet (+), higher relative abundance in salmon fed the insect meal diet; Segment (+), higher relative abundance in the distal intestine; *foxp3* (+) / *plin2* (+), positive correlation between microbial clade relative abundance and gene expression levels. (b) Representative taxa showing higher relative abundances in salmon fed the reference diet. (c) Representative taxa showing higher relative abundances in salmon fed the insect meal diet. The relative abundances of representative taxa in the feeds are shown as grey dots in panels b and c. As the number of taxa showing significant associations with diet was too high to be properly displayed on the heatmap, we filtered the results to keep those with a *q*-value < 0.0001. Complete results are available in our accompanying R Markdown reports. Taxa not assigned at the genus level are prepended with letters indicating whether the taxonomic assignment was made at the phylum(p_), order (o_), or family (f_) level. Abbreviations: REF, reference diet; IM, insect meal diet; PI, proximal intestine; DI, distal intestine; FDR, false discovery rate; N.not.zero, number of observations that are not zero.

tween intestinal segments were minor in the digesta and negligible in the mucosa. The diet effects were essentially the same when evaluated using samples from different intestinal segments. Taken together, these results suggest that it may be sufficient to collect digesta and mucosa samples from one intestinal segment (e.g., the distal intestine) when conducting a diet-microbiota study in fish with limited resources.

Microbial overlap was low between the intestine and water but high between the intestine and feeds. Water and feed are considered two environmental sources of microbiota which can be transferred to the fish intestine. In line with previous studies in salmon (46–48) and other fish species (49–51), we found that microbial overlap between the intestine and water was low in the present study of salmon in freshwater. This may be explained by the fact that dur-

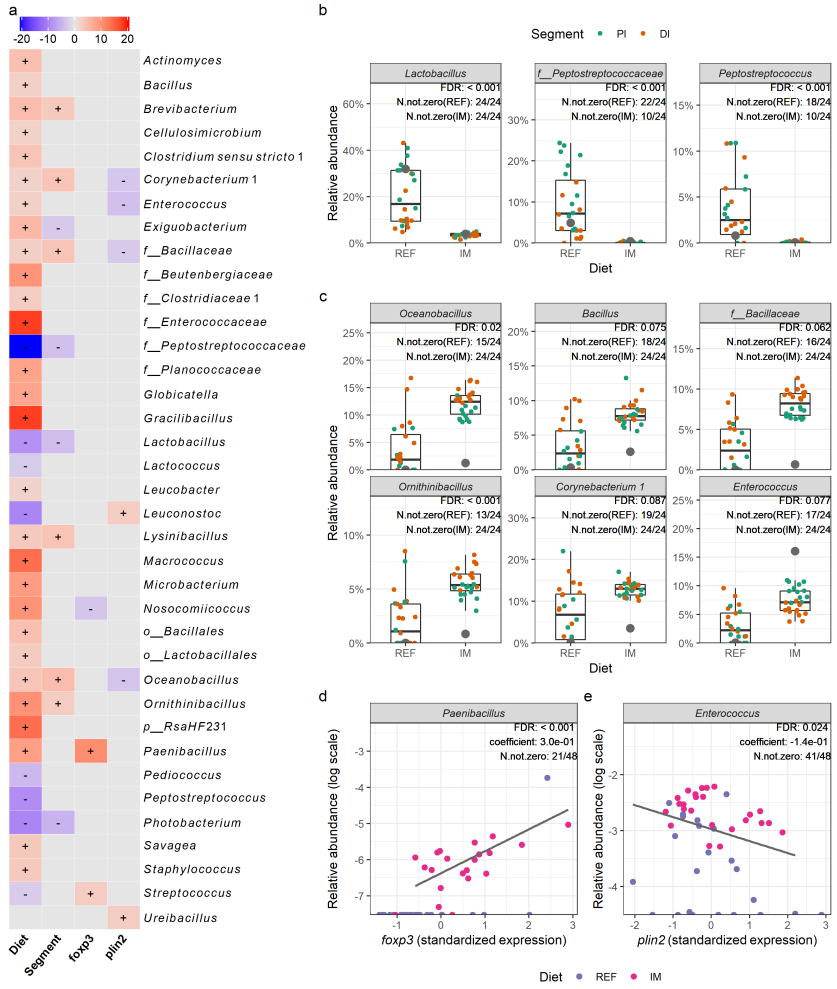

Fig. 7. Significant associations between sample metadata and microbial clades in the mucosa. (a) Heatmap summarizing significant associations between sample metadata and microbial clades in the mucosa. Color key: -log(*q*-value) * sign(coefficient). Cells that denote significant associations are colored in red or blue and overlaid with a plus (+) or minus (-) sign that indicates the direction of association: Diet (+), higher relative abundance in salmon fed the insect meal diet; Segment (+), higher relative abundance in the distal intestine; *foxp3* (+) / *plin2* (+), positive correlation between microbial clade relative abundance and gene expression levels. (b) Representative taxa showing higher relative abundances in salmon fed the reference diet. (c) Representative taxa showing higher relative abundances in salmon fed the insect meal diet. (d) Positive correlation between the relative abundance of *Paenibacillus* and *foxp3* expression levels in the intestine. (e) Negative correlation between the relative abundance of *Enterococcus* and *plin2* expression levels in the intestine. The relative abundances of representative taxa in the feeds are shown as grey dots in panels b and c. Taxa not assigned at the genus level are prepended with letters indicating whether the taxonomic assignment was made at the phylum(p_), order (o_), or family (f_) level. Abbreviations: REF, reference diet; IM, insect meal diet; PI, proximal intestine; DI, distal intestine; FDR, false discovery rate; N.not.zero, number of observations that are not zero.

ing their freshwater stage, salmon drink little water to accommodate osmoregulation needs in a hypo-osmotic environment, which greatly limits the intake of microbes from the surrounding water environment. Conversely, we found a high overlap between microbiota in the intestine and the feeds. Contradicting results have been reported in the literature regarding microbial overlaps between the fish intestine and formulated feeds (52–55). As discussed earlier, the feed microbiota detected by amplicon sequencing may have primarily originated from inactive microbes. Therefore, feed microbiota can be a confounding factor of the observed diet effects. Given that the influence of feed microbiota on the observed diet effects is unequal across experimental groups as opposed to the water microbiota, we strongly recommend collecting feed samples when designing a sequencing-based, diet-microbiota study in fish.

Associations between microbial clades and host gene expressions. The close relationship between microbiota and the intestinal immune system is well established (56). Interaction between microbiota and lipid metabolism in the intestine has also been documented (57, 58). Here we found a positive correlation between the *Paenibacillus* relative abundance and *foxp3* expression level in the intestine, suggesting a putative link between the enrichment of *Paenibacillus* and increased expression of *foxp3* in salmon fed the insect meal diet. In addition, we found negative correlations between relative abundances of unclassified *Bacillaceae*, *Corynebacterium 1*, *Enterococcus*, *Oceanobacillus*, and unclassified *RsaHF231*, and the expression level of *plin2* in the intestine. This may suggest that the reduction in steatosis in the proximal intestine of salmon fed the insect meal diet might be related to the enrichment of these taxa, either a cause or consequence. However, as microbiome data are sparse and noisy, association analysis is more meaningful when the sample size is much larger than it in this study. Given the limited sample size, our results should be interpreted as exploratory. Further research is required to test if these bacteria taxa are indeed involved in the immune modulation and lipid metabolism in the salmon intestine.

Conclusions

Our work showed that the insect meal diet markedly modulated the Atlantic salmon intestinal microbiota. Overall, the microbial diversity was lower in the digesta of salmon fed the insect meal diet but higher in the mucosa. A group of bacterial genera, dominated by members of the *Bacillaceae* family, was enriched in salmon fed the insect meal diet. These results support our previous findings from a study of Atlantic salmon in seawater. We also found that microbiota in the intestine closely resembled that of the feed but was distinct from the water microbiota. Notably, bacterial genera associated with the diet effects were present in the feed samples as well. We conclude that salmon fed the insect meal diets show consistent changes in the intestinal microbiota. The next challenge is to evaluate the extent to which these alterations are attributable to feed microbiota and dietary nutrients and what these changes mean for fish physiology and health.

Methods

Experimental fish, diet and sampling. An 8-week freshwater feeding trial was conducted at Cargill AquaNutrition experimental facility at Dirdal, Norway. A total of 800 Atlantic salmon with a mean initial body weight of 49 g (1.5 g SEM) were randomly assigned into 8 fiberglass tanks (450 L, 100 fish per tank) supplied with running freshwater. Quadruplicate tanks of fish were fed either a reference diet with a combination of fish meal, soy protein concentrate, and wheat gluten as protein sources, or an insect meal diet wherein 85% of the protein was supplied by black soldier fly larvae meal, replacing most of the fish meal and soy protein concentrate (Table 2). The black soldier fly larvae were grown on feed substrates containing organic waste streams. After eight days of growing, the larvae were harvested and partially defatted before being dried and ground to make the insect meal (Protix Biosystems BV, Dongen, The Netherlands). The diets were formulated and produced by Cargill (Dirdal, Norway) and stored at -20 °C until use. The fish were fed continuously by automatic disk feeders under a photoperiod regimen of 24 h daylight. Uneaten feeds were collected from tank outlets and registered daily. During the feeding trial, the water temperature was 13.7 ± 0.1 °C, and the dissolved oxygen concentration of the inlet and outlet water was 11.9 ± 1.2 and 8.7 ± 0.5 mg/L, respectively. Further details on the nutritional composition of the insect meal and diets have been reported elsewhere (27, 59).

Table 2. Formulation of the experimental diets.

Ingredients (g/100 g)	REF	IM
Fishmeal LT94	35.0	6.0
Insect meal	0	60.0
Soy protein concentrate	29.6	5.0
Wheat gluten	14.3	14.4
Fish oil	4.6	6.9
Rapeseed Oil	12.0	4.8
Vitamin & mineral premix	0.3	0.3
Yttrium	0.2	0.2
Miscellaneous	4.0	2.4
Chemical composition		
Dry matter (%)	94	96
Crude lipid (%)	18	22
Crude protein (%)	47	44
Carbohydrates (%)	11	12
Ash (%)	8	7
Gross energy (MJ/Kg dry matter)	22	23
TBARS (nmol/g)	7	17

Abbreviations: REF, reference diet; IM, insect meal diet; TBARS, Thiobarbituric acid reactive substances.

Sample collection. At the termination of the feeding trial, 3 fish were randomly taken from each tank (i.e., 12 fish per treatment), anesthetized with tricaine methanesulfonate (MS222®; Argent Chemical Laboratories, Redmond, WA, USA), and euthanized by a sharp blow to the head. After cleaning the exterior of each fish with 70% ethanol, the proximal and distal intestine were aseptically removed from the abdominal cavity, placed in sterile Petri dishes, and opened longitudinally. Only fish with digesta along the whole intestine were sampled to ensure that the intestine had been exposed to the diets. The intestinal digesta was gently removed and transferred into a 1.5 mL sterile Eppendorf tube using a spatula and snap-frozen in liquid N_2 for the profiling of digesta-associated intestinal microbiota. The intestinal tissue was rinsed in sterile phosphate-buffered saline 3 times to remove traces of remaining digesta. After rinsing, the intestinal tissue was cut into 3 pieces for histological evaluation (fixed in 4% phosphate-buffered formaldehyde solution for 24 h and transferred to 70% ethanol for storage), gene expression analysis (preserved in RNAlater solution and stored at -20 °C), and profiling of mucosa-associated intestinal microbiota (snap-frozen in liquid N_2), respectively. In addition, 300 mL water was taken from each tank, pre-filtered through a 0.8

μm sterile syringe filter (Acrodisc®, Pall Corporation, New York, USA), and vacuum-filtered onto a 0.2 μm sterile nitrocellulose filter (Nalgene™, Thermo Scientific, USA). The filter containing enriched bacteria was folded, placed into an 8 mL sterile tube, and snap-frozen in liquid N_2 to profile microbial community in water. The collection of microbiota samples was performed near a gas burner to secure aseptic conditions. Tools were cleaned and decontaminated by 70% ethanol sprays and flaming before the subsequent sampling was carried out. The samples for microbiota profiling were transported in dry ice and stored at -80 °C until DNA extraction.

DNA extraction. Total DNA was extracted from ~100 mg digesta, mucosa, and feed using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) as previously described (39), except that 2 mL prefilled PowerBead tubes (glass beads, 0.1 mm; Cat no. 13118-50, Qiagen) were used for the bead beating. To extract DNA from water samples, the frozen filter was allowed to soften on ice and rolled into a cylinder with the white filter membrane facing outward using two sets of sterile forceps. The filter was then inserted into an 8 mL sterile tube containing the double amount of ASL buffer and glass beads used in the prefilled PowerBead tubes. The tube was secured horizontally to a mixer mill (Retsch GmbH, Germany; model, MM 301) and shaken vigorously at the frequency of 30 Hz for 5 min (2.5 min, pause and invert the tube, 2.5 min). After shaking, the tube was centrifuged at 4000 g for 1 min, and 2.6 mL supernatant was collected and evenly aliquoted into two 1.5 mL Eppendorf tubes. The DNA was extracted from the supernatant aliquots and pooled afterward, following the protocol as previously described (39). For quality control purposes, a companion "blank extraction" sample was added to each batch of sample DNA extraction by omitting the input material, whereas an additional mock sample (ZymoBIOMICS™, Zymo Research, California, USA; catalog no., D6300) was included for each DNA extraction kit as a positive control. The mock consists of 8 bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica*, *Lactobacillus fermentum*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis*) and 2 yeasts (*Saccharomyces cerevisiae*, *Cryptococcus neoformans*).

Library preparation and sequencing. The V1-2 hypervariable regions of the bacterial 16S rRNA gene were amplified using the primer set 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 338R (5'-GCW GCC WCC CGT AGG WGT-3') (60). The PCR was run in a total reaction volume of 25 μL containing 12.5 μL of Phusion® High-Fidelity PCR Master Mix (Thermo Scientific, CA, USA; catalog no., F531L), 10.5 μL molecular grade H_2O, 1 μL DNA template, and 0.5 μL of each primer (10 μM). The amplification program was set as follows: initial denaturation at 98 °C for 3 min; 35 cycles of denaturation at 98 °C for 15 s, annealing decreasing from 63 °C to 53 °C in 10 cycles for 30 s followed by 25 cycles at 53 °C for 30 s, and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 10 min. The PCR was run in duplicate incorporating negative PCR controls, which were generated by replacing the template DNA with molecular grade H_2O. The duplicate PCR products were pooled and examined by a 1.5% agarose gel electrophoresis before cleanup.

The sequencing was carried out on a Miseq platform following the Illumina 16S metagenomic sequencing library preparation protocol (61). Briefly, the PCR products were cleaned using the Agencourt AMPure XP system (Beckman Coulter, Indiana, USA; catalog no., A63881), multiplexed by dual indexing using the Nextera XT Index Kit (Illumina, California, USA; catalog no., FC-131-1096) and purified again using the AMPure beads. After the second clean-up, representative libraries were selected and analyzed using the Agilent DNA 1000 Kit (Agilent Technologies, California, USA; catalog no., 5067-1505) to verify the library size. Cleaned libraries were quantified using the Invitrogen Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, California, USA; catalog no., Q32854), diluted to 4 nM in 10 mM Tris (pH 8.5) and finally pooled in an equal volume. Negative controls with library concentrations lower than 4 nM were pooled in equal volume directly. Due to the low diversity of amplicon library, 15% Illumina generated PhiX control (catalog no., FC-110-3001) was spiked in by combining 510 μL amplicon library with 90 μL PhiX control library. The library was loaded at 6 pM and sequenced using the Miseq Reagent Kit v3 (600-cycle) (Illumina; catalog no., MS-102-3003).

Due to technical challenges in obtaining high-quality PCR products for mucosa samples, the digesta samples were first amplified and sequenced. The PCR conditions for mucosa samples were optimized by diluting the DNA templates (1:5) to reduce the influence of PCR inhibitors. The mucosa samples were then sequenced in a second run together with feed and water samples. To assess potential batch effects between sequencing runs, 8 representative digesta samples were also sequenced in the second run to serve as technical replicates.

Sequence data processing. The raw sequence data from each run were separately processed by the DADA2 (version 1.18) in R (version 4.0.5) (62) to infer amplicon sequence variants (ASVs) (63). Specifically, the demultiplexed paired-ended reads were trimmed off the primer sequences (first 20 bps of forward reads and first 18 bps of reverse reads), truncated at the position where the median Phred quality score crashed (forward reads at position 260 bp and reverse reads at position 188 bp for the first run; forward reads at position 290 bp and reverse reads at position 248 bp for the second run) and filtered off low-quality reads. After the trimming and filtering, run-specific error rates were estimated, and the ASVs were inferred from each sample independently. The chimeras were removed using the "consensus" method after merging the forward and reverse reads. The resulting feature table and representative sequences from each run were imported into QIIME2 (version 2020.11) (64) and merged. The taxonomy was assigned by a scikit-learn naive Bayes machine-learning classifier (65), which was trained on the SILVA 132 99% OTUs (66) that were trimmed to only include V1-V2 regions

of the 16S rRNA gene. Taxa identified as chloroplasts or mitochondria were excluded from the feature table. The feature table was conservatively filtered to remove ASVs that had no phylum-level taxonomic assignments or appeared in only one biological sample. Contaminating ASVs were identified and removed based on two suggested criteria: contaminants are often found in negative controls and inversely correlate with sample DNA concentration (67), which was quantified by qPCR as previously described (32). The ASVs filtered from the feature table were also removed from the representative sequences, which were then clustered into OTUs at 97% similarity using the VSEARCH *de novo* clustering method (68). The resulting OTU table and representative sequences were used for the downstream data analysis. The phylogeny was constructed by inserting the representative sequences into a reference phylogenetic tree built on the SILVA 128 database using SEPP (69). The alpha-diversity indices were computed by rarefying the OTU table at a subsampling depth of 10 345 sequences. To compare beta-diversity, we performed robust Aitchison PCA using the QIIME2 library DEICODE (70), which is a form of Aitchison distance that is robust to high levels of sparsity in the microbiome data via matrix completion. For downstream data visualization and statistical analyses, QIIME2 artifacts were imported into R using the qiime2R package (71) and a phyloseq (72) object was assembled. As the technical replicates showed strong batch effects between the sequencing runs, which could not be effectively removed by existing batch effect correction methods such as RUVSeq (73) and ComBat-seq (74), we performed the downstream data analysis independently for samples sequenced in different runs.

Statistics. Differences in the alpha-diversity indices were compared by linear mixed-effects models using the R package afex (75), which runs the lme4 (76) under the hood to fit mixed-effects models. Predictor variables in the models include the fixed effects Diet + Segment + Diet x Segment, and the random effects FishID + Tank. The models were validated by visual inspections of residual diagnostic plots generated by the R package ggResidpanel (77). The statistical significance of fixed predictors was estimated by Type III ANOVA with Kenward-Roger's approximation (78) of denominator degrees of freedom. When the interaction between the main effects was significant, conditional contrasts for the main effects were made using the R package emmeans (79). To compare differences in the beta-diversity, we performed the PERMANOVA (80) in PRIMER v7 (Primer-E Ltd., Plymouth, UK) using the same predictors included in the linear mixed-effects models. Terms with negative estimates for components of variation were sequentially removed from the model via term pooling, starting with the one showing the smallest mean squares. At each step, the model was reassessed whether more terms needed to be removed or not. Conditional contrasts for the main effects were constructed when their interaction was significant. Monte Carlo p values were computed as well when the unique permutations for the terms in the PERMANOVA were small (< 100). The homogeneity of multivariate dispersions among groups was visually assessed with boxplots and was formally tested by the permutation test, PERMDISP (81), using the R package vegan (82). Per-feature tests for the association between specific microbial clade and sample metadata were done using the R package MaAsLin2 (version 1.4.0) (83). The feature table was collapsed at the genus level and bacterial taxa of low prevalence (present in < 25% of samples) were excluded before running the association analysis. Predictor variables included in the association testing are fixed factors Diet + Segment + *foxp3* (qPCR) + *plin2* (qPCR), and the random effects FishID + Tank. Multiple comparisons were adjusted by the Holm (84) or Benjamini-Hochberg (85) method where applicable. Differences were regarded as significant for $p < 0.05$ or FDR-corrected $q < 0.1$.

Declarations

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Authors' contributions. T.M.K. and Å.K. conceived and designed the study. Y.L., K.G., and Å.K. participated in the sample collection. Y.L., K.G. and A.J.-T. carried out the laboratory works. Y.L. performed the data analysis and completed the first draft of the manuscript. All the authors read, revised, and approved the final version of the manuscript.

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Availability of data and materials. The raw 16S rRNA gene sequencing data are deposited at the NCBI SRA database under the BioProject PRJNA730696. The code for reproducing our results is available at the GitHub repository (https://github.com/yanxianl/Li_AqFl1-Microbiota_2021).

Ethics approval and consent to participate. The experiment was conducted in compliance with the Norwegian Animal Welfare Act 10/07/2009 and the EU Directive on the Protection of Animals used for Scientific Purposes (2010/63/EU).

Consent for publication. Not applicable.

Competing interests. The authors declare that they have no competing interests.

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Supplementary Note 1: Supplemental tables

- **Table S1. Contaminating ASVs removed from the feature table (available at the GitHub repository: https://github.com/yanxianl/Li_AqF11-Microbiota_2021/tree/master/result/table).**

Supplementary Note 2: Supplemental figures

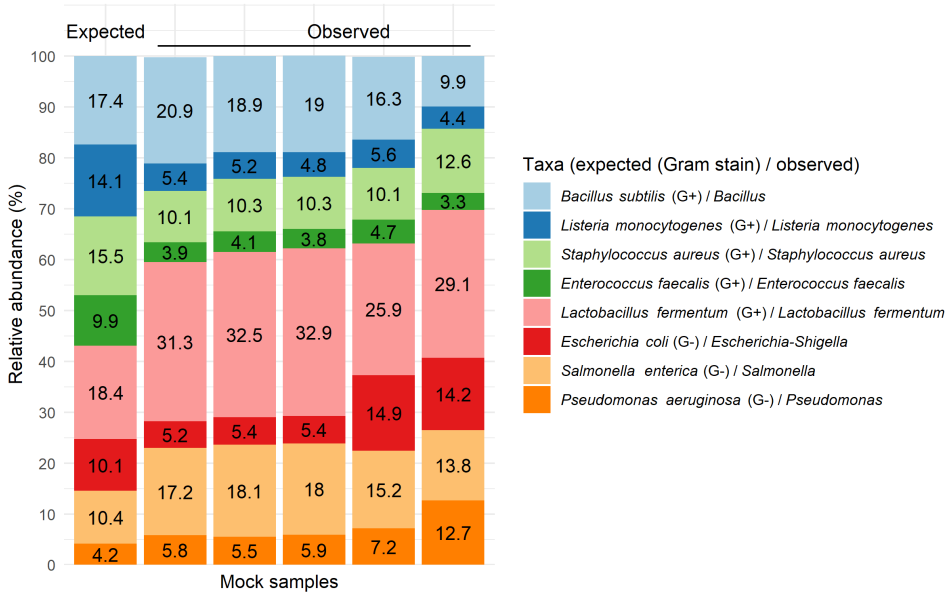

Fig. S1. The expected and observed taxonomic composition of the sequenced ZymoBIOMICS mock samples.

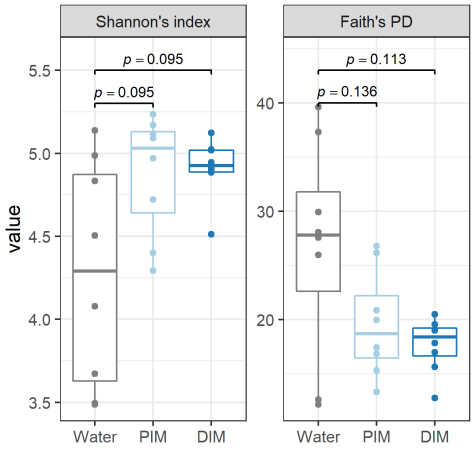

Fig. S2. Comparison of alpha-diversity between paired water and intestinal mucosa samples. Note that alpha-diversity indices of intestinal mucosa samples from the same tank were aggregated before running paired *t*-test. Abbreviations: PIM, proximal intestine mucosa; DIM, distal intestine mucosa; PD, phylogenetic diversity.

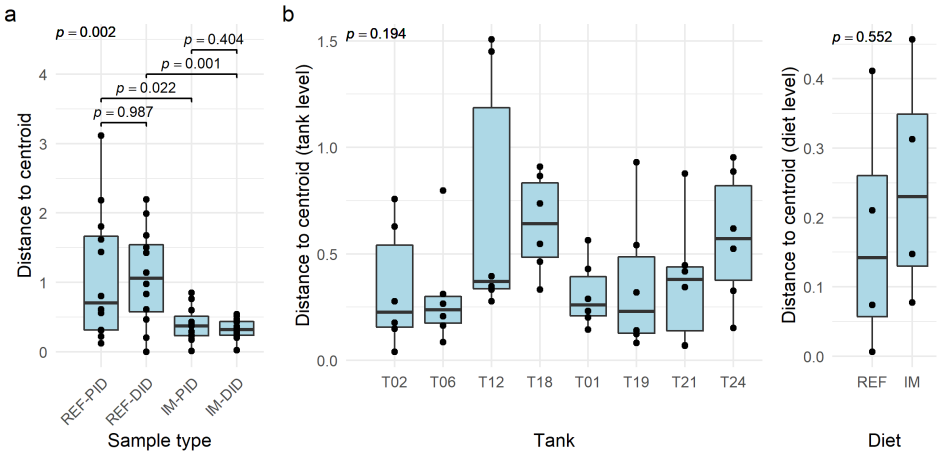

Fig. S3. Tests for homogeneity of multivariate dispersions (PERMDISP) in digesta (a) and mucosa (b) samples. (a) The PERMANOVA suggested little evidence of tank effect for digesta samples, thus we used individual fish as the statistical unit when running the PERDISP. (b) The PERDISP, however, was carried out on tank and diet level for mucosa samples because of a significant tank effect. Abbreviations: REF, reference diet; IM, insect meal diet; PID, proximal intestine digesta; DID, distal intestine digesta.

III

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Total replacement of fish meal with black soldier fly (*Hermetia illucens*) larvae meal does not compromise the gut health of Atlantic salmon (*Salmo salar*)

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ABSTRACT

Limited availability of sustainable feed ingredients is a significant concern in salmon aquaculture. Insects may become an important, sustainable resource for expanding the raw material repertoire. Herein, we present data from a 16-week seawater feeding trial with Atlantic salmon (initial body weight, 1.4 kg) fed either a reference diet with a combination of fish meal, soy protein concentrate, pea protein concentrate, corn gluten and wheat gluten as protein source, or a test diet wherein all the fish meal and most of the pea protein concentrate were replaced by black soldier fly larvae meal. The gut health of fish was evaluated using endpoints including organ and tissue indices, histopathology variables and gene expression indicative of lipid metabolism, immune responses, barrier functions and detoxification/stress responses. A higher relative weight of distal intestine was found in fish fed the insect meal diet. Steatosis of enterocytes was observed in the proximal and mid intestine in both diet groups, albeit, less severe in the proximal intestine of fish fed the insect meal diet. Inflammatory morphological changes, similar to those induced in the distal intestine by standard soybean meal, were present in all the examined intestinal segments, with a higher degree of submucosa cellularity in the proximal intestine of insect meal diet fed fish, the only notable diet effect. Few differentially expressed genes were identified in the proximal or distal intestine. In summary, total replacement of fish meal with black soldier fly larvae meal did not compromise the gut health of Atlantic salmon.

1. Introduction

Marine ingredients in the Norwegian salmon diet have gradually been replaced by plant sources, decreasing from ~90% in 1990 to ~25% in 2016. Among the plant-based protein sources, soy protein concentrate accounted for 19.2% of the total diet ingredients followed by wheat gluten (9.0%), corn gluten (3.4%), horse beans (2.0%), pea protein concentrate (1.4%), faba beans (1.3%), sunflower meal (1.2%) and other marginally used plant proteins (2.7%) (Aas et al., 2018). While the future availability of plant proteins is guaranteed in the short-term (Shepherd et al., 2017), there is a need for new nutrient sources in Norwegian salmon aquaculture as the production volume is expected to grow. Moreover, as the world population is projected to reach 9.8 billion in 2050 (UN, 2017), global food production must maximize the nutritional output for human consumption and minimize the input of resources, with the lowest possible impact on the environment (Ytrestøyl et al., 2015). Hence, the salmon feed producers need to reduce their dependency on terrestrial plant products that may be used directly for human consumption, and seek new, sustainable feed ingredients for the future salmon aquaculture.

Insects possess an outstanding capacity to upgrade low-quality organic material, require minimal water and cultivable land, and emit little greenhouse gases (van Huis, 2013). At present, exploiting insects as feed ingredients is not in direct competition with food production. Black soldier fly (BSF; *Hermetia illucens*) is being produced at industrial scale in Europe due to its exceptionally good nutritional value and suitability for massive production. On a dry matter basis, BSF larvae contain about 42% protein and 35% lipid (Newton et al., 1977). In terms of protein quality, BSF larvae contains a favorable essential amino acid profile closer to fishmeal than that of soybean meal (Barroso

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et al., 2014). Moreover, the fat level and fatty acid profile are diet-dependent, allowing for control using different feed substrates (St-Hilaire et al., 2007a, 2007b). The potential of BSF larvae as an alternative feed ingredient for fish has been evaluated in several omnivorous and carnivorous species including Atlantic salmon (Belghit et al., 2018; Bondari and Sheppard, 1987; Borgogno et al., 2017; Devic et al., 2017; Hu et al., 2017; Kroeckel et al., 2012; Li et al., 2016; Li et al., 2017; Lock et al., 2016; Magalhaes et al., 2017; Renna et al., 2017; Sealey et al., 2011; St-Hilaire et al., 2007a, 2007b). The optimal substitution level of fishmeal in the diet by BSF larvae meal varies considerably in different studies ranging from 25% to 100%, possibly due to differences in the larvae meal quality, fish species and diet formulation. While the nutritional value of BSF larvae meal has been extensively studied, its impact on fish health, the gut health in particular, has not been investigated.

The present study was part of a larger investigation consisting of a freshwater and seawater feeding trial that aimed to reveal the nutritional value and possible health effects for Atlantic salmon, of a protein-rich insect meal (IM) produced from BSF larvae. In the 8-week freshwater trial, pre-smolt salmon were fed either a reference diet or a test diet wherein 85% of the dietary protein was supplied by BSF larvae meal. The gut health of fish was evaluated using endpoints including organ and tissue indices, histopathology variables and gene expressions (Li et al., 2019). Results from the freshwater trial showed no indications that dietary inclusion of insect meal may affect the gut health of Atlantic salmon negatively. The insect meal diet seemed to reduce excessive lipid deposition in the pyloric caeca enterocytes and stimulate xenobiotic metabolism (Li et al., 2019). The present study focuses on the gut health in the seawater-phase salmon fed BSF larvae meal for 16 weeks. Post-smolt Atlantic salmon was fed either a reference diet with a combination of fish meal, soy protein concentrate, pea protein concentrate, corn gluten and wheat gluten as protein sources, or a test diet wherein all the fish meal and most of pea protein concentrate were replaced by BSF larvae meal. The gut health of seawater-phase salmon fed a commercially-relevant reference diet and an insect meal test diet was evaluated using the same endpoints measured in the freshwater trial (Li et al., 2019).

2. Materials and methods

2.1. Diets and fish husbandry

A feeding trial with seawater-phase Atlantic salmon (initial body weight 1.40 kg, S.D. = 0.043 kg) was conducted at the Gildeskål Research Station (GIFAS), Nordland, Norway, in accordance with laws regulating the experimentation with live animals in Norway. Fish were fed either a commercially-relevant reference diet (REF) with a combination of fish meal, soy protein concentrate, pea protein concentrate, corn gluten and wheat gluten as protein source, or an insect meal diet (IM) wherein all the fish meal and most of the pea protein concentrate were replaced by BSF larvae meal (Table 1). The insect meal was produced from BSF larvae by Protix Biosystems BV (Dongen, The Netherlands). The larvae were grown on media partially containing seaweed (*Ascophyllum nodosum*) mixed with organic plant-derived waste (60:40). At the end of an eight-day growth period, the larvae were mechanically separated from the feeding media, washed and partially defatted before being dried and ground to produce the insect meal. Each diet was randomly allocated to triplicate net pens (5 × 5 × 5 m; 125 m^3) each containing 90 fish. Fish were fed by hand until apparent satiation twice daily (or once due to the light conditions). The feeding trial lasted for 16 weeks. Within this period, the salmon reached a mean weight of 3.7 kg that is suitable for sensory testing. Further details on the insect meal, diet composition and fish husbandry were reported elsewhere (Belghit et al., 2019).

Table 1
Formulation and proximate composition of the experimental diets (previously published in Belghit et al., 2019).

	REF	IM
Ingredients (% wet-weight)		
Fishmeal LT94	10	0.0
Black soldier fly larva meal[a]	0.0	14.75
Soy protein concentrate	25	25
Corn gluten meal	7.5	7.5
Wheat gluten meal	3.35	6.88
Pea protein concentrate 55	8.8	2.84
Fish oil	10.18	14.76
Rapeseed oil	20.95	14.73
Binder	12.32	11.24
Additives[b]	1.89	2.29
Yttrium	1.0	1.0
Chemical composition (wet-weight basis)		
Dry matter (%)	93	95
Crude Protein (%)	38	39
Crude Lipid (%)	29	29
Ash (%)	4.6	4.5
Carbohydrates (%)	11.6	11.4
Gross energy (MJ/kg)	24.6	25.0
TBARS (nmol/g)	3.0	4.9

REF, reference diet; IM, insect meal diet; TBARS, Thiobarbituric acid reactive substances.

[a] Partially defatted. Produced by the Protix Biosystems BV (Dongen, The Netherlands).

[b] Supplemented to meet the nutrient requirements of salmon, mostly consist of vitamin/mineral mix, amino acids (methionine and lysine) and phosphorus.

2.2. Sample collection

At the termination of the feeding trial, fish were randomly taken from the net pens, anesthetized with tricaine methanesulfonate (MS222®; Argent Chemical Laboratories, Redmond, WA, USA) and euthanized by a sharp blow to the head. Body weight was registered for all the fish sampled. From 6 fish per net pen, the whole digestive tract was dissected, cleaned free of attached adipose tissue and opened longitudinally. Only fish with chyme present along the whole intestine were sampled to ensure exposure to the diets until the point of sampling. The chyme was gently removed using a spatula. The emptied intestine was divided into proximal (PI), mid (MI) and distal (DI) segments and weighed, respectively. The gut tissue was rinsed in phosphate buffered saline three times to remove traces of remaining chyme and cut into pieces for RNA extraction and histological evaluation. The gut tissue for RNA extraction was preserved in RNAlater solution at room temperature for < 12 h, incubated at 4 °C for 48 h and stored at −20 °C after arrival at the lab, whereas the gut tissue for the latter purpose was fixed in 4% phosphate-buffered formaldehyde solution for 24 h and transferred to 70% ethanol for storage at room temperature.

2.3. Organosomatic indices

Organosomatic indices (OSI) of the PI, MI and DI were calculated as percentages of the weight of intestinal segments relative to the fish body weight; OSI = 100 * TW/BW, where TW is the tissue weight and BW is the fish body weight.

2.4. Histology

After fixation, PI, MI, and DI samples were processed according to standard histological techniques to produce sections of 3 μm thickness from each intestinal segment and stained with hematoxylin and eosin. The sections were then examined blindly with a light microscope paying attention to typical inflammatory morphological changes commonly observed in salmonid intestine: that is, shortening and fusion of

mucosal folds, cellular infiltration within the lamina propria and submucosa, reduced enterocyte vacuolization and nucleus position disparity (Baeverfjord and Krogdahl, 1996). Normally, little to no vacuolization is present in the enterocytes of the PI and MI whereas enterocytes of the DI show varying degrees of supranuclear vacuolization that diminishes or disappears during inflammation. Shortening and fusion of mucosal folds are usually absent when signs of inflammation, such as immune cell infiltration within the lamina propria and submucosa, are observed in the PI and MI. Therefore, mucosal fold morphology (height and fusion) was only evaluated for the severity of inflammation in the DI. For each histological characteristic evaluated, a value of normal, mild, moderate, marked or severe was assigned.

2.5. Quantitative real-time PCR

Real-time qPCR assays were performed following the MIQE guidelines (Bustin et al., 2009) as described in the freshwater feeding trial (Li et al., 2019). In brief, total RNA was extracted from PI and DI samples with a mean A_{260}/A_{280} ratio of 2.2 (S.D. = 0.01). The RNA integrity was evaluated by agarose gel electrophoresis using the NorthernMax®-Gly sample loading dye (catalog no., AM8551; Ambion, Austin, TX, USA). Based on the gel electrophoresis results, the RNA integrity of 24 representative samples was further confirmed by the 2100 Bioanalyzer using the 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA, USA). The average RIN (RNA integrity number) value for the selected samples was 9.5 (S.D. = 0.38). The cDNA synthesis was performed using 1.0 μg total RNA from all samples using a Superscript™ IV VILO™ cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). A negative control was set up by omitting RNA and the obtained cDNA was diluted 1:10 before use. The qPCR assays were performed using the LightCycler 96 (Roche Applied Science, Basel, Switzerland) and a 10-μL reaction volume was used, which contained 2 μL of PCR-grade water, 2 μL diluted cDNA template, 5 μL LightCycler 480 SYBR Green I Master (Roche Applied Science) and 0.5 μL (10 μM) of each forward and reverse primer. Samples were run in duplicates in addition to a no-reverse-transcription control and a no-template control for each gene. A three-step qPCR programme was applied incorporating an enzyme activation step at 95 °C (5 min) and 45 cycles of 95 °C (10 s), 55–63 °C (10 s) and 72 °C (15 s). Quantification cycle (Cq) values were determined using the second derivative method. Beta-actin (*actb*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), RNA polymerase 2 (*rnapo2*) and hypoxanthine phosphoribosyltransferase 1 (*hprt1*) were evaluated for use as reference genes according to their stability across and within the treatments as described by (Kortner et al., 2011). The expression of target genes in the PI and DI were normalized to the geometric mean of the 4 reference genes evaluated. The mean normalized expression of the target genes was calculated from raw Cq values (Muller et al., 2002). The genes profiled and the primers used for the qPCR assays are given in Table S1.

2.6. Statistics

Statistical analyses and creation of graphs were performed in R 3.5.2 (R Core Team, 2013). The *tidyverse* package (Wickham, 2017) was used to import, tidy, transform and visualize data. After exploratory analyses, continuous response variables were fitted by linear mixed effect model via the *lme4* package (Bates et al., 2015), treating diet as fixed effect and net pen as random effect. The model diagnostics were performed by plotting residuals against the fitted values and against each covariate in the model to assess homogeneity, by making a QQ-plot to check normality and by detecting influential observations using the *influence.ME* package (Nieuwenhuis et al., 2012). The p value of diet effect was obtained via parametric bootstrap comparisons using the *pbkrtest* package (Halekoh and Højsgaard, 2014). When the fitted models were singular, the Welch's *t*-test was run to compare group means and the normality assumption was visually checked via QQ-plots. For ordinal response variables, data were fitted by cumulative link mixed model via the *ordinal* package (Christensen, 2019), treating diet as fixed effect and net pen as random effect. The random effect was dropped when the full model produced singular fits or huge Hessian numbers, or when the random effect was not significant. The proportional odds assumption was checked by comparing models against ones that relax this assumption (i.e., allow nominal/scale effect) via likelihood-ratio tests. The random effect was visually inspected via conditional modes with 95% confidence intervals based on the conditional variance. The statistical model outputs were tidied using the *broom* package (Robinson and Hayes, 2019) when needed. Multiple comparisons were adjusted by the Holm-Bonferroni correction (controlling family-wise error rate) or Benjamini-Hochberg procedure (controlling false discovery rate) where applicable. Differences were regarded as significant when $p < .05$. Plots were generated using *ComplexHeatmap* (Gu et al., 2016), *ggplot2* of the *tidyverse* and extension packages of *ggplot2* including *cowplot* (Wilke, 2019), *ggpubr* (Kassambara, 2018) and *ggsignif* (Ahlmann-Eltze, 2019). Multiple figure panels were combined using the *cowplot* or *gridExtra* package (Auguie, 2017).

3. Results

To aid readers in interpreting data reported here, results on general fish performance and nutrients utilization, which have been published elsewhere (Belghit et al., 2019), are summarized below.

Both diets were readily accepted by the salmon throughout the whole feeding trial. No differences between the diet groups were recorded for feed intake, feed conversion ratio, body weight gain, protein productive value or whole-body proximate composition. Condition factor, hepatosomatic and viscerosomatic indices were not affected by dietary replacement of fish meal with IM. In line with absence of diet effect on the proteinase activity (trypsin and leucine aminopeptidase) and total bile salts level in the chyme, the apparent digestibility of crude protein, crude lipid, amino acids and fatty acids was not affected by dietary IM inclusion.

3.1. Somatic indices of intestinal sections

No significant diet effect was observed for PI-somatic index or MI-somatic index. However, DI-somatic index was significantly higher in fish fed the IM diet ($p < .05$) (Fig. 1).

3.2. Histological appearance

Enterocyte hypervacuolization, suggestive of excessive lipid accumulation (steatosis), was observed in the PI and MI in both diet groups (Fig. 2). It was, however, less severe in the PI of fish fed the IM diet ($p < .05$). Typical signs of enteritis commonly observed in salmonid intestine fed soybean meal diets, including shortening and fusion of mucosal folds (only evaluated for DI), cellular infiltration within submucosa and lamina propria and reduced enterocyte vacuolization (only applicable to DI), were observed in all the intestinal segments in both diet groups (Fig. 2). The only significant diet effect was a higher degree of submucosa cellularity in the PI of fish fed the IM diet ($p < .05$).

3.3. Gene expression

In total, we profiled 36 genes related to immune modulation, lipid metabolism, barrier function and xenobiotic metabolism in the intestine. The diet effect on the gene expression profile was quite minor in the PI and DI. In the PI, matrix metalloproteinase 13 (*mmp13*), a marker gene involved in tissue reorganization, was the only differential expressed gene which showed lower expression levels in fish fed the IM diet ($p < .05$) (Fig. 3). In the DI, choline kinase (*chk*), a marker gene involved in de novo synthesis of phosphatidylcholine, was the only differential expressed gene which showed lower expression levels in

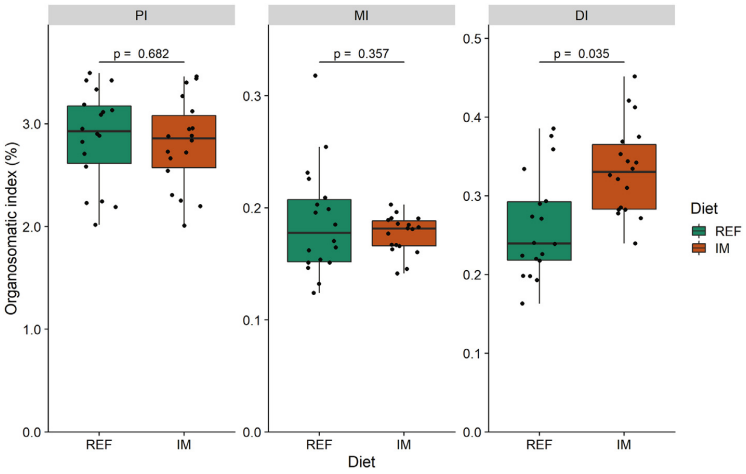

Fig. 1. Somatic indices of intestinal sections of Atlantic salmon fed the experimental diets. The boxplots are in the style of Tukey. PI, proximal intestine; MI, mid intestine; DI, distal intestine; REF, reference diet; IM, insect meal diet.

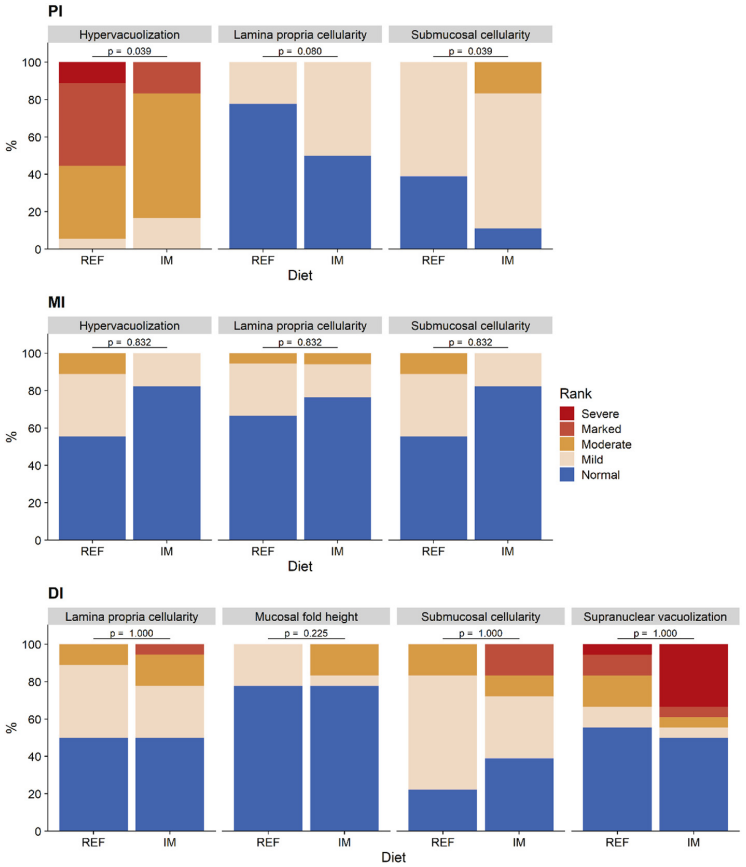

Fig. 2. Contingency chart showing percentages of sampled fish scored normal, mild, moderate, marked and severe regarding enterocyte hypervacuolization (steatosis) and inflammation in different gut segments. PI, proximal intestine; MI, mid intestine; DI, distal intestine; REF, reference diet; IM, insect meal diet.

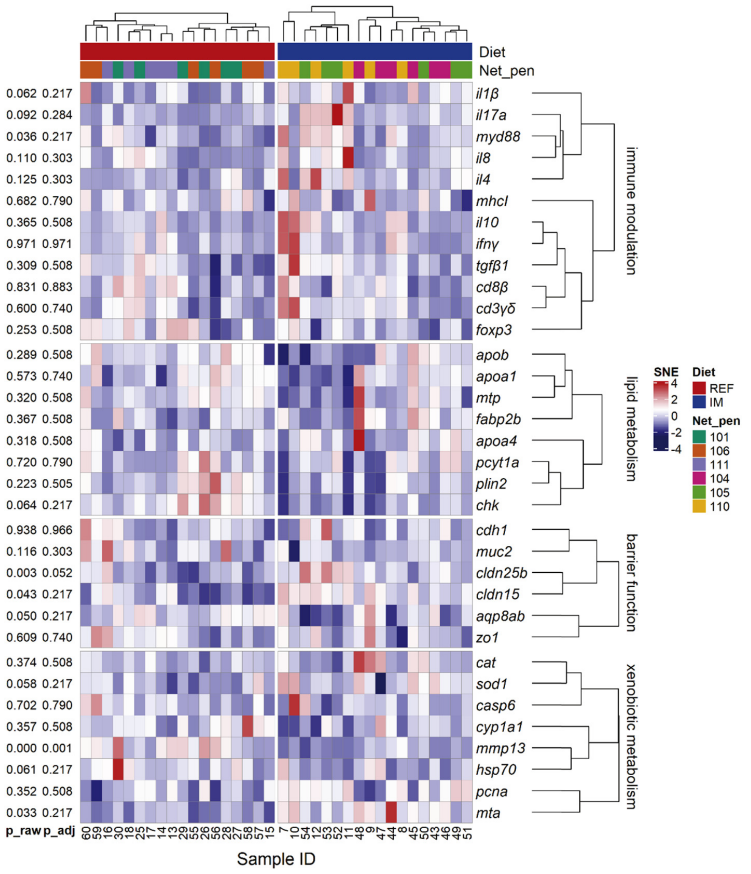

Fig. 3. Gene expression profile in the proximal intestine of Atlantic salmon fed the experimental diets. Data in the same row was scaled (each data point was subtracted by the row mean and divided by the standard deviation). Samples (columns) were clustered within each diet based on the Euclidean distance and genes (rows) were clustered within each functional category based on the Spearman's rank-order correlation. The Ward's minimum variance method was used for the linkage of clusters. For cells in the same row, the deeper the red color, the higher is the gene expression in the respective sample; similarly, the deeper the blue color, the lower is the gene expression in the respective sample. The raw (p_raw) and FDR-adjusted (p_adj) *p* value of diet effect for each gene are shown on the left side of the heatmap. The annotations for the samples (diet and net pen) are given on the top of the heatmap. A supplementary figure showing the normalized expression data before scaling is available as Fig. S1 which displays the data as boxplots overlaid by individual data point. Abbreviations: SNE, scaled normalized expression; REF, reference diet; IM, insect meal diet; see Table S1 for explanations of gene abbreviations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fish fed the IM diet as well ($p < .05$) (Fig. 4).

4. Discussion

In the present study, total replacement of fish meal with BSF larvae meal was associated with a lower degree of steatosis in the proximal intestine and a higher relative weight of distal intestine. Furthermore, replacing fish meal with insect meal in the diet of salmon was associated with increased submucosa cellularity in the proximal intestine.

In our previous experiment, pre-smolt Atlantic salmon were fed a test diet for 8 weeks wherein BSF larvae meal accounted for 60% of the total diet ingredients, replacing most of the fish meal and soy protein concentrate in the reference diet (Li et al., 2019). Gene expression analysis showed increased amount of transcripts indicative of uptake of fatty acids (*cd36*, *fabp2*) and cholesterol (*npc1l1*), immune tolerance (*foxp3*), stress response (*hsp70*) and detoxification activity (*cyp1a1*, *mta*, *sod* and *cat*) in the intestine of fish fed the insect meal diet (Li et al., 2019). Given the much lower inclusion level of insect meal in the present study (15% in the diet), it is not surprising that few genes showed differential expressions. Despite the substantial difference on inclusion level of insect meal between the previous study (Li et al., 2019) and the current trial, both studies showed that insect meal diet was associated with lower enterocyte steatosis in the proximal intestine and increased the relative weight of distal intestine.

Enterocyte steatosis is thought to represent a lipid transport or metabolism disorder in enterocytes which in severe cases may be accompanied by accumulations of lipidic materials inside the gut lumen and referred to as lipid malabsorption, eventually resulting in steatorrhea and the so-called “floating feces” on the surface of sea cages (Hanche-Olsen et al., 2013; Penn, 2011). In contrast to the freshwater trial where the enterocyte steatosis was confined to the proximal intestine (Li et al., 2019), it was observed in both proximal and mid intestine in the present seawater trial. Moreover, all the sampled fish showed varying degrees of steatosis in the proximal intestine enterocytes. The higher prevalence and severity of the enterocyte steatosis is possibly related to a higher feed intake of the seawater-phase salmon (Belghit et al., 2018; Belghit et al., 2019), which may exceed the capacity of enterocytes to transport the absorbed nutrients out of cytoplasm. Consistent with our previous finding in the freshwater trial (Li et al., 2019), fish fed the insect meal diet showed a lower degree of enterocyte steatosis in the proximal intestine, which is in line with a lower but insignificant expression level of *plin2*, a surface marker of lipid droplets (Heid et al., 1998). One should be reminded that there were no macroscopic appearances of lipid malabsorption in any fish at the time of sampling, and no apparent indications of reduced fish health as a result of the steatosis. Also, the analysis of total lipid content, lipid class and lipid droplet size and number in the liver showed no diet effect (Belghit et al., 2019).

Consistent with results from the freshwater trial (Li et al., 2019), increased relative weight of distal intestine was also observed in the

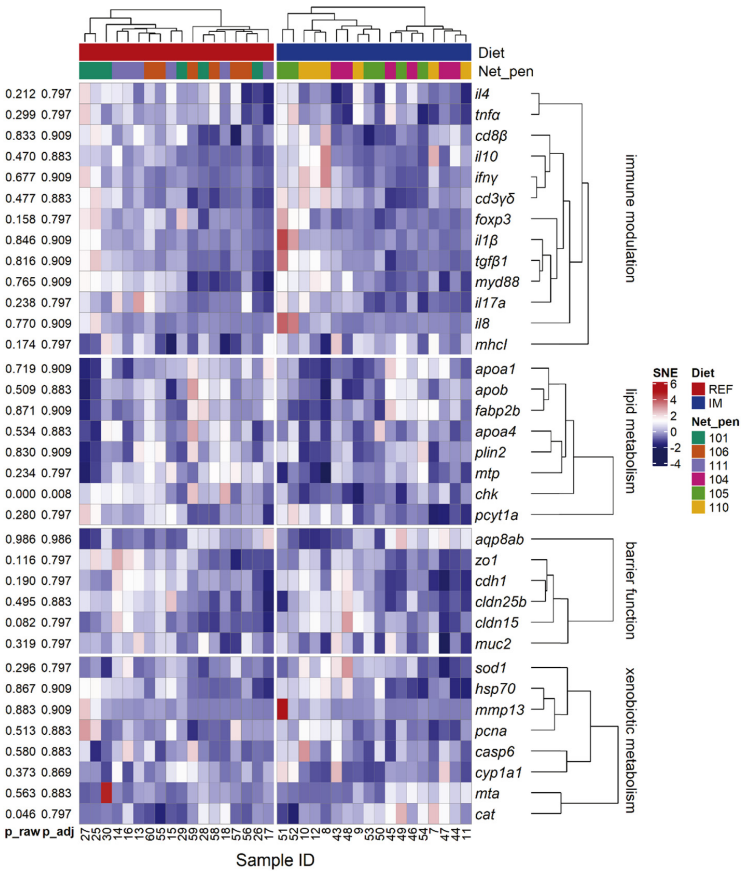

Fig. 4. Gene expression profile in the distal intestine of Atlantic salmon fed the experimental diets. See Fig. 3 for explanations of the graph and abbreviations. A supplementary figure showing the normalized expression data before scaling is available as Fig. S2 which displays the data as boxplots overlaid by individual data point.

present seawater trial. Findings from experiments with chickens may be relevant in comparison. Dietary inclusion of BSF larvae meal (7.3% or 14.6%) was reported to increase the length of jejunum in laying hens (Bovera et al., 2018; Cutrignelli et al., 2018). In another study, laying hens fed a diet containing 17% BSF larvae meal for 21 weeks showed higher concentrations of short chain fatty acids (SCFAs) in the caecal content, including acetate, propionate, isobutyrate, butyrate, isovalerate and valerate. Notably, butyrate nearly tripled in its concentration increasing from 1.5 to 4.4 mmol/L (Borrelli et al., 2017). Butyrate is an important energy source for intestinal epithelial cells. It was estimated to provide 60–70% energy for colonic epithelium in human (Roediger, 1980) and is known to stimulate the proliferation of mucosal cells in colon (Kripke et al., 1989; Mortensen et al., 1999; Souleimani and Asselin, 1993; Whitehead et al., 1986). Whether dietary inclusion of BSF larvae meal may increase the production of SCFAs in the distal intestine of salmon and thus contribute to the increased organ weight remains further elucidation.

Opposed to the absence of gut inflammation in the freshwater trial (Li et al., 2019), signs of inflammation were observed in both diet groups in all the gut segments examined, which is a rare case. While gut inflammation has also been reported in farmed salmon fed commercial diets, it's usually only present in the distal intestine (Chikwati et al., 2018). The exception was when a parasitic infection occurs, such as tapeworm or nematode infection, causing inflammation throughout the whole intestine (Chikwati et al., 2018; Murphy et al., 2010). However, no parasitic infection was noted during the feeding trial or at the time of sampling. Given that the feeding trial was commenced with fish already at sizes averaging 1.4 kg, and no basal gut health assessment was conducted prior to start of the trial, it is hard to rule out historical exposure to inflammation-inducing diets and/or parasites. It is thus a good practice to conduct a basal gut health evaluation of experimental fish (> 100 g) before assignment to feed groups to minimize pre-existing gut health disorders that may diminish trial outcomes and goals.

Recent studies on the nutritional value of BSF larvae meal for rainbow trout (*Oncorhynchus mykiss*) (10.5%, 21%) (Cardinaletti et al., 2019), clownfish (*Amphiprion ocellaris*) (20%, 40%, 60%) (Vargas-Abúndez et al., 2019) and zebrafish (*Danio rerio*) (25%, 50%) (Zarantoniello et al., 2019) have not revealed signs of gut inflammation. Furthermore, its inclusion increased the expression of *foxp3*, a master transcription factor for the differentiation of naïve CD4 T cells into regulatory T cells, in the proximal and distal intestine of salmon in our freshwater trial (Li et al., 2019). In the present seawater trial, however, increased submucosa cellularity was found in the proximal intestine of salmon fed the insect meal diet. Possible explanations are: 1) Atlantic salmon prey on insects in the freshwater before they finish smoltification and migrate to the sea. Hence, the gut immune system of

salmon might have a higher tolerance of insect ingredients in the freshwater than in the seawater. 2) The increased submucosa cellularity was possibly, already present in the fish prior to start of the trial but the experimental diets improved the gut health in the proximal intestine to differing levels, with the reference diet performing better than the insect meal diet in reducing the severity of inflammatory changes. It should be noted that none of the proinflammatory marker genes profiled in the proximal intestine showed differential expressions. Neither did we observe comprised gut functions as a result of the increased submucosa cellularity.

In conclusion, total replacement of fish meal with black soldier fly larvae meal did not compromise the gut health of Atlantic salmon. Dietary insect meal inclusion seemed to reduce excessive lipid deposition within enterocytes (steatosis) in the proximal intestine. Possible interactions between insect meal inclusion and the development of gut inflammation in seawater-phase salmon is worth of attention in future studies.

Data and code availability

The data and code used for the statistical analyses and creation of figures are deposited at the GitHub repository (https://github.com/yanxianl/AquaFly-SeawaterGutHealth-Aquaculture-2019).

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Declaration of Competing Interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.734967.

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Animal Microbiome

RESEARCH ARTICLE Open Access

Differential response of digesta- and mucosa-associated intestinal microbiota to dietary insect meal during the seawater phase of Atlantic salmon

Yanxian Li[1*†], Leonardo Bruni[2†], Alexander Jaramillo-Torres[1], Karina Gajardo[1], Trond M. Kortner[1] and Åshild Krogdahl[1]

Abstract

Background: Intestinal digesta is commonly used for studying responses of microbiota to dietary shifts, yet evidence is accumulating that it represents an incomplete view of the intestinal microbiota. The present work aims to investigate the differences between digesta- and mucosa-associated intestinal microbiota in Atlantic salmon (*Salmo salar*) and how they may respond differently to dietary perturbations. In a 16-week seawater feeding trial, Atlantic salmon were fed either a commercially-relevant reference diet or an insect meal diet containing ~ 15% black soldier fly (*Hermetia illucens*) larvae meal. The digesta- and mucosa-associated distal intestinal microbiota were profiled by 16S rRNA gene sequencing.

Results: Regardless of diet, we observed substantial differences between digesta- and mucosa-associated intestinal microbiota. Microbial richness and diversity were much higher in the digesta than the mucosa. The insect meal diet altered the distal intestinal microbiota resulting in higher microbial richness and diversity. The diet effect, however, depended on the sample origin. Digesta-associated intestinal microbiota showed more pronounced changes than the mucosa-associated microbiota. Multivariate association analyses identified two mucosa-enriched taxa, *Brevinema andersonii* and *Spirochaetaceae*, associated with the expression of genes related to immune responses and barrier function in the distal intestine, respectively.

Conclusions: Our data show that salmon intestinal digesta and mucosa harbor microbial communities with clear differences. While feeding insects increased microbial richness and diversity in both digesta- and mucosa-associated intestinal microbiota, mucosa-associated intestinal microbiota seems more resilient to variations in the diet composition. To fully unveil the response of intestinal microbiota to dietary changes, concurrent profiling of digesta- and mucosa-associated intestinal microbiota is recommended whenever feasible. Specific taxa enriched in the intestinal mucosa are associated to gene expression related to immune responses and barrier function. Detailed studies are needed on the ecological and functional significance of taxa associated to intestinal microbiota dwelling on the mucosa.

Keywords: Atlantic salmon, Diet, Black soldier fly, Microbiota, Digesta, Mucosa

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Background

The global population is projected to reach 9.7 billion in 2050 [1], requiring an increase in the food supply by 25–70% [2]. Producing more safe and high-quality food in a sustainable way to meet the global population growth is a great challenge for our generation. Fish are considered as nutritionally valuable part of the human diet and play an important role in the global food supply [3, 4]. The average annual growth rate of world food fish consumption in the period 2019–2030 is projected to be 1.4%, reaching 28 million tonnes live weight in 2030 [5]. Atlantic salmon (*Salmo salar*) is the most produced marine fish species and one of the most economically important farmed fish worldwide [6]. While Atlantic salmon are strictly carnivorous in the wild, farmed Atlantic salmon have experienced a substantial shift in the diet composition due to a limited supply of marine ingredients. Marine ingredients used for Norwegian farmed Atlantic salmon have gradually been replaced by plant sources, decreasing from ~ 90% in 1990 to ~ 25% in 2016 [7]. Due to concerns on the economic, environmental and social sustainability of the current raw materials for Atlantic salmon farming [6], more sustainable alternative feed ingredients, such as insects [8] and single-cell organisms (bacteria, yeasts and microalgae) [9], have been developed and used. One of the insect species with the greatest potential as an alternative feed ingredient for salmon aquaculture is black soldier fly (BSF; *Hermetia illucens*), which is now produced at industrial scale in Europe. In terms of protein quality, BSF larvae have a favorable essential amino acid profile closer to fishmeal than that of soybean meal [10]. The nutritional value of BSF larvae meal has been extensively evaluated in various fish species including Atlantic salmon [11–21]. However, how dietary BSF larvae meal may influence the intestinal health, function and microbiota of fish remains largely unexplored.

It is now well established that intestinal microbiota plays a pivotal role in host development and physiology, from being an essential element for the development of normal gut functions and immunity [22, 23] to modulating lipid metabolism and energy balance [24, 25]. Recent advances in sequencing technologies have transformed our ability to study the composition and dynamics of fish intestinal microbiota, leading to increasing interest in selective manipulation of intestinal microbiota. Diet is one of the key factors in shaping the intestinal microbiota. While long-term dietary habits have a considerable effect on the structure and activity of host intestinal microbiota [26–28], short-term dietary change also alters the intestinal microbiota in a rapid and reproducible way [29]. Different dietary components selectively promote or suppress the growth of certain microbial clades, which in turn could inflict important effects on the host health and disease resistance [30, 31]. The use of alternative feed ingredients may not only affect the nutrient utilization, fish growth, health, welfare and product quality, but also intestinal microbiota in Atlantic salmon [32–34]. While studies in mammals and fish have revealed substantial differences between the digesta- and mucosa-associated intestinal microbiota [32, 35–38], most studies investigating diet effects on the intestinal microbiota of fish have sampled the digesta only or a mixture of digesta and mucosa. Evidence is accumulating that digesta- and mucosa-associated intestinal microbiota in fish respond differently to dietary changes [32, 39–42]. Profiling only one of or a mixture of digesta- and mucosa-associated microbiota may obscure the response of intestinal microbiota to dietary changes.

Characterizing intestinal microbiota and its associations with host responses is an essential step towards identifying key microbial clades promoting fish health and welfare. Ultimately, a milestone in the fish microbiota research would be knowing how to selectively manipulate the microbiota to improve the growth performance, disease resistance and health status of farmed fish. The main aims of the present study were (i) to compare distal intestinal microbiota of Atlantic salmon fed a commercially relevant diet or an insect meal diet, (ii) to further explore the dissimilarity between digesta- and mucosa-associated microbiota and the differences in their response to dietary changes, and (iii) to identify associations between microbial clades and host responses. This work was part of a larger study consisting of a freshwater and seawater feeding trial that aimed to investigate the nutritional value and possible health effects for Atlantic salmon of a protein-rich insect meal produced from BSF larvae. The results presented herein focus on the intestinal microbiota in seawater phase Atlantic salmon fed an insect meal diet containing ~ 15% BSF larvae meal for 16 weeks.

Results

To aid readers in interpreting the data we report here, results on the feed utilization, growth performance, fillet quality, intestinal histopathology and gene expression, which have been reported elsewhere [43–45], are summarized as the following. In brief, there was lack of evidence that the insect meal diet negatively affected the feed utilization, growth performance or fillet quality of Atlantic salmon. Profiling of genes related to lipid metabolism, immune responses, barrier functions and stress responses in the proximal and distal intestine showed little evidence of diet effect. Histopathological examination of intestinal segments showed enterocyte steatosis in the proximal and mid intestine in both diet groups, but it was less severe in the proximal intestine of fish fed the insect meal diet.

Hereafter, different sample groups are named based on the combination of diet (REF vs. IM) and sample origin (DID vs. DIM). Hence, in addition to the extraction blanks, library blanks and mock, we have four different sample types, i.e., REF-DID, REF-DIM, IM-DID and IM-DIM.

qPCR

Since Cq values of most mucosa DNA templates were out of the linear range of the standard curve, the raw Cq value was used as a proxy of 16S rRNA gene quantity in the diluted DNA templates (Fig. S1). On average, REF-DID showed the highest 16S rRNA gene quantities (mean Cq = 24.7), followed by the mocks (mean Cq = 26.1) and IM-DID (mean Cq = 28.4). Irrespective of diet, mucosa DNA templates (REF-DIM, IM-DIM) showed similar 16S rRNA gene quantities (mean Cq = 30) that were close to extraction blanks (mean Cq = 32.4).

Characteristics of the sequence data

The high-throughput sequencing generated a total number of 7.5 million raw reads for biological samples. The median of raw reads per sample was 96,231, with the minimum and maximum value being 14,940 and 151, 916, respectively. After the sequence denoising and ASV filtering, a total number of 1620 unique ASVs was generated. The mean percentage of chloroplasts and mitochondria removed from the ASV table before filtering out contaminants was 7.4 and 0.2%, respectively. The mean percentage of chloroplast varied considerably between digesta (7.1%) and mucosa (0.2%) samples and between diets within digesta samples, e.g. REF-DID (24.9%) and IM-DID (4.4%). The number of effective sequences retained for the downstream data analysis was 3.6 million. The median of effective sequences per sample was 46,372, with the minimum and maximum value being 951 and 106,591, respectively.

Taxonomic composition

All the eight bacterial species included in the mock were successfully identified at genus level with *E. faecalis, L. fermentum, L. monocytogenes* and *S. aureus* further being annotated at the species level (Fig. S2A). At the genus level, the average Pearson's *r* between the expected and observed taxonomic profile of the mock was 0.33, whereas the Pearson's *r* between the observed taxonomic profile of the mock was 0.98. The relative abundance of most Gram-positive bacteria, *L. monocytogenes* and *E. faecalis* in particular, were underestimated. In contrast, the relative abundance of Gram-negative bacteria was overestimated. Most ASVs (97.5–99.9%) in the extraction and library blanks were classified as *Pseudomonas* (Fig. S2B), which was the main contaminating taxon removed from the biological samples. Other contaminating ASVs removed from the biological samples were classified as *Curtobacterium*, *Jeotgalicoccus*, *Modestobacter*, *Cutibacterium*, *Hymenobacter*, *Brevundimonas*, *Micrococcus*, *Sphingomonas*, *Devosia*, *Sphingomonas aurantiaca* and *Marinobacter adhaerens*. The exact sequence and taxonomy of the contaminating ASVs and their relative abundance in the extraction and library blanks are available in Table S1.

The taxonomic composition of mucosa samples showed higher similarity than that of the digesta samples, which were more diet-dependent (Fig. 1). At the phylum level, the dominant taxa of mucosa samples for both diets were *Spirochaetes* (REF-DIM, 72%; IM-DIM, 47%) (mean relative abundance), *Proteobacteria* (REF-DIM, 21%; IM-DIM, 23%), *Firmicutes* (REF-DIM, 1%; IM-DIM, 11%), *Tenericutes* (REF-DIM, 4%; IM-DIM, 8%) and *Actinobacteria* (REF-DIM, 1%; IM-DIM, 9%). For digesta samples, the dominant taxa of REF-DID were *Tenericutes* (33%), *Proteobacteria* (31%), *Firmicutes* (25%) and *Spirochaetes* (9%), whereas IM-DID was dominated by *Firmicutes* (45%), *Actinobacteria* (25%), *Proteobacteria* (17%), *Tenericutes* (7%) and *RsaHF231* (4%) (Fig. 1a). At the genus level, the dominant taxa of mucosa samples for both diets were *Brevinema* (REF-DIM, 52%; IM-DIM, 25%), *Spirochaetaceae* (REF-DIM, 20%; IM-DIM, 22%), *Aliivibrio* (REF-DIM, 18%; IM-DIM, 18%) and *Mycoplasma* (REF-DIM, 4%; IM-DIM, 8%). For digesta samples, the dominant taxa of REF-DID were *Mycoplasma* (33%), *Aliivibrio* (20%), *Photobacterium* (10%), *Brevinema* (6%) and *Lactobacillus* (5%), whereas IM-DID was dominated by *Aliivibrio* (15%), *Lactobacillales* (14%), *Corynebacterium 1* (13%), *Bacillus* (8%), *Mycoplasma* (7%) and *Actinomyces* (5%) (Fig. 1b).

Core ASVs

In total, 339 ASVs were identified as core ASVs based on their prevalence in each sample type (Fig. 2; Table S2). Three ASVs, classified as *Aliivibrio*, *Brevinema andersonii*, and *Mycoplasma* respectively, were identified as core ASVs in all the sample types. The *Brevinema andersonii* ASV was universally present in all the samples. Additionally, 11 ASVs were identified as core ASVs for digesta samples (REF-DID and IM-DID), which were classified as *Geobacillus* (1 ASV), *Lactobacillus* (3 ASVs), *Mycoplasma* (2 ASVs), *Photobacterium* (3 ASVs), *Streptococcus* (1 ASV) and *Weissella* (1 ASV). Two additional core ASVs were identified for the mucosa samples (REF-DIM and IM-DIM), which were classified as *Brevinema andersonii* and *Spirochaetaceae*, respectively. Six additional core ASVs were identified for fish fed the insect meal diet (IM-DID and IM-DIM), which were classified as *Actinomyces*, *Corynebacterium 1*, *Corynebacterium aurimucosum* ATCC 70097, *Lactobacillales*, *RsaHF23* and *Spirochaetaceae*, respectively. No

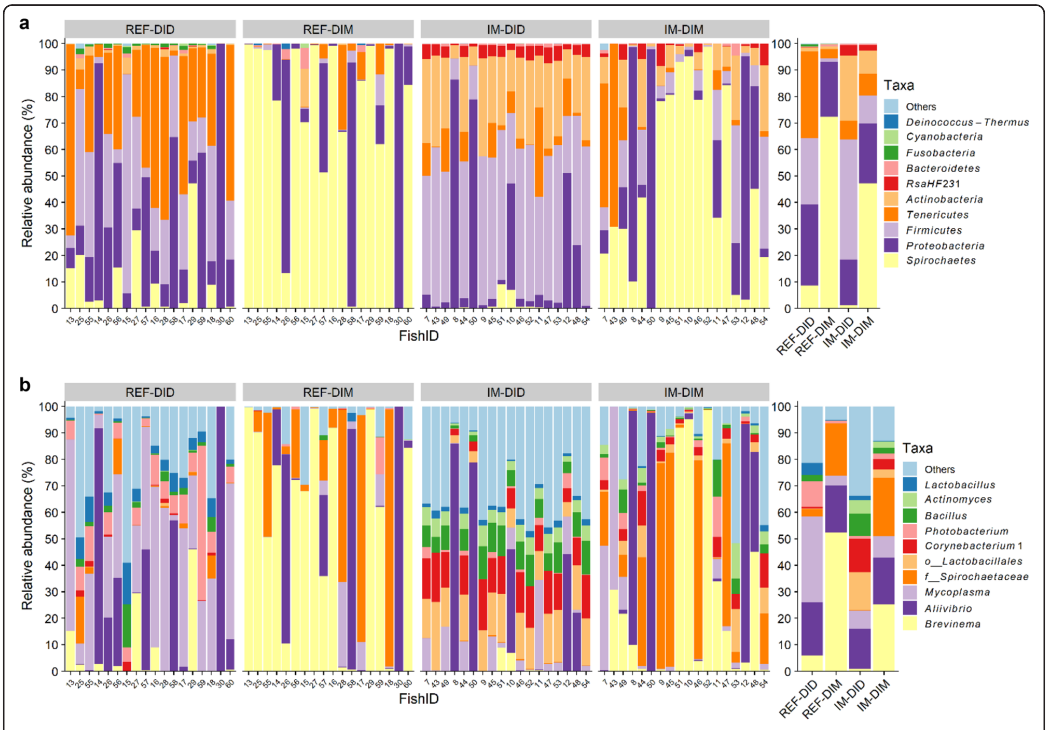

Fig. 1 Top 10 most abundant taxa of all samples at phylum (**a**) and genus (**b**) level. The samples are grouped by the sample type. The mean relative abundance of each taxon within the same sample type is displayed on the right side. *o__*, order; *f__*, family; REF, reference diet; IM, insect meal diet; DID, distal intestine digesta; DIM, distal intestine mucosa

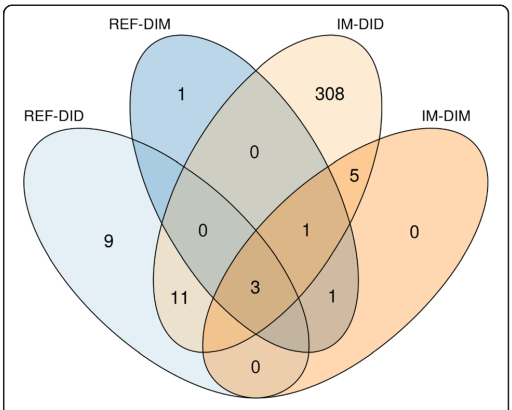

Fig. 2 Venn's diagram showing the shared and unique core ASVs in each sample type. The core ASVs were computed using a prevalence threshold at 80%. REF, reference diet; IM, insect meal diet; DID, distal intestine digesta; DIM, distal intestine mucosa

additional core ASVs were identified for fish fed the reference diet (REF-DID and REF-DIM). Lastly, 308 ASVs were found to be more prevalent in IM-DID than in any other sample type.

Alpha-diversity

Regardless of diet, all the alpha-diversity indices were higher in digesta samples than mucosa samples ($p < 0.05$) (Fig. 3). Independent of sample origin, all the alpha-diversity indices were higher in fish fed the IM diet than those fed the REF diet ($p < 0.05$). A significant interaction between the diet and sample origin effect was detected for the observed ASVs ($p < 0.001$) and Faith's phylogenetic diversity ($p < 0.001$), both of which showed a stronger diet effect in digesta samples than mucosa samples.

Beta-diversity

The PCoA plots built on the Jaccard and unweighted UniFrac distance matrix showed clear separations of samples belonging to different dietary groups and sample origins (Fig. 4a-b). However, the average distance

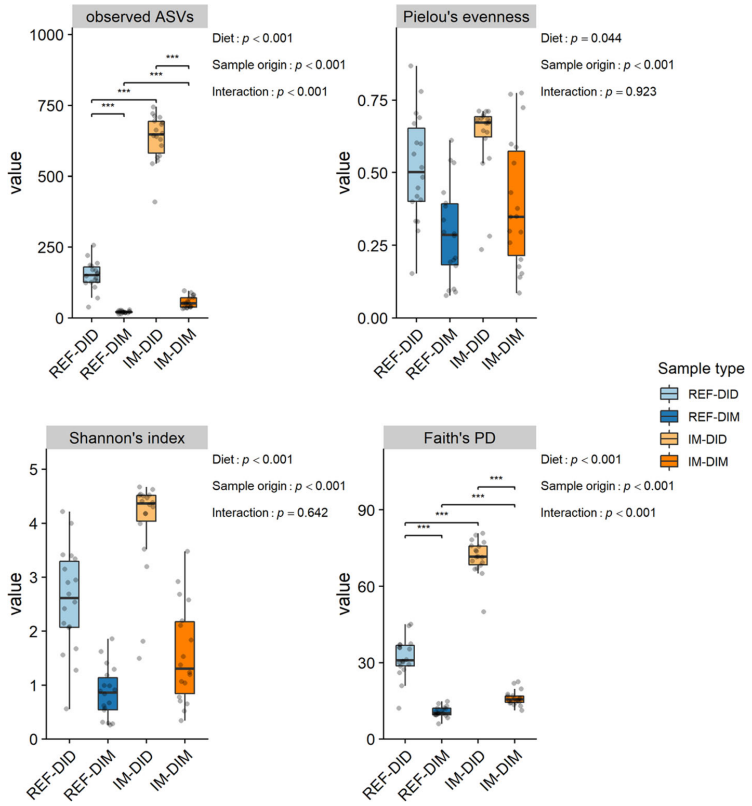

Fig. 3 The sample origin and diet effects on the alpha-diversity of distal intestinal microbiota in seawater phase Atlantic salmon. The *p* value of the main effects and their interaction are displayed on the top-right corner of each sub-plot. Asterisks denote statistically significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). PD, phylogenetic diversity; REF, reference diet; IM, insect meal diet; DID, distal intestine digesta; DIM, distal intestine mucosa

between samples from different dietary groups was dependent on sample origin. Specifically, mucosa samples from different dietary groups formed clusters close to each other, whereas digesta samples from different dietary groups were far apart. The PCoA plots built on the Aitchison and PHILR transformed Euclidean distance matrix also showed separations of samples belonging to different dietary groups and sample origins (Fig. 4c-d). Again, the average distance between samples from different dietary groups was dependent on sample origin. Mucosa samples from different dietary groups formed clusters boarding (Fig. 4c) or overlapping (Fig. 4d) each other, whereas digesta samples from different dietary groups were more clearly separated.

The PERMANOVA and its following conditional contrasts largely confirmed the PCoA results. Regardless of the distance matrix used, both main factors had significant effects on the beta-diversity and their interaction was significant as well ($p < 0.05$) (Table 2). Results on the tests of homogeneity of multivariate dispersions are shown in Table 3. For Jaccard distance, significant differences in the multivariate dispersions were observed between digesta and mucosa samples for both diets (REF-DID VS. REF-DIM, $p = 0.045$; IM-DID VS. IM-DIM, $p = 0.002$), and between diets for digesta samples (REF-DID VS. IM-DID, $p = 0.002$). For unweighted UniFrac distance, IM-DID showed lower multivariate dispersions than other sample types resulting in significant differences compared to REF-DID ($p = 0.002$) and IM-DIM ($p = 0.002$). For Aitchison distance, REF-DIM showed lower multivariate dispersions than other sample types resulting in significant differences compared to REF-DID ($p = 0.046$) and IM-DIM ($p = 0.046$). For PHILR transformed Euclidean distance, the differences in the

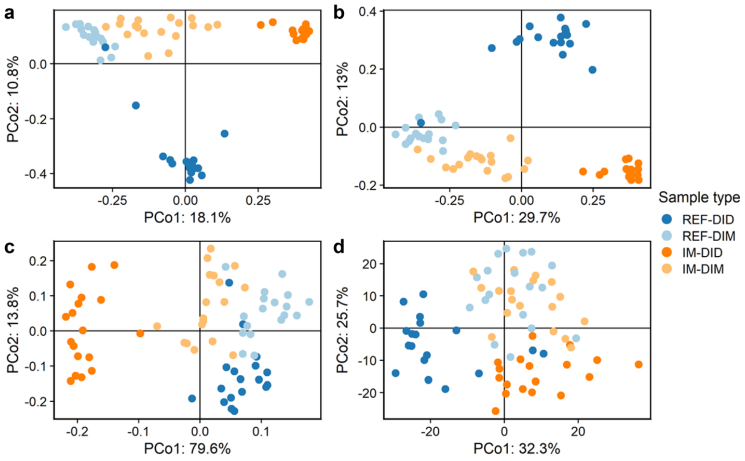

Fig. 4 The sample origin and diet effects on the beta-diversity of distal intestinal microbiota in seawater phase Atlantic salmon. The PCoA plots were built on Jaccard (**a**), unweighted UniFrac (**b**), Aitchison (**c**) and phylogenetic isometric log-ratio (PHILR) transformed Euclidean (**d**) distance matrix, respectively. PCo, principle coordinate; REF, reference diet; IM, insect meal diet; DID, distal intestine digesta; DIM, distal intestine mucosa

multivariate dispersions among the sample types were not significant ($p > 0.05$).

Significant associations between microbial clades and sample metadata

The multivariate association analysis identified 53 taxa showing significant associations with the metadata of interest (Fig. 5a). The diagnostic plots showing the raw data underlying the significant associations are shown in Figs. S3–8. Forty-seven differentially abundant taxa were identified for the sample origin effect, 45 of which, including *Bacillus*, *Enterococcus*, *Flavobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Mycoplasma*, *Peptostreptococcus*, *Photobacterium*, *Staphylococcus*, *Streptococcus*, *Vagococcus* and *Weissella*, showed lower relative abundances in the mucosa than the digesta (Fig. S3). In contrast, two taxa belonging to the *Spirochaetes* phylum, *Brevinema andersonii* and *Spirochaetaceae*, were enriched in the mucosa (Fig. 5b). Thirty-six differentially abundant taxa were identified for the diet effect, 26 of which showed increased relative abundances in fish fed the IM diet (Fig. S4). Among these 26 taxa, some were enriched in both intestinal digesta and mucosa which included *Actinomyces*, *Bacillaceae*, *Bacillus*, *Beutenbergiaceae*, *Brevibacterium*, *Corynebacterium 1*, *Enterococcus*, *Lactobacillales*, *Microbacterium*, *Oceanobacillus* and *RsaHF231* (partially illustrated as Fig. 5c). For the histological scores, the relative abundance of *Sphingobacteriaceae* and *RsaHF231* were found to increase and decrease, respectively, in fish scored abnormal regarding lamina propria cellularity (LPC) in distal intestine (Fig. S5). The relative abundance of *Acinetobacter* and *Pseudomonas* were negatively correlated with the distal intestine somatic index (DISI) (Fig. S6). Six taxa, including *Actinomyces*, *Brevinema andersonii*, *Kurthia*, *Lysobacter*, *Microbacterium* and the *Sphingobacteriaceae*, were found to associate with the expression of genes related to immune responses (Fig. S7). Notably, the relative abundance of *Brevinema andersonii* showed a clear positive correlation with the expression levels of immune genes (Fig. 5d), which decreased as the PC1 of the PCA increased. Furthermore, 3 taxa including *Cellulosimicrobium*, *Glutamicibacter* and the *Spirochaetaceae* were found to associate with the expression of genes related to barrier functions (Fig. S8). The relative abundance of the *Spirochaetaceae* showed a negative correlation with the expression levels of barrier function relevant genes (Fig. 5e), which decreased as the PC1 of the PCA increased.

Discussion

Core microbiota

In accordance with previous studies in Atlantic salmon [33, 46–51], *Aliivibrio*, *Brevinema andersonii* and *Mycoplasma* were identified as core microbiota in the present study. *Aliivibrio* is commonly found in the seawater phase Atlantic salmon intestine [48–50, 52–56] and has been identified as a core taxon of both wild and captive Atlantic salmon [47, 49, 50]. Provided its common presence in seawater, *Aliivibrio* may have originated from the surrounding water and colonized the intestinal mucosa as Atlantic salmon constantly drink seawater to

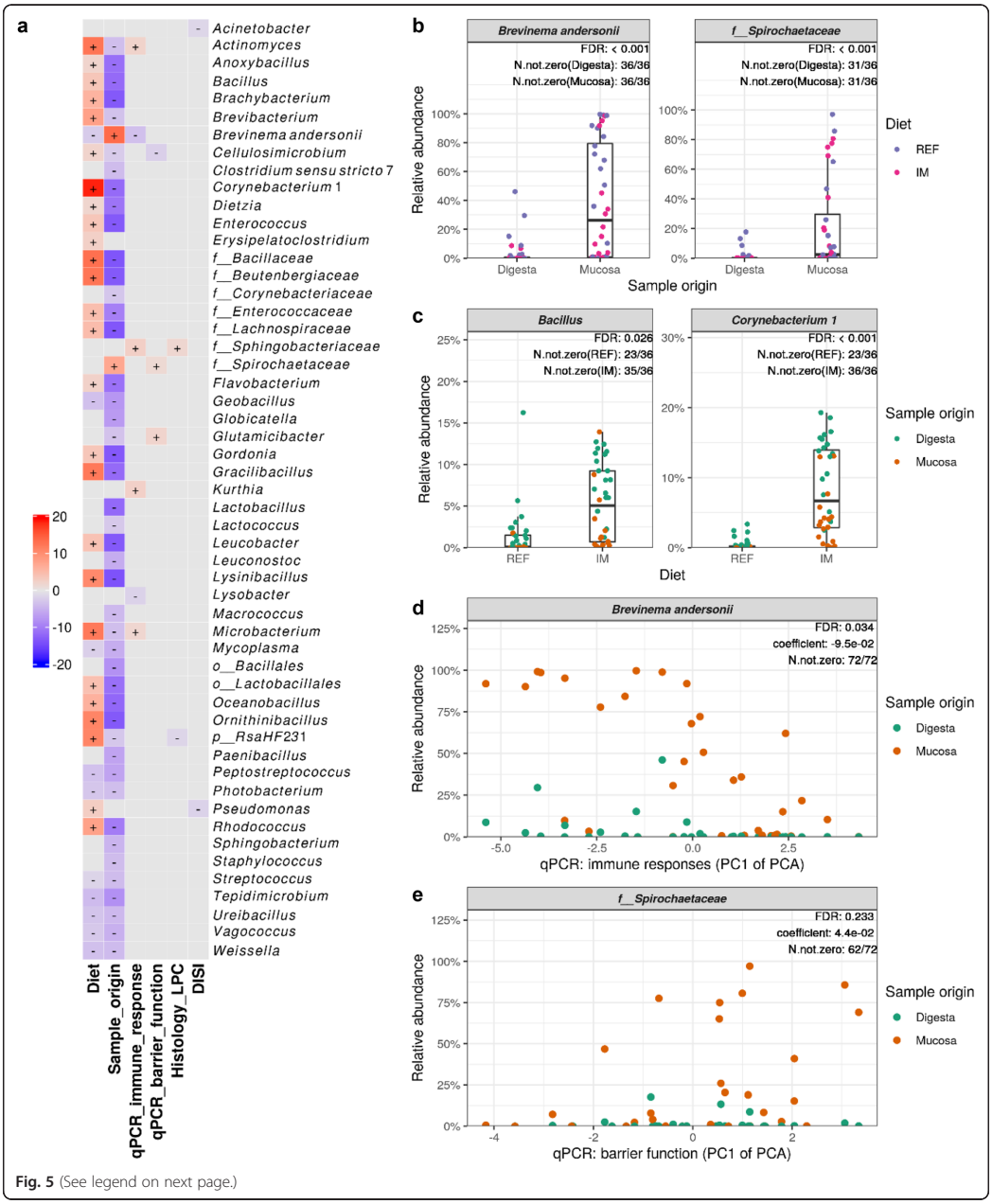

Fig. 5 (See legend on next page.)

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Fig. 5 Significant associations between microbial clades and 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: Diet (+), higher abundance in salmon fed the IM diet; Sample_origin (+), higher abundance in mucosa samples; Histology_LPC (+), higher abundance in salmon scored abnormal regarding lamina propria cellularity (LPC) in the distal intestine; DISI (+), positive correlation between microbial clade abundance and distal intestine somatic index (DISI); qPCR_immune_response (+) / qPCR_barrier_function (+), negative correlation between microbial clade abundance and the gene expression levels. **b** Taxa that are more abundant in the intestinal mucosa than the digesta. **c** Representative taxa showing increased relative abundances in both intestinal digesta and mucosa of salmon fed the IM diet. **d** The positive correlation between the relative abundance of *Brevinema andersonii* and immune gene expression levels in the distal intestine. Note that the expression levels of the immune genes decreased as the PC1 of the PCA increased. **e** The negative correlation between the relative abundance of the *Spirochaetaceae* and the expression levels of barrier function relevant genes. Also note that the expression levels of the barrier function relevant genes decreased as the PC1 of the PCA increased. *p__*, phylum; *o__*, order; *f__*, family; FDR, false discovery rate; N.not.zero, number of observations that are not zero; REF, reference diet; IM, insect meal diet

prevent dehydration in a hyperosmotic environment. Currently, *Aliivibrio* comprises of four closely related species including *Aliivibrio fischeri*, *Aliivibrio logei*, *Aliivibrio salmonicida* and *Aliivibrio wodanis*, which were split from the *Vibrio* genus and reclassified as *Aliivibrio* in 2007 [57]. Strains of *A. fischeri* and *A. logei* have been described as bioluminescent symbionts of certain fishes and squids [58], whereas *A. salmonicida* and *A. wodanis* have been identified as pathogens for Atlantic salmon causing cold-water vibriosis [59] and 'winter ulcer' [60], respectively. We identified 7 *Aliivibrio* ASVs in this study, four of which, including the core *Aliivibrio* ASV, were closely related and clustered with unknow *Aliivibrio* species in the reference database. Among the known *Aliivibrio* species, *A. logei* is most closely related to the core *Aliivibrio* ASV, which was also the predominant *Aliivibrio* ASV found in the present study. One of the *Aliivibrio* ASVs, which was detected at very low abundances (< 0.00015%), was closely related to *A. wodanis*. These observations coincide with previous findings in Arctic seawater-farmed Atlantic salmon [54], suggesting that *Aliivibrio* in the salmon intestine mostly comprises of commensal species.

Though *Spirochaetes* has typically been found in low abundances in the Atlantic salmon intestine [32, 36, 40, 52, 61], two recent studies have identified *Brevinema andersonii* as a core taxon of both digesta- and mucosa-associated intestinal microbiota in seawater phase Atlantic salmon [48, 49]. Notably, *Brevinema andersonii* is also a predominant taxon in the digesta and mucosa in one of the studies [49]. *Brevinema andersonii* was initially isolated from short-tailed shrews (*Blarina brevicauda*) and white-footed mice (*Peromyscus leucopus*) as an infectious pathogen [62]. This taxon has also been found in the intestine and gill tissue of rainbow trout (*Oncorhynchus mykiss*) [63], and intestinal digesta of Senegalese sole (*Solea senegalensis*) [64].

Mycoplasma is widely distributed in nature and well known for its minute size and lack of cell wall. It seems to be particularly well-adapted to Atlantic salmon intestine [65]. Like *Aliivibrio*, it has been frequently identified as a core taxon of both wild and captive Atlantic salmon [33, 46, 48–51]. Notably, it was found to be more abundant in marine adults than in freshwater juvenile Atlantic salmon [50] and sporadically predominate intestinal microbial community in the digesta [33, 49, 50, 54, 66] and mucosa [48] reaching > 90% of total reads in extreme cases. Due to its small compact genome and limited biosynthesis capacities, *Mycoplasma* typically forms obligate parasitic or commensal relationships with its host to obtain necessary nutrients such as amino acids, fatty acids and sterols [67]. Recent shotgun-metagenomic sequencing of the Atlantic salmon *Mycoplasma* revealed that it is closely related to *Mycoplasma penetrans* [33, 68]. It was suggested that the presence of riboflavin encoding genes and lack of pathogenicity factors in the metagenome-assembled *Mycoplasma* genome is indicative of a symbiotic relationship between the *Mycoplasma* and Atlantic salmon [68].

Sample origin effect

In line with previous findings in mammals and fish [32, 35–38], we observed substantial differences between digesta- and mucosa-associated microbiota. The microbial richness and diversity were much higher in the digesta than the mucosa, as previously observed in seawater phase Atlantic salmon [32, 36, 49]. Furthermore, most of the bacterial taxa in the distal intestine, including those commonly found in the Atlantic salmon intestine such as *Bacillus*, *Enterococcus*, *Flavobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Mycoplasma*, *Peptostreptococcus*, *Photobacterium*, *Staphylococcus*, *Streptococcus*, *Vagococcus* and *Weissella*, were less abundant in the mucosa than in the digesta. These results are suggestive of a selection pressure from the host that determines which microbial clades colonize and flourish in the intestinal mucus layer [69]. In this study, two taxa belonging to the *Spirochaetes* phylum, *Brevinema*

andersonii and *Spirochaetaceae*, were more abundant in the distal intestine mucosa than the digesta. As aforementioned, *Spirochaetes* were typically found in low abundances in the Atlantic salmon intestine. Yet a recent study also showed that irrespective of diets *Brevinema andersonii* seemed to be more abundant in the intestinal mucosa than the digesta of seawater phase Atlantic salmon [49]. Known for high motility and chemotactic attraction to mucin, some *Spirochaetes* can penetrate the mucus and associate with the intestinal mucosa [70–72]. Further work is required to confirm whether these taxa are consistently enriched in the intestinal mucus layer of seawater phase Atlantic salmon.

Diet effect

Diet is one of the key factors in shaping the fish intestinal microbiota. In agreement with previous findings in rainbow trout [42, 73, 74] and laying hens [75, 76], we found that the insect meal diet altered the distal intestinal microbiota assemblage resulting in higher microbial richness and diversity. Our findings, showing that the insect meal diet increased the relative abundance of *Actinomyces, Bacillus, Brevibacterium, Corynebacterium 1 and Enterococcus,* are in accord with recent studies in rainbow trout fed diets containing 30% BSF larvae meal [42, 74]. Importantly, these results were partly confirmed in other studies employing fluorescence in situ hybridization for targeted profiling of changes in the intestinal microbiota. Specifically, increased absolute abundance of *Lactobacillus*/*Enterococcus* was found in rainbow trout fed 20% dietary BSF larvae meal [77], whereas increased absolute abundance of *Bacillus*, *Enterococcus* and *Lactobacillus* was documented in Siberian sturgeon (*Acipenser baerii*) fed 15% BSF larvae meal [78].

The increases in the relative abundance of specific microbial clades in Atlantic salmon fed the insect meal diet may be explained by feed-borne microbiota and/or feed composition. Bacterial taxa, including *Actinomyces*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Enterococcus*, *Oceanobacillus* and *RsaHF231*, have been found in BSF whole larvae or larvae intestine [79–82]. The fact that *RsaHF231* has not been documented in fish before indicates that these bacterial taxa may have partially originated from BSF larvae meal. Our results from the freshwater feeding trial showed that these bacterial taxa were also enriched in the intestinal digesta and mucosa of Atlantic salmon smolts fed an insect meal diet containing 60% soldier fly larvae meal. Importantly, these bacterial taxa were also detected in the feed pellets which contained considerable amount of bacterial DNA (unpublished data). Given the hydrothermal treatments the feed pellets underwent during the extrusion, the feed-borne microbiota profiled by the DNA sequencing techniques could have largely originated from dead bacteria and bacterial spores rather than living bacteria. As sequencing-based methods cannot differentiate between living and dead cells, future studies should investigate to what extent the feed-borne microbiota may contribute to, or confound the observed diet effects on intestinal microbiota, using methods that distinguish living and dead bacteria such as viability PCR and RNA sequencing [83]. On the other hand, unique nutrients in the insect meal diet such as chitin, an essential component of the insect exoskeleton, may have selectively promoted the growth of certain intestinal microbes. Many bacterial species belonging to *Bacillus* can produce chitinase [84]. *Bacillus* and *Lactobacillus* were two of the predominant taxa in the intestinal mucosa of Atlantic salmon fed a 5% chitin diet, the former of which displayed the highest in vitro chitinase activity [85].

Significant interactions between diet and sample origin effect

We observed in the present study that the diet effect on the intestinal microbial community richness and structure was dependent on the sample origin, with mucosa-associated intestinal microbiota showing higher resilience to the dietary change. Our results corroborate previous findings in rainbow trout revealing that mucosa-associated intestinal microbiota was less influenced by dietary inclusion of 30% BSF larvae meal compared to digesta-associated intestinal microbiota [41, 42]. Results from molecular-based studies on salmonid intestinal microbiota hitherto suggest that diet modulates digesta- and mucosa-associated intestinal microbiota to varying degrees with the latter generally being more resilient to dietary interventions [32, 39–42, 48]. As such, current practices of profiling only one of or a mixture of digesta- and mucosa-associated microbiota may obscure the response of intestinal microbiota to dietary changes. To fully unveil the response of intestinal microbiota to dietary changes, we recommend concurrent profiling of digesta- and mucosa-associated intestinal microbiota whenever it is feasible.

Significant associations between microbial clades and sample metadata

To our knowledge, only a few studies have carried out association analysis between intestinal microbial clades and host responses in Atlantic salmon. As such, our results should be treated as preliminary observations and critically evaluated in later studies. Herein, we highlight the significant associations between two mucosa-enriched taxa and host gene expressions in the intestine. Specifically, *Brevinema andersonii*, part of the core microbiota, was associated with the expression of genes related to pro- and anti-inflammatory responses whereas

the *Spirochaetaceae* was associated with the expression of genes related to barrier function. Intestinal microbiota is well known to modulate the local immune responses and intestinal epithelial barrier function [86]. Furthermore, it is hypothesized that mucosa-associated microbiota plays a more crucial role in shaping the host immunity in that it can interact both directly and indirectly with intestinal epithelial barrier whereas digesta-associated microbiota can only interact indirectly [69]. Taken together, further research should be undertaken to investigate the potential ecological and functional significance of these two taxa for seawater phase Atlantic salmon.

Quality control: use of mock and negative controls

As in any field of research, conducting a well-controlled microbiome study requires great care in the experiment design such as setting up appropriate experimental controls. The use of mock as a positive control allows for critical evaluation and optimization of microbiota profiling workflow. That all the bacterial taxa in the mock were correctly identified at the genus level indicates that the current workflow is reliable for the taxonomic profiling of intestinal microbiota. Furthermore, the taxonomic profile of mock from different DNA extraction batches was fairly similar, suggesting that the results generated by the current workflow are also largely reproducible. However, the low concordance between the expected and observed relative abundance of bacterial taxa in the mock is reminiscent of the fact that bias is introduced at different steps of the marker-gene survey [87–89], among which DNA extraction and PCR amplification are the two largest sources of bias due to preferential extraction and amplification of some microbial clades over others. In line with previous observations that Gram-positive bacteria may be more subjective to incomplete lysis during DNA extraction due to their tough cell walls [90, 91], the recovery of most Gram-positive bacteria in the mock was lower than the expected. The insufficient lysing of Gram-positive bacteria in the mock was largely mitigated in our later experiments by using a mixture of beads with different sizes for the bead beating during DNA extraction (unpublished data). The bias in the marker-gene sequencing experiments, as reflected in the observed taxonomic profile of the mock, highlights the necessity of validating such results by absolute quantification techniques such as cultivation (if possible), qPCR, flow cytometry and fluorescence in situ hybridization.

Reagent contamination is a common issue in molecular-based studies of microbial communities. The main contaminating taxon identified in this study is *Pseudomonas*, which has been reported as a common reagent contaminant in numerous studies [92–98]. Given the dominance of *Pseudomonas* in the negative controls of both DNA extraction and PCR, most of the observed contamination has likely derived from PCR reagents such as molecular-grade water [99–101]. Notably, *Pseudomonas* has also been isolated from intestinal digesta and mucosa of Atlantic salmon by traditional culturing approaches [85, 102–104], and reported as a member of Atlantic salmon core microbiota in culture-independent studies [32, 36, 46, 47, 51, 105]. Due to the low taxonomic resolution of amplicon sequencing, it is difficult to discern contaminating taxa from true signals solely based on taxonomic labels. The inclusion of negative controls, coupled with quantifications of microbial DNA concentration in the samples, has enabled fast and reliable identification of contaminating taxa in this study. Besides *Pseudomonas*, other common reagent contaminants, including *Bradyrhizobium*, *Burkholderia*, *Comamonas*, *Methylobacterium*, *Propionibacterium*, *Ralstonia*, *Sphingomonas* and *Stenotrophomonas* [97, 99, 101, 106–110], have also been frequently reported as members of Atlantic salmon intestinal microbiota, indicating that existing studies of Atlantic salmon intestinal microbiota may have been plagued with reagent contamination that is hard to ascertain due to lack of negative controls. As reagent contamination is unavoidable, study-specific and can critically influence sequencing-based microbiome analyses [99, 111, 112], negative controls should always be included and sequenced in microbiome studies especially when dealing with low microbial biomass samples like intestinal mucosa.

Conclusions

In summary, we confirmed previous findings in mammals and fish that intestinal digesta and mucosa harbor microbial communities with clear differences. Regardless of diet, microbial richness and diversity were much higher in the digesta than the mucosa. The insect meal diet altered the distal intestinal microbiota assemblage resulting in higher microbial richness and diversity. The diet effect was however dependent on the sample origin, with mucosa-associated intestinal microbiota being more resilient to the dietary change. To fully unveil the response of intestinal microbiota to dietary changes, concurrent profiling of digesta- and mucosa-associated intestinal microbiota is recommended whenever feasible. Lastly, we identified two mucosa-enriched taxa, *Brevinema andersonii* and *Spirochaetaceae*, which were associated with the expression in the distal intestine of genes related to immune responses and barrier function, respectively. As mucosa-associated microbiota could play a more critical role in shaping the host metabolism, their potential functional significance for seawater phase Atlantic salmon merits further investigations.

Methods

Experimental fish, diet and sampling

A 16-week seawater feeding trial with Atlantic salmon (initial body weight = 1.40 kg, S.D. = 0.043 kg) was conducted at the Gildeskål Research Station (GIFAS), Nordland, Norway. The experimental fish were randomly assigned into 6 adjacent square net pens (5 × 5 m) with a depth of 5 m, each containing 90 fish. The fish were fed, in triplicate net pens, either a commercially-relevant reference diet (REF) with a combination of fish meal, soy protein concentrate, pea protein concentrate, corn gluten and wheat gluten as the protein source, or an insect meal diet (IM) wherein all the fish meal and most of the pea protein concentrate were replaced by insect meal produced from BSF larvae. Formulation and proximate composition of the experimental diets are shown in Table 1. The diets were formulated to be isonitrogenous (39% crude protein), isolipidic (29% crude lipid) and isoenergetic (25 MJ/kg DM gross energy), and to meet the nutrient requirements of Atlantic salmon. The diets were extruded, dried and vacuum coated with oils, producing feed pellets with a diameter size of 3.5 mm (Cargill, Dirdal, Norway). The insect meal was produced by Protix Biosystems BV (Dongen, The Netherlands). The fly larvae were grown on feed substrates containing seaweed (*Ascophyllum nodosum*) and vegetable wastes (60:40). After 8 days of growing, the larvae were harvested and partially defatted before being dried and ground to make the insect meal. The insect meal contains about 52% crude protein and 18% crude lipid. Fish were fed by hand until apparent satiation once or twice daily depending on the duration of daylight. During the feeding trial, the water temperature was 8.3 ± 3.7 °C, dissolved oxygen 8.9 ± 1 mg/L and salinity 31.6 ± 0.8 ‰. Further details on the nutritional composition of the insect meal and diets have been reported elsewhere [45].

Table 1 Formulation and proximate composition of the experimental diets

	REF	IM
Ingredients (% wet-weight)		
Fishmeal LT94	10	0
Black soldier fly larva meal[a]	0	14,75
Soy protein concentrate	25	25
Corn gluten meal	7,5	7,5
Wheat gluten meal	3,35	6,88
Pea protein concentrate 55	8,8	2,84
Fish oil	10,18	14,76
Rapeseed oil	20,95	14,73
Binder	12,32	11,24
Additives	1,89	2,29
Chemical composition (wet-weight basis)		
Dry matter (%)	93	95
Crude Protein (%)	38	39
Crude Lipid (%)	29	29
Ash (%)	4,6	4,5
Carbohydrates (%)	11,6	11,4
Gross energy (MJ/kg)	24,6	25
TBARS (nmol/g)	3	4,9

REF reference diet, *IM* insect meal diet, *TBARS* Thiobarbituric acid reactive substances

[a]Partially defatted. Crude protein: 52%, crude lipid: 18%. Produced by the Protix Biosystems BV (Dongen, The Netherlands)

At the termination of the feeding trial, the average body weight of fish reached 3.7 kg. Six fish were randomly taken from each net pen, anesthetized with tricaine methanesulfonate (MS222®; Argent Chemical Laboratories, Redmond, WA, USA) and euthanized by a sharp blow to the head. After cleaning the exterior of each fish with 70% ethanol, the distal intestine, i.e., the segment from the increase in intestinal diameter and the appearance of transverse luminal folds to the anus, was aseptically removed from the abdominal cavity, placed in a sterile Petri dish and opened longitudinally. Only fish with digesta along the whole intestine were sampled to ensure that the intestine had been exposed to the diets. The intestinal digesta was gently scraped and collected into a 50 mL skirted sterile centrifuge tube and mixed thoroughly using a spatula. An aliquot of the homogenate was then transferred into a 1.5 mL sterile Eppendorf tube and snap-frozen in liquid N_2 for the profiling of digesta-associated intestinal microbiota. A tissue section from the mid part of the distal intestine was excised and rinsed in sterile phosphate-buffered saline 3 times to remove traces of the remaining digesta. After rinsing, the intestinal tissue was longitudinally cut into 3 pieces for histological evaluation (fixed in 4% phosphate-buffered formaldehyde solution for 24 h and transferred to 70% ethanol for storage), RNA extraction (preserved in RNAlater solution and stored at − 20 °C) and profiling of mucosa-associated intestinal microbiota (snap-frozen in liquid N_2), respectively. The collection of microbiota samples was performed near a gas burner to secure aseptic conditions. After the sampling of each fish, tools were cleaned and decontaminated by a 70% ethanol spray and flaming. Microbiota samples of the distal intestine digesta (DID) and mucosa (DIM) were transported in dry ice and stored at − 80 °C until DNA extraction.

DNA extraction

Total DNA was extracted from ~ 200 mg distal intestine digesta or mucosa using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany; catalog no., 51,504) with some modifications to the manufacturer's specifications as described before [32], except that 2 mL prefilled bead

tubes (Qiagen; catalog no., 13,118–50) were used for the bead beating. For quality control purposes, a companion "blank extraction" sample was added to each batch of sample DNA extraction by omitting the input material, whereas an additional microbial community standard (ZymoBIOMICS™, Zymo Research, California, USA; catalog no., D6300), i.e. mock, was included for each DNA extraction kit as a positive control. The mock consists of 8 bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica*, *Lactobacillus fermentum*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis*) and 2 yeasts (*Saccharomyces cerevisiae*, *Cryptococcus neoformans*).

Amplicon PCR

The V1–2 hypervariable regions of the bacterial 16S rRNA gene were amplified using the primer set 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 338R (5′-GCW GCC WCC CGT AGG WGT-3′) [113]. The PCR was run in a total reaction volume of 25 μL containing 12.5 μL of Phusion® High-Fidelity PCR Master Mix (Thermo Scientific, CA, USA; catalog no., F531L), 10.9 μL molecular grade H_2O, 1 μL DNA template and 0.3 μL of each primer (10 μM). The amplification program was set as follows: initial denaturation at 98 °C for 3 min; 35 cycles of denaturation at 98 °C for 15 s, annealing decreasing from 63 °C to 53 °C in 10 cycles for 30 s followed by 25 cycles at 53 °C for 30 s, and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 10 min. For samples with faint or invisible bands in the agarose gel after PCR, the PCR condition was optimized by applying serial dilutions to the DNA templates to reduce the influence of PCR inhibitors. All the digesta samples were diluted 1:2 in buffer ATE (10 mM Tris-Cl, pH 8.3, with 0.1 mM EDTA and 0.04% NaN3) whereas all the mucosa samples were diluted 1:32. The formal amplicon PCR was run in duplicate incorporating two negative PCR controls, which were generated by replacing the template DNA with molecular grade H_2O. The duplicate PCR products were then pooled and examined by a 1.5% agarose gel electrophoresis.

Quantification of 16S rRNA gene by qPCR

To assist in identifying contaminating sequences, 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′) that has been used for bacterial DNA quantification in previous studies [114, 115]. The assays were carried out using the LightCycler 96 (Roche Applied Science, Basel, Switzerland) in a 10 μL reaction volume, which contained 2 μL of PCR-grade water, 1 μL diluted DNA template, 5 μL LightCycler 480 SYBR Green I Master Mix (Roche Applied Science) and 1 μL (3 μM) of each primer. Samples, together with the extraction blanks and mock, were run in duplicate in addition to Femto™ bacterial DNA standards (Zymo Research; catalog no., E2006) and a no-template control of the qPCR assay. The qPCR program encompassed an initial enzyme activation step at 95 °C for 2 min, 45 three-step cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 15 s, and a melting curve analysis at the end. Quantification cycle (Cq) values were determined using the second derivative method [116]. The specificity of qPCR amplification was confirmed by evaluating the melting curve of qPCR products and the band pattern on the agarose gel after electrophoresis. The inter-plate calibration factor was calculated following the method described in [117], using the bacterial DNA standards as inter-plate calibrators.

Sequencing

The sequencing was carried out on a Miseq platform following the Illumina 16S metagenomic sequencing library preparation protocol [118]. Briefly, the PCR products were cleaned using the Agencourt AMPure XP system (Beckman Coulter, Indiana, USA; catalog no., A63881), multiplexed by dual indexing using the Nextera XT Index Kit (Illumina, California, USA; catalog no., FC-131-1096) and purified again using the AMPure beads. After the second clean-up, representative libraries were selected and analyzed using the Agilent DNA 1000 Kit (Agilent Technologies, California, USA; catalog no., 5067–1505) to verify the library size. Cleaned libraries were quantified using the Invitrogen Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, California, USA; catalog no., Q32854), diluted to 4 nM in 10 mM Tris (pH 8.5) and finally pooled in an equal volume. Negative controls with library concentrations lower than 4 nM were pooled in equal volume directly. Due to the low diversity of amplicon library, 15% Illumina generated PhiX control (catalog no., FC-110-3001) was spiked in by combining 510 μL amplicon library with 90 μL PhiX control library. The library was loaded at 6 pM and sequenced using the Miseq Reagent Kit v3 (600-cycle) (Illumina; catalog no., MS-102-3003).

Sequence data processing

The raw sequence data were processed by the DADA2 1.14 in R 3.6.3 [119] to infer amplicon sequence variants (ASVs) [120]. Specifically, the demultiplexed paired-ended reads were trimmed off the primer sequences (forward reads, first 20 bps; reverse reads, first 18 bps), truncated at the position where the median Phred quality score crashed (forward reads, at position 290 bp; reverse reads, at position 248 bp) and filtered off low-

Table 2 PERMANOVA results and subsequent conditional contrasts

Distance matrix	Main effects		Interaction	Conditional contrasts			
	Diet	Sample origin		REF-DID VS. IM-DID	REF-DIM VS. IM-DIM	REF-DID VS. REF-DIM	IM-DID VS. IM-DIM
Jaccard	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Unweighted UniFrac	0.001[a]	0.001	0.001	0.001[a]	0.001	0.001	0.001
Aitchison	0.001	0.003	0.004	0.002	0.004	0.004[a]	0.002[a]
PHILR (Euclidean)[b]	0.001	0.001	0.001	0.001	0.005	0.001	0.001

REF reference diet, *IM* insect meal diet, DID distal intestine digesta, *DIM* distal intestine mucosa
[a]Monte Carlo *p* value
[b]Phylogenetic isometric log-ratio transformed Euclidean distance

quality reads. After trimming and filtering, the run-specific error rates were estimated and the ASVs were inferred by pooling reads from all the samples sequenced in the same run. The chimeras were removed using the "pooled" method after merging the reads. The resulting raw ASV table and representative sequences were imported into QIIME2 (version, 2020.2) [121]. The taxonomy was assigned by a scikit-learn naive Bayes machine-learning classifier [122], which was trained on the SILVA 132 99% OTUs [123] that were trimmed to only include the regions of 16S rRNA gene amplified by our primers. ASVs identified as chloroplasts or mitochondria were excluded from the ASV table. The ASV table was conservatively filtered to remove ASVs that had no phylum-level taxonomic assignment or appeared in only one biological sample. Contaminating ASVs were identified based on two suggested criteria: contaminants are often found in negative controls and inversely correlate with sample DNA concentration [98]. The ASVs filtered from the raw ASV table were also removed from the representative sequences, which were then inserted into a reference phylogenetic tree built on the SILVA 128 database using SEPP [124]. The alpha rarefaction curves and the core metrics results were generated with a sampling depth of 10,000 and 2047 sequences per sample, respectively (Fig. S9). For downstream data analysis and visualization, QIIME2 artifacts were imported into R using the *qiime2R* package [125] and a *phyloseq* [126] object was assembled from the sample metadata, ASV table, taxonomy and phylogenetic tree. The core ASVs were calculated using a prevalence threshold at 80% and visualized by the Venn's diagram. The alpha-diversity indices, including observed ASVs, Pielou's evenness, Shannon's index and Faith's phylogenetic diversity (PD), were computed via the R packages *microbiome* [127] and *picante* [128]. For beta-diversity analyses, we used distance matrices including Jaccard distance, unweighted UniFrac distance, Aitchison distance and phylogenetic isometric log-ratio (PHILR) transformed Euclidean distance. Since rarefying remains to be the best solution for unweighted distance matrices [129], the Jaccard distance and unweighted UniFrac distance were computed in QIIME2 using the rarefied ASV table. The compositionality-aware distance matrices, Aitchison distance and PHILR transformed Euclidean distance, were calculated using the unrarefied ASV table. The Aitchison distance was computed by the *DEICODE* plugin in QIIME2, a form of Aitchison distance that is robust to high levels of sparsity by using the matrix completion to handle the excessive zeros in the microbiome data [130]. The PHILR transform of the ASV table was performed in R using the *philr* package [131]. The selected distance matrices were explored and visualized by the principal coordinates analysis (PCoA).

Table 3 Test of homogeneity of multivariate dispersions among groups

Distance matrix	Conditional contrasts			
	REF-DID VS. IM-DID	REF-DIM VS. IM-DIM	REF-DID VS. REF-DIM	IM-DID VS. IM-DIM
Jaccard	0.002	0.087	0.045	0.002
Unweighted UniFrac	0.002	0.711	0.200	0.002
Aitchison	0.453	0.046	0.046	0.369
PHILR (Euclidean)[a]	0.240	0.266	0.240	0.266

REF reference diet, *IM* insect meal diet, *DID* distal intestine digesta, *DIM* distal intestine mucosa
[a]Phylogenetic isometric log-ratio transformed Euclidean distance

Multivariate association analysis

To reduce the multiple testing burden, the ASV table was collapsed at the genus level before running the multivariate association analysis. Bacterial taxa of very low abundance (< 0.01%) or low prevalence (present in < 25% of samples) were removed from the feature table. The microbial clades were then tested for significant associations with metadata of interest by MaAsLin2 (version, 0.99.12) (https://huttenhower.sph.harvard.edu/maaslin2) in R, using the default parameters. The results of the analysis are the associations of specific microbial clades with metadata, deconfounding the influence of other factors included in the model. Association was

considered significant when the *q*-value was below 0.25. Metadata included in the multivariate association testing are fixed factors Diet + Sample origin + distal intestine somatic index (DISI) + lamina propria cellularity (histological scores) + immune response (qPCR) + barrier function (qPCR), and random factors FishID + NetPen. FishID was nested in NetPen, and NetPen nested in Diet. The methodological approach to these parameters was reported in a previous study [44]. Lamina propria cellularity reflects the severity of inflammation in the distal intestine. Based on the degree of cellular infiltration within the lamina propria, a value of normal, mild, moderate, marked or severe was assigned. To make the data appropriate for the association testing, the highly skewed five-category scores were collapsed into more balanced binary data, i.e., normal and abnormal. The immune-related genes included for the association testing were myeloid differentiation factor 88 (*myd88*), interleukin 1β (*il1β*), interleukin 8 (*il8*), cluster of differentiation 3 γδ (*cd3γδ*), transforming growth factor β1 (*tgfβ1*), interferon γ (*ifnγ*), interleukin 17A (*il17a*), forkhead box P3 (*foxp3*) and interleukin 10 (*il10*), whose expression levels were higher in the distal intestine of fish assigned abnormal regarding lamina propria cellularity. 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. For genes relevant to the barrier function, which included claudin-15 (*cldn15*), claudin-25b (*cldn25b*), zonula occludens 1 (*zo1*), E-cadherin / cadherin 1 (*cdh1*) and mucin-2 (*muc2*), we also used the PC1 of the PCA for the association testing based on the same considerations.

Statistics

All the statistical analyses were run in R except for the PERMANOVA, which was run in PRIMER (version 7; PRIMER-e). The differences in the alpha-diversity indices were compared using linear mixed-effects models via the *lme4* package [132]. Predictor variables in the models included the fixed effects Diet + Sample origin + Diet x Sample origin, and the random effects FishID + NetPen. The models were validated by visual inspections of residual diagnostic plots generated by the *ggResidpanel* package [133]. The statistical significance of fixed predictors was estimated by Type III *ANOVA* with Kenward-Roger's approximation of denominator degrees of freedom via the *lmerTest* package [134]. When the interaction between the main effects was significant, conditional contrasts for the main effects were made via the *emmeans* package [135]. To compare the differences in beta-diversity, we performed the PERMANOVA [136] using the same predictors included in the linear mixed-effects models. Terms with negative estimates for components of variation were sequentially removed from the model via term pooling, starting with the one showing the smallest mean squares. At each step, the model was reassessed whether more terms needed to be removed or not. Conditional contrasts for the main effects were constructed when their interaction was significant. Monte Carlo *p* values were computed as well when the unique permutations for the terms in the PERMANOVA were small (< 100). The homogeneity of multivariate dispersions among groups was visually assessed with boxplots and was formally tested by the permutation test, PERMDISP [137], via the R package *vegan* [138]. Multiple comparisons were adjusted by the Benjamini-Hochberg procedure where applicable. Differences were regarded as significant when $p < 0.05$.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-020-00071-3.

Additional file 1: Figure S1. Quantification of bacterial 16S rRNA gene in different sample types using qPCR. Since the Cq values of most mucosa-associated samples were out of the linear range of the standard curve, the Cq value was used as a proxy of 16S rRNA gene quantity which is reliable for the screening of contaminant sequences. Data are presented as mean ± 1 standard deviation overlaying the raw data points. Abbreviations: REF, reference diet; IM, insect meal diet; DID, distal intestine digesta; DIM, distal intestine mucosa. **Figure S2.** Taxonomic profile of the mock (A) and contaminating features in the negative controls (B). The lowest level of taxonomic ranks was displayed for each taxon. EB, extraction blank; LB, library blank. **Figure S3**. Microbial clades showing significant associations with sample origin. *p__*, phylum; *o__*, order; *f__*, family; FDR, false discovery rate; N.not.zero, number of non-zero observations; REF, reference diet; IM, insect meal diet. **Figure S4.** Microbial clades showing significant associations with diet. *p__*, phylum; *o__*, order; *f__*, family; FDR, false discovery rate; N.not.zero, number of non-zero observations; REF, reference diet; IM, insect meal diet. **Figure S5.** Microbial clades showing significant associations with histological scores on lamina propria cellularity in the distal intestine. *p__*, phylum; *f__*, family; FDR, false discovery rate; N.not.zero, number of non-zero observations. **Figure S6.** Microbial clades showing significant associations with distal intestine somatic index (DISI). FDR, false discovery rate; N.not.zero, number of non-zero observations. **Figure S7.** Microbial clades showing significant associations with immune gene expressions in the distal intestine. Since the expression levels of immune genes were highly correlated, we ran a principle component analysis (PCA) and used the first principle component (PC1) for the association testing to avoid multicollinearity and reduce the number of association testing. Note that the expression levels of immune genes decrease as the PC1 increases from left to right. Hence, a positive correlation coefficient denotes a negative association between the microbial clade and immune gene expressions, and vice versa. *f__*, family; FDR, false discovery rate; N.not.zero, number of non-zero observations. **Figure S8.** Microbial clades showing significant associations with expressions of barrier function related genes in the distal intestine. Since the expression levels of barrier function related genes were highly correlated, we ran a principle component analysis (PCA) and used the first principle component (PC1) for the association testing to avoid multicollinearity and reduce the number of association testing. Note that the expression levels of barrier function related genes decrease as the PC1 increases from left to right. Hence, a positive correlation coefficient denotes a negative association between the microbial clade and barrier function related gene expressions, and vice versa. *f__*, family; FDR,

false discovery rate; N.not.zero, number of non-zero observations. **Figure S9.** Rarefaction curves based on Observed ASVs for the different sample types. The rarefaction analysis showed that mucosa samples (REF-DIM, IM-DIM) reached the saturation phase at a subsampling depth of 2000 sequences whereas digesta samples (REF-DID, IM-DID) reached the saturation phase at a subsampling depth of 9500 sequences. To preserve a maximum number of samples for the downstream data analysis, we rarefied the ASV table to 2047 sequences per sample which left out 2 samples. To ensure that the subsampling depth of 2047 sequences per sample produced reliable comparisons of microbial communities, we computed compositionality-aware distance matrices, the Aitchison distance and PHILR transformed Euclidean distance, which do not require rarefying and use all the sequences in the samples.

Additional file 2: Table S1. Contaminating features removed from the ASV table.

Additional file 3: Table S2. The prevalence of core ASVs in different sample types.

Abbreviations

ASVs: amplicon sequence variants; BSF: black soldier fly; Cq: quantification cycle; DID: distal intestine digesta; DIM: distal intestine mucosa; DISI: distal intestine somatic index; IM: insect meal; LPC: lamina propria cellularity; PC1: principal component one; PCA: principal component analysis; PCoA: principal coordinates analysis; PD: phylogenetic diversity; PERM ANOVA: permutational multivariate analysis of variance; PHILR: phylogenetic isometric log-ratio; REF: reference

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Authors' contributions

TMK and ÅK concepted and designed the study. YL, LB and KG participated in the sample collection. YL, LB and AJ-T carried out the laboratory works. YL performed the bioinformatics, statistical analyses and data visualization. YL and LB completed the first draft of the manuscript. All the authors read, revised and approved the final version of the manuscript.

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Availability of data and materials

The raw 16S rRNA gene sequencing data are deposited at the NCBI SRA database under the BioProject PRJNA555355. Other raw data and code for reproducing our results are available from the GitHub repository (https://github.com/yanxianl/Li_AqFl2-Microbiota_ASM_2020).

Ethics approval and consent to participate

The experiment was conducted in compliance with the Norwegian Animal Welfare Act 10/07/2009 and the EU Directive on the Protection of Animals used for Scientific Purposes (2010/63/EU).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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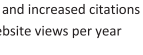

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