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Feed additives elicit changes in the structure of the intestinal bacterial community of Atlantic salmon

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Preface

This dissertation is submitted in partial fulfillment of the requirements for the degree of Philosophiae Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University, Bodø, Norway. The studies included in this dissertation represent original research conducted at the faculty over the period of 3.5 years from September 2015 to March 2019. This PhD project was funded by the Nordland County Council and FBA, Nord University.

The project team consisted of the following members:

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Kiron Viswanath, Professor, FBA, Nord University: principal supervisor

Jorge M.O. Fernandes, Professor, FBA, Nord University: co-supervisor

Shruti Gupta

Bodø, 11th March, 2019

Dedication

I want to dedicate this dissertation to my late father Shri Satish Chandra Gupta (1961 - 2008). He had always motivated me to pursue my passion for scientific research.

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Lastly, I would like to thank my family. The following part is written in Hindi since it is for them. मैं मेरे परिवार को उनके अचूक स्नेह, भावनात्मक समर्थन और आशीर्वाद के लिए धन्यवाद देना चाहती हूं, जिनके बिना मुझे यह उपलब्धि हासिल नहीं होती।

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List of abbreviations

16S rRNA	-	16S ribosomal RNA
GIT	-	Gastrointestinal tract
OTUs	-	Operational taxonomic units
NGS	-	Next-generation sequencing
PCR	-	Polymerase chain reaction
TLRs	-	Toll-like receptors
SCFA	-	Short chain fatty acid
LAB	-	Lactic acid bacteria
AlgOS	-	Alginate oligosaccharide
SDB network	-	Single-domain bacterial network

Abstract

A stable and resilient intestinal microbiota is indispensable for maintaining host health and well-being. Dietary components can modify the diversity and stability of the intestinal microbiota of vertebrates including fish. Atlantic salmon is one of the high-value fishes that is farmed mainly in Norway, Scotland, Canada, Chile and Tasmania. This anadromous fish is intensively farmed, an operation that is associated with the risk of transmission of pathogens that cause diseases. Although vaccination has helped to reduce much of the microbial diseases, antibiotics are still offered to farmed fish. Since antibiotic exposure can cause adverse effects on the intestinal microbes there is a need to develop alternative health management strategies such as offering the fish with different dietary supplements like probiotics and prebiotics to maintain a well-balanced bacterial community. The aim of the thesis was to investigate the effect of dietary supplements (pro- and prebiotics) and antibiotics on the intestinal microbiota of Atlantic salmon employing high-throughput 16S rRNA gene amplicon sequencing. Furthermore, intestinal microbe-microbe interactions under the influence of different feeds were also assessed.

Intestinal mucus-associated community is representative of the resident microbes in the host. Molecular profiling showed that the resident intestinal bacteria of Atlantic salmon consists of two predominant phyla viz., Proteobacteria and Tenericutes followed by Spirochaetes, Firmicutes, Actinobacteria and Bacteriodetes. The results indicated that antibiotic intake can increase the bacterial diversity in the mucus of the distal intestine and shift the composition and abundance of the dominant bacterial phyla in the fish. Probiotic supplementation increased the bacterial diversity in the mucus. It reduced the abundance of Proteobacteria but promoted the dominance of intestinal *Lactobacillus* and certain members of the phyla Tenericutes, Spirochaetes, and Actinobacteria. On the other hand, a prebiotic–macroalgae-derived oligosaccharide–reduced the bacterial diversity and shifted the composition of the bacteria, mainly through the changes in the abundance of Proteobacteria and Spirochaetes. Furthermore, investigations on microbe-microbe association indicated that antibiotics and probiotics can affect the bacterial interactions differentially.

Taken together, these findings provide insights into the influence of different dietary components on the diversity and composition of the intestinal bacteria of Atlantic salmon.

This baseline information will be valuable for future studies that explore the dietary manipulations of the intestinal microbiota of the fish and the microbial interactions between antibiotic- and probiotic-modulated intestinal microbes and the host.

Sammendrag

Ulike fôrtilsetninger kan modifisere både diversitet og stabilitet til mikrobiotaen i tarmen hos virveldyr, som også inkluderer fisk. En stabil og motstandsdyktig microbiota er viktig for å sikre god helse hos verten. Atlanterhavslaks er en av art som i hovedsak blir oppdrettet i Norge, Skottland, Canada, Chile og Tasmania. Dette er en anadrom art som produseres i intensivt oppdrett med relativ høy tetthet, noe som øker risikoen for overføring av sykdom forårsaket av patogener. Bruk av antibiotika er redusert til et minimum i produksjon av laks siden all fisk som settes på sjø blir vaksinert mot bakterielle sykdommer. Det er flere grunner til at bruken av antibiotika skal holdes på et minimum, og en av dem er negative effekter på mikrobiota i tarm. Det er derfor ønskelig å benytte forebyggende strategier, som eksempelvis bruk av probiotiske og prebiotiske fôrtilsetninger for å opprettholde et velbalansert bakterielt samfunn.

Målet med denne avhandlingen er å undersøke effekten av fôrtilsetninger (pro- og prebiotika) og antibiotika på microbiota i tarm hos atlantisk laks. Endringer i mikrobiomet ble studert ved bruk av «high-throughput 16S rRNA gene amplicon sequencing». Effekter av ulike fôr på interaksjoner mellom ulike mikrobepopulasjoner ble også studert.

Bakterisamfunn som finnes i tarmens slimhinne er vanligvis de man anser som naturlig tilstedeværende og er vanligvis ikke skadelige. Molekylær profiliering viste at disse tarmbakteriene var dominert av to phyla viz. Proteobakter og Tenericuter, etterfulgt av Spirochaeter, Firmicuter, Actinobacterier og Bacteriodeter. Resultatene indikerte at inntak av antibiotika økte både diversitet av bakterier isolert fra slimlaget i baktarm, samt sammensettingen og mengden av de dominante bakterielle phyla i fisken. Fôr med probiotiska økte det bakterielle mangfoldet i baktarmens slimhinne. Mengden av Proteobakter ble redusert mens en økning ble observert for *Lactobacillus* og visse medlemmer av phyla Tenericuter, Spirochaeter og Actinobacterier. Laks fôret med et prebiotika fremstilt fra makroalger viste redusert mangfold og endret sammensetting av bakteriene, gjennom en økning i Proteobakterer og Spirochaeter. Videre undersøkelser av mikrobe-mikrobe samspilletindikerte at antibiotika og probiotika hadde ulike effekter på samspillet mellom ulike bakteriegrupper.

Resultatene fra denne avhandlingen gir ny kunnskap om effekter av ulike fôrtilsetninger på diversitet og sammensetning av mikrobiota i tarm hos laks. Resultatene fra avhandlingen kan brukes som kunnskapsgrunnlag for fremtidige studier hvor hensikten er å undersøke effekter av ulike fôringredienser på endringer i tarm mikrobiota hos fisk, samt studier for å undersøke samspillet mellom vert og mikrobiota, eller samspillet mellom ulike bakteriegrupper som blir manipulert enten gjennom bruk av antibiotika, probiotka eller prebiotika.

1. Introduction

1.1 Microbiota

Microbes are inevitable components of ecosystems, whether it be aquatic, terrestrial or existing along with plants or animals. They fall into four basic categories: bacteria, archaea, eukaryotes (which include fungi), and viruses (Ishiguro et al. 2018). An ecological assemblage of microbes (commensal, symbiotic and pathogenic) present on and within multicellular organisms is referred to as microbiota (Marchesi and Ravel 2015). These microbes, their genomes and gene products, their entire habitat and the surrounding environmental conditions is collectively referred to as the microbiome (Marchesi and Ravel 2015). The microbial ecosystem of a human being consists of more than 100 trillion microbes (Wang et al. 2017). Altogether the microbiota makes up to 1-2 kg of our body weight, and they interact among themselves and function as a new organ (Baquero and Nombela 2012) that helps an individual to maintain a good health status. Microbiota can be found in many sites of the body–skin, respiratory tract, gastrointestinal tract (GIT), urogenital tract, etc. However, the composition of the microbiota in different body sites varies significantly (Ursell et al. 2012). The intestine of the vertebrates, including fish, represents the most extensive interface between the host and the environment, and is the organ where much of the host-microbiota interactions occur (Thursby and Juge 2017, Wang et al. 2018a). Over the past decade, scientists have been using metagenomics to study the genomic DNA from an assemblage of microbes associated with humans; to understand the genes, genomes and the functions of its members. Similarly, the microbiota of plants (mostly in roots) and animals (largely in the gut) are also colonized by diverse microbes that enhance the functional capacities of the host (Hacquard et al. 2015). These microbial communities have co-evolved along with their hosts, over thousands of years, to establish a complex and symbiotic relationship with the host (Thursby and Juge 2017). The microbes and their hosts form an ecological unit due to their intimate relationship and therefore can be considered as holobionts (van de Guchte et al. 2018). The abundance of the microbial communities within the microbiota is highly dependent on the type of host, metabolic environment and the microbe-microbe interactions (Bjork et al. 2018). The composition of the microbiota is unique to each individual, and its establishment depends on various factors, e.g., diet, genetics, health status and geographical location of the host (Rodríguez et al. 2015). While each individual has a distinct microbiota, the metabolic

functions of the microbes could be similar, which may have a bearing on the host health (Lozupone et al. 2012).

1.2 The gut-microbiota and its influence on the host

Of all the sites in and on our body, the GIT has the most dense, diverse, and dynamic collection of microorganisms (Ishiguro et al. 2018). The microbiota residing in the gut is referred to as the gut microbiota. Although "gut microbiota" should denote the microbial community present throughout the digestive tract, this term is more commonly associated with colonic fecal microbiota (mostly in human studies), since the microorganisms present in the feces (representative of gut microbiota) are most widely and well-studied due to their direct impact on the host health.

The balance between commensal and pathogenic microbes is maintained to establish intestinal homeostasis. Reduced cooperation due to loss of beneficial microbes and/or expansion of pathobionts and/or loss of diversity may lead to a pathological state known as "gut dysbiosis". During healthy, homeostatic conditions the gut microbiota is composed of diverse microorganisms that benefit the host. However, environmental factors including diets and episodes of antibiotic exposure can lead to modifications of the gut microbial community profile (Petersen and Round, 2014).

The intestinal microbes interact with the body systems, thereby influencing immunological, neurological and endocrinological functions in the host. For instance, the pattern recognition receptors of the host immune system recognize antigens of pathobionts and activate the intracellular signaling pathways to generate immune responses (Thaiss et al. 2016). The metabolic and immune ability of the host majorly depends on complex interactions between the intestinal microbiota and host cells. These interactions begin at birth and shape the host fitness throughout life (Chen et al. 2018, Portune et al. 2016). Intestinal microbes can affect key aspects of the evolutionary fitness of the host, such as lifespan, fecundity, and developmental time, while the host, in turn, molds the gut microbiome (Gould et al. 2018) and provides an ideal niche for the microbiota to thrive. The microbiota shapes the host immune system, which in turn affects the composition of the microbiota (Brown et al. 2013).

In mammals, the diversity of microbes in the different regions of the GIT varies significantly; low in the esophagus and stomach and high in the colon. The gut microbiota is made up of diverse microbes, and bacteria are the most dominant microorganisms. The gut bacteria maintain an intimate relationship with the gut mucosa and impart substantial functions in a healthy individual–they are involved, among other functions, in the production of vitamin, synthesis of amino acid, biotransformation of bile acids and production of bacterial metabolites (Prakash et al. 2011). Humans lack the intestinal enzymes to break down nondigestible dietary substrates like carbohydrates (Rowland et al. 2018); most anaerobic bacteria residing in the large intestine have incredible capacities for fermenting such substrates, and this fermentation produces bacterial metabolites such as SCFAs and gases (H_2, CO_2, and CH_4); acetate, butyrate, and propionate are the major short-chain fatty acids (Wong et al. 2006). Intestinal bacteria also metabolise polyphenols to produce hydroxyphenyl-acetic acids and hydroxyphenylpropionic acids (Rowland et al. 2018). The generated metabolites support the growth of other microbes too.

Beyond its role in digestion, the gut microbiota ensures protection from pathogens through both immune- and non-immune-mediated mechanisms. In general, the immune-mediated mechanisms involve the components of the mucus layer (e.g., mucins and antimicrobial peptides) or the intestinal epithelium (e.g., toll-like receptors, TLRs). On the other hand, the gut microbiota can guard the host from pathogen invasion through mechanisms that do not require immune-mediated systems. For instance, certain commensal strains (e.g., *Enterococcus faecalis* and *Escherichia coli* strain Nissle 1917) produce bacteriocins or proteins that act as bacteriostatic or bactericidal molecules, while others may inhibit bacterial pathogens from colonizing in the intestine through nutrient competition (Ubeda et al. 2017). Moreover, the gut microbiota also aids in maintaining the integrity of the intestinal barrier and structure of the GIT. For example, microbial cell wall peptidoglycan stimulates the TLR2-mediated signaling that helps regulate the permeability of intestinal epithelial tight junctions (Cario et al. 2007).

The gut microbes are key to many aspects of host intestinal homeostasis as well as metabolic and immune functions (Blaser 2014, Valdes et al. 2018). For instance, members of the commensal *Lactobacilli* can enhance intestinal epithelial development and homeostasis in Drosophila and mouse models (Jones et al. 2013). The host immune tolerance to commensal

bacteria is induced via microbe-associated molecular pattern recognition systems for example when commensal bacteria interacts with epithelial cells, only TLRs are activated and their co-receptor molecules stimulate homeostatic functions (Ohland and Jobin 2014, Rumbo et al. 2006). Commensal bacteria can also have a positive effect on the $CD4^+$T cell differentiation into Th1, Th2, Tregs and Th17 cell types, so that each cell type secretes a special set of cytokines that help regulate the immune tolerance (Wu and Wu 2012). In addition, the proper regulation and balance of antigen presenting cells and $CD4^+$ T cells are vital to maintain the immune homeostasis and to protect the body from infections (Wu and Wu 2012). Studies have provided evidence that the gut microbiota plays a pivotal role in regulating the development of antigen presenting cells and T cells; monocolonization of commensal *Escherichia coli* in germ-free pigs increased the number of intestinal dendritic cells (Haverson et al. 2007), *Bacteroides fragilis* was shown to induce the development of a systemic Th1 cells in germ-free mice (Mazmanian et al. 2005). In summary, the microbes residing in the GIT are essential for immune homeostasis, and structural and morphological development of the host GIT (Figure 1).

As described for humans and other animals, the gut microbiota of fish can also contribute to digestion and nutrition, as well as maintenance of immune homeostasis. However, we have limited information on these aspects in fish, including dietary manipulations of gut microbiota, even though this knowledge could be applied to target the health of the farmed fish species.

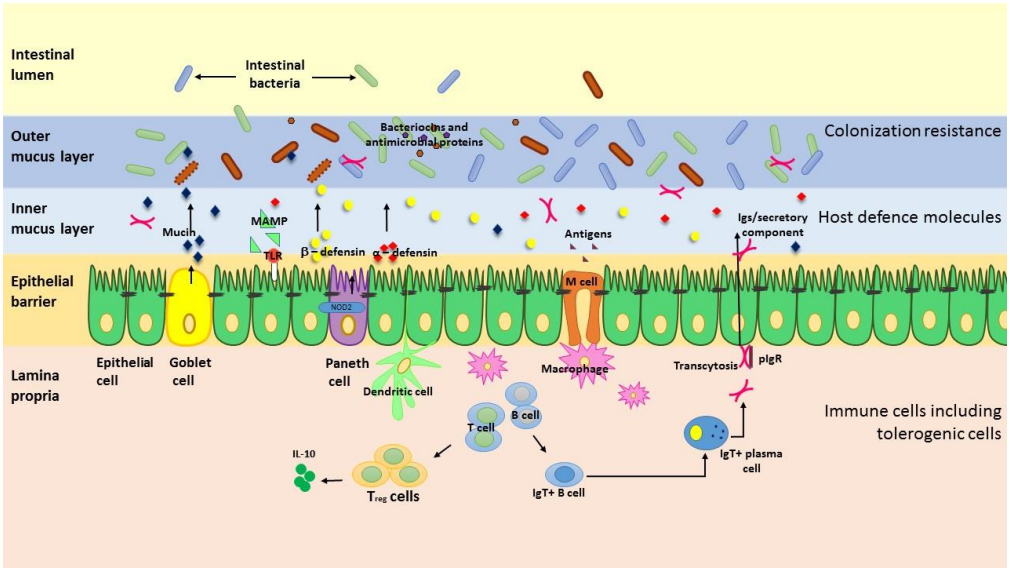

Figure 1. Microbiota maintains the intestinal structure and immune homeostasis.

Microbial colonization influences the different immune cells present in the intestine and vice-versa. Some immune mechanisms work in concert with the intestinal microbiota and contribute to intestinal homeostasis. Goblet cells secrete mucus containing mucins to maintain epithelial barrier integrity. Different epithelial cells secrete antimicrobial proteins. AMPs, mucins and other host defense molecules such as immunoglobulins (Igs) and secretory components help protect the host from bacterial invasion. Microbial metabolites and antigens help in the accumulation of regulatory T (Tregs) cells and IgT-producing plasma cells. The concept of this illustration is derived from Hooper and Macpherson (2010).

1.3 Factors influencing the gut microbiota

Several factors including host genotype, lifestyle, and environmental conditions affect the host gut microbiota (Figure 2). Its composition and diversity, as well as functions and metabolic activities impact the host health (Ghanbari et al. 2015, Kho and Lal 2018). This section will provide specific examples that demonstrate the influence of different factors on host gut microbiota.

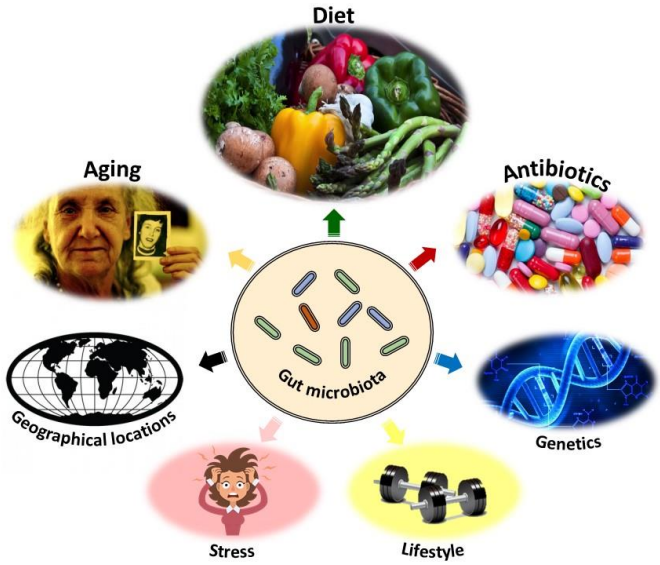

Figure 2. Factors that shape the gut microbes.

Diet is the main factor that alters the composition of the gut microbial communities. Other factors like aging/life stage, host genetics, geographical locations, stress, medicines and lifestyle can also influence the structure and functions of the gut microbiota. Image source: Google Images.

Host-specific factors that drive the variation in the fish and mammalian gut microbiota composition and diversity include age, sexual maturity, and genetics (Egerton et al. 2018, Kho and Lal 2018). Developmental stage-based differences in microbiota diversity and composition have been reported previously. For instance, fish embryonic microbial communities have lower richness and diversity compared to hatchlings (Lokesh et al. 2018) and gut microbial communities of various life-stages are different (Llewellyn et al. 2016). In humans, gut microbiota composition shifts throughout the life; from birth to adult stage (18-60), and then to the old age (over 60). In the early stages of development, the gut microbiota, in general, is less diverse and is dominated mainly by two phyla, Firmicutes and Actinobacteria. Firmicutes are dominant among the bacterial communities of adults, while at old age Proteobacteria are also dominant (Odamaki et al. 2016).

The genetic background of the host also determines the gut microbiota composition. Studies have reported differences in the community composition based on host genetics; family-specific bacterial groups were identified in rainbow trout, *Oncorhynchus mykiss*

(Navarrete et al. 2012) and seven Cervinae (a subfamily of deer) at different phylogenetic levels share common microbial communities (Li et al. 2018).

Environmental factors, including geographic location, antibiotic intake, and feeding habits influence the composition of the gut microbiota (Clapp et al. 2017). Feed components can significantly affect the structure and composition of the gut microbiota (Riaz Rajoka et al. 2017, Hervert-Hernández and Goñi 2011, Wong et al. 2015). Carnivorous fish generally have lower gut bacterial diversity compared to omnivorous and herbivorous fish (Wang et al. 2018a). The most abundant bacteria of carnivorous fish species include *Clostridium*, *Cetobacterium*, and *Halomonas*, whereas in omnivorous *Cetobacterium* and *Halomonas* are most abundant, and in herbivorous fish *Leptotrichia*, *Clostridium* and *Citrobacter* are the most abundant genera (Li et al. 2014a, Liu et al. 2016a, Kashinskaya et al. 2018).

Geographical locations also affect the gut microbial population (Senghor et al. 2018); the gut microbiota of elderly women living in different parts of South Korea were not similar (Shin et al. 2016), and the gut microbiota composition of two Central Africa Republic ethnic groups were similar, but they were different from those of US Americans (Gomez et al. 2016). In fish too, habitats influence the composition of microbiota; gut microbial communities of Atlantic salmon from different locations were dissimilar (Llewellyn et al. 2016, Dehler et al. 2017). Furthermore, environmental factors such as drugs, pollutants, and pathogens may stress the gut microbiota. Several studies have reported that antibiotics can also alter the composition of the gut microbiota. For instance, in humans, after 7-day treatment of fluoroquinolones and β-lactams, the microbial diversity decreased; however, these antibiotics increased the ratio of Bacteroidetes to Firmicutes (Panda et al. 2014). In channel catfish, *Ictalurus punctatus* and C57BL/6 mice, another antibiotic, florfenicol, increased the abundance of intestinal Proteobacteria and Bacteroidetes (Wang et al. 2019, Li et al. 2017). Environmental pollutants, microplastics and endocrine disrupting chemicals altered the gut microbiota composition of male zebrafish (Jin et al. 2018, Liu et al. 2016b). In a feeding study on mice, a high-fat diet significantly reduced the number of intestinal lactic acid bacteria (Liu et al. 2011). Another study on mice reported that black raspberry-rich diet modified the gut bacterial diversity and composition (Tu et al. 2018). In humans, the abundance of intestinal commensal *Bifidobacterium* and *Lactobacillus* increased with the intake of whey and pea protein extract, while whey alone lowered the growth of pathogenic *Bacteroides fragilis* and *Clostridium*

perfringens (Romond et al. 1998, Meddah et al. 2001, Dominika et al. 2011). Intestinal enterotypes in humans known primarily by the levels of Bacteroides, Prevotella, and Ruminococcus are strongly associated with long-term consumption of specific food types (Arumugam et al. 2011). Other studies found that human microbiota may be either dominated by Bacteroides/Clostridiales or by Prevotella (Nakayama et al. 2015, Wu et al. 2011). Protein and animal fat intake is associated with Bacteroides, whereas carbohydrate consumption is linked to Prevotella (Wu et al. 2011). The same study also reported changes in the microbiome composition within 24 h of introduction of a high-fat and low-fiber or low-fat and high-fiber diet. Diet, in particular, can directly affect the nutrients that are available to intestinal microbes (Salonen and de Vos 2014, Louis et al. 2016, Sonnenburg and Backhed 2016). These findings ascertain the ability of diet to significantly modify the diversity and composition of the gut microbiota, and therefore, diet can be considered as the most reasonable manipulator of gut microbiota.

1.4 Strategies to modulate the gut microbiota

Improving host health through modulation of the gut microbiota is an evolving strategy that is part of an inclusive approach to lifestyle-wellness (Prescott et al. 2016). Homeostasis in the gut is essential to facilitate normal functions, and, from this perspective, dietary manipulation may represent a strategy to ensure a healthy intestinal microbial community and contribute to the welfare of the host. Several experimental interventions have been used to maneuver the gut microbial communities; employing antibiotics, dietary probiotics, prebiotics and synbiotics (combination of pro- and prebiotics) (Toward et al. 2012, Ramos et al. 2013, Unno et al. 2015, Becattini et al. 2016, Abbasi et al. 2018, Zhou et al. 2018).

1.4.1 Antibiotics

Antibiotics are antimicrobial substances that are used to treat bacterial infections, either by killing pathogenic bacteria or by inhibiting their growth. Antibiotics are considered as a double-edged sword, since they can also have a detrimental effect on the commensal gut microbial communities of the host. Several studies have confirmed that antibiotics can tremendously influence the composition and diversity of the gut microbiota in humans and other animals (Jernberg et al. 2007, Buffie et al. 2012, Carlson et al. 2017, Looft et al. 2014).

Their mode of actions, dose, and target bacteria can alter the gut microbial communities differentially (Ianiro et al. 2016). Studies have shown that broad-spectrum antibiotics (e.g., ampicillin, gentamicin, amoxicillin) exert long-lasting effects on the microbiota of human infants, with an increase in members of Proteobacteria and decrease in members of Actinobacteria (Fouhy et al. 2012, Mangin et al. 2010). Studies have reported that antibiotics can impair infant microbiota even before birth. For example, in a Canadian cohort, infants whose mothers received intrapartum antibiotic prophylaxis exhibited a long-lasting dysbiosis, with an increase in Enterococci and Clostridia and decrease in Bacteroides (Azad et al. 2016). Furthermore, in a European cohort, 6-week-old babies whose mothers received antibiotics–perinatal and/or during breastfeeding–had a lower abundance of Bacteroides compared to babies fed formula (Fallani et al. 2010). The widespread use of antibiotics poses many health risks. These include the increasing bacterial resistance to antibiotics and the detrimental effects of antibiotics on the commensal microbiota (Casals-Pascual et al. 2018, Langdon et al. 2016). The perturbations in microbiota caused by antibiotics can negatively affect the host health in numerous manners and for long periods (Langdon et al. 2016). Until and unless alternatives are in place to contain bacterial infections, strategies are required to minimize the negative consequences of antibiotic administration.

1.4.2 Probiotics

Probiotics are live bacterial supplements that can confer health benefits to host after they consume the right amount of recommended microorganisms (FAO and WHO 2006). The microorganisms must be characterized and have a scientifically-demonstrated beneficial effect on host health. The health benefits occur through immunomodulation, stimulation of intestinal epithelial cell proliferation and differentiation and protection of intestinal barrier (Thomas and Versalovic 2010, Hemarajata and Versalovic 2013, Gareau et al. 2010). Probiotic bacteria can have both direct and indirect effects on the gut microbiota composition, diversity and global host metabolic functions (Scott et al. 2015). They produce antimicrobial compounds that suppress the growth of specific microbes through competing for their receptors and binding sites (Spinler et al. 2008, O'Shea et al. 2012), thus modulating the gut microbiota (Collado et al. 2007). The most commonly used probiotics are members of bacterial genera *Bifidobacterium* and *Lactobacillus*; they are included in a variety of products, including

foods, dietary supplements or drugs (Valdes et al. 2018, O'Toole and Cooney 2008). Other potential probiotics that are employed to prevent human diseases include bacteria *Bacillus*, *Escherichia*, and *Propionibacterium*, and yeasts, mainly *Saccharomyces* (Azad et al. 2018). The effects of probiotics on the modulation of the gut microbiota is now being studied using new techniques like high-throughput metagenomic sequencing. For example, a study analysed the fecal microbiota of 6-month-old infants fed with daily supplements of a *Lactobacillus* strain (*L. rhamnosus*) and reported that the abundance of the supplemented bacteria, as well as other known probiotic species, increased in infants (Cox et al. 2010). Another study that employed metatranscriptomic analyses demonstrated that probiotics-treated mice had significantly altered expression of microbiota-encoded enzymes (McNulty et al. 2011). In fish farms, probiotics are used for different applications, for example, as growth promoters, pathogen inhibitors, digestive helpers and as an alternative to antibiotics (Sayes et al. 2018). The most common probiotics employed in aquafeeds include bacteria such as *Lactobacillus*, *Bacillus*, *Lactococcus*, *pseudomonads*, and the yeast, *Saccharomyces cerevisiae* (Merrifield et al. 2010b). Previous studies have shown that dietary supplementation of probiotics can modulate fish gut microbiota–intestine of antibiotic-treated rainbow trout offered *Bacillus* sp*. and Enterococcus* sp. were highly colonized by the fed probiotic (Merrifield et al. 2010a); intestinal microbiota of juvenile rainbow trout was shaped by multi-strain probiotics (Ramos et al. 2013).

These examples indicate that probiotics not only affect the composition of the gut microbiota but also modulate their global metabolic function. Although the increased awareness of the potential of beneficial microorganisms has spurred probiotic research, the demonstrated effects need extensive validation prior to commercial acceptance.

1.4.3 Prebiotics

Prebiotics are used as tools for microbiota modulation. They are feed additives that are “selectively utilized by the host microorganisms conferring a health benefit”, as defined by Gibson et al. (2017). They are intended to evoke beneficial health effects on the host through manipulation of the gut microbial communities and by the production of microbial metabolites. Vandeputte et al. (2017) reported the selective effects of prebiotics on certain microorganisms (e.g., *Bifidobacterium*, *Anaerostipes,* and *Bilophila*). Prebiotics serves as

nutrients for beneficial microbes (administered probiotic strains and resident microbes) harboured in the host. The most common dietary prebiotics consumed by humans are the non-digestible oligosaccharides, fructans, and galactans (Rastall and Gibson 2015). On the other hand, although fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), mannanoligosaccharides (MOS), oligofructose, inulin, xylooligosaccharides (XOS), arabinoxylooligosaccharides (AXOS) and few other candidates have been employed in aquafeeds, further research is warranted (Ringø et al. 2016). In-depth research on prebiotics for humans has helped us recognize the broader impact of these supplements on the microbial community. For instance, commensal intestinal lactic acid bacteria ferment fructans and produce lactate and acetate, which are used as energy source by many other intestinal bacteria including *Eubacterium*, *Roseburia*, and *Faecalibacterium*, that produce butyrate (Ríos-Covián et al. 2016). This process is known as cross-feeding, during which the byproducts of fermentation of a polysaccharide by one bacteria provide substrates for the growth of other bacterial populations present in the community (Belenguer et al. 2006). Among the fermentation by-products of prebiotics, SCFAs are studied most intensively, though they are not the only biologically active products derived from gut microbiota fermentation.

1.5 Fish-associated microbiota altered by diets and dietary components

Research is progressing to explore and engineer the gut microbiota of experimental model organisms including laboratory mice and insects, using high-throughput techniques. Although research on humans often overshadows the studies of aquatic animals, aquaculture-associated microbiota that affect the health of the host deserves attention because the industry is poised to provide quality food to future generations. The rapid developments in this field of research have helped to characterize the gut microbiota of main farmed aquatic groups like carps, salmonids, and tilapia. The fish microbiota is diverse and includes protists, bacteria, archaea, fungi and viruses (Merrifield and Rodiles 2015).

Research into the gut microbiota of fish started in the early 20th century (Reed and Spence 1929, Gibbons 1933); recently this field has grown significantly due to the expansion of the aquaculture industry. During the 1950s and ’60s, more studies contributed to our understanding of the fish gut microbiota; for example by investigating the effects of fasting on the intestinal microbial communities of different fish species (Margolis 1953, Colwell 1962).

In the following decades, researchers became pragmatic about the fish gut microbiota studies and investigated diet-induced changes (Sera and Ishida 1972, Dhanasiri et al. 2011) and the differences in the gut microbiota of farmed fish (Gilmour et al. 1976). Recent focus is on the nutritional manipulations and modifications of the gut microbiota of farmed fish. Compared to human and land animal studies, fewer reports describe the effect of dietary components on the gut microbiota of farmed fish. With the development of new molecular techniques, the research on fish gut microbiota has expanded dramatically. Nevertheless, information regarding diversity, composition and species-level functions of fish gut microbiota should be collected to develop effective strategies to keep pathogens at bay and to improve the growth and health of farmed fish (Wang et al. 2018a).

An update on the gut microbiota of fish: Bacteria are the dominant microbes in the gut of the fish (Cahill 1990), and thus far they are in the research spotlight. The first reviews on the gut microbiota of fish were published in the early 1990s (Cahill 1990, Ringø et al. 1995); based on the culture-dependent methods they concluded that the diversity of bacteria in the fish gut is low and the microorganisms are often derived from the surrounding environment or diet. Now we know that in fish, only < 0.1% of the total gut bacterial community are cultivable (Zhou et al. 2014). The next-generation sequencing (NGS) methods are frequently used for amplicon sequencing of bacterial biomarker genes to characterize fish-associated gut bacterial communities.

In fish, Proteobacteria that make ~90% of the intestinal microbiota are found to be the prominent microbial phylum, followed by Bacteroidetes and Firmicutes (Ghanbari et al. 2015). In a review, Nayak (2010) pointed out that gut microbes of freshwater and saltwater fish differ significantly, with bacteria such as *Aeromonas*, *Pseudomonas* and *Bacteroides* majorly colonizing the gut of freshwater species and *Vibrio*, *Pseudomonas*, *Achromobacter*, *Corynebacterium*, *Alteromonas*, *Flavobacterium* and *Micrococcus* predominating the gut of marine species. Egerton et al. (2018) stated that the three most frequently reported dominant bacterial genera are *Vibrio*, *Photobacterium*, and *Clostridium*–based on over 30 studies on a variety of fish. The density, diversity, and community composition of the microbiota change along the length of the fish gut (Clements et al. 2014). For example, a dietary intervention study on Atlantic cod, *Gadus morhua*, revealed not only diet-induced differences in the gut microbiota but also variations in the dominant bacterial species in the fore-, -mid- and the

hindgut of the fish (Ringø et al. 2006b). Similarly, region-based variations in the dominant bacteria of farmed Atlantic salmon were also reported (Hovda et al. 2007). Diet is one of the most important factors that shapes the diversity and composition of the gut microbiota. The following sections will cover the impact of the dietary components–antibiotics, probiotics, and prebiotics on the gut microbiota of fish.

1.5.1 Effect of antibiotics on fish gut microbiota

Antibiotic treatment can cause an imbalance in the gut microbiota, which may stimulate the development of/or aggravate certain intestinal diseases. Many studies have reported the effect of antibiotics on the intestinal microbiota of fish. For instance, in channel catfish (*Ictalurus punctatus*) florfenicol-medicated feed reduced the intestinal microbial composition and diversity, with an increase in abundance of Proteobacteria and Bacteroidetes, and pathogenic *Plesiomonas* spp. (Wang et al. 2019). In zebrafish (*Danio rerio*) long-term exposure to oxytetracycline caused a decrease in the gut microbial species richness and Shannon diversity (Almeida et al. 2019). He et al. (2010) reported a significant decrease in the intestinal autochthonous bacterial diversity of juvenile hybrid tilapia (*Oreochromis niloticus* ♀ × O. *aureus* ♂) offered dietary flavomycin and florfenicol. Navarrete et al. (2008) showed that oxytetracycline administration reduces bacterial diversity of the intestinal microbiota of juvenile Atlantic salmon (*Salmo salar*). In mosquitofish (*Gambusia affinis*), rifampicin exposure lowered the skin and gut microbial community diversity and altered the microbial composition (Carlson et al. 2017). He et al. (2017b) showed that supplementation of antibiotic olaquindox alters zebrafish intestinal microbial community composition and diversity, and increased the pathogen susceptibility of the fish. Amoxicillin administration significantly decreased the diversity of intestinal bacterial communities with a slight increase in *Lactobacillus* in adult zebrafish (Deprey and Uno 2016). Thus there is ample evidence to suggest that antibiotics can alter the intestinal microbiota of fish.

1.5.2 Effect of probiotics on fish gut microbiota

Manipulation of intestinal microbiota through dietary supplementation of probiotics is an approach to improve the intestinal health, growth performance and well being of farmed fishes (Andani et al. 2012, Han et al. 2015). Many bacterial candidates (such as lactic acid

bacteria (LAB), Bacillii and *Pseudomonas*) have been evaluated for their ability to manipulate the gut microbial community of several fish species. However, a particular probiotic candidate that may be effective in one fish may be ineffective in another species due to physiological status and rearing conditions of the fish (Lazado et al. 2015).

Dietary administration of probiotics can affect the intestinal microbiota of fishes. For instance, rainbow trout fed dietary probiotics (after antibiotic treatment) had the supplemented organisms as the dominant bacteria in the mucosa and digesta (Merrifield et al. 2010a). Furthermore, a fed probiotic (*Bacillus subtilis*) persisted in the gut of ornamental fishes (*Poecilia* sp. and *Xiphophorus* sp.) during the feeding period, when administered at concentrations of 10^6 to 10^8 cells g^{-1} (Ghosh et al. 2008). Dietary administration of probiotic *Lactococcus* (*L. lactis* MM1) increased the species richness and alpha diversity (Shannon index) in the gut microbiota of juvenile grouper, *Epinephelus coioides* (Sun et al. 2012). Another study on the same fish, but fed *Bacillus clausii* reported that the probiotic did not affect the gut bacterial diversity of the fish (Yang et al. 2012), suggesting that the additives differ in their effect on the gut microbial communities in fish. Studies on black molly (*Poecilia sphenops*) and Javanese carp (*Puntius gonionotus*) have also reported the modulation of the microbiota due to dietary supplementation of probiotics (Schmidt et al. 2017) and its influence on SCFA production in the fish intestine (Allameh et al. 2017). These examples indicate that probiotics can modulate the intestinal microbiota of fish.

1.5.3 Effect of prebiotics on fish gut microbiota

In fish, prebiotics improves disease resistance by modulating the non-specific immune responses through altering the gut microbiota, improving mineral uptake and increasing the production of bacterial metabolites (Burr et al. 2005). At present, substrates that qualify as prebiotics include only certain dietary fibers and non-digestible carbohydrates because not all of them bring specific changes in the gut microbiota. Moreover, it is known that the effect of prebiotics on the gut microbiota may differ depending on its dose and its ability to exert influence on the intestine (Biggs et al. 2007). Oligofructose prebiotics are non-digestible oligosaccharides composed of fructose and glucose units (Swanson et al. 2002) that can be fermented by certain intestinal bacterial genera such as *Lactobacillus* and *Bifidobacterium*, creating a favourable environment for the growth of certain bacteria. Hoseinifar et al. (2011)

investigated the effects of dietary oligofructose on the culturable autochthonous intestinal microbiota of beluga sturgeon (*Huso huso*); although viable culturable autochthonous levels were not affected, LAB levels in the intestine were significantly elevated in fish fed 20g prebiotic per kg feed. In hybrid tilapia, scFOS feeding caused a marginal and non-significant increase in the intestinal bacteria (particularly *Vibrio parahaemolyticus*, *A. hydrophila*, *Lactobacillus* spp. and *Streptococcus faecalis*) (Lv et al. 2007). MOS is another prebiotic, and this glucomannoprotein complex is mainly obtained from the cell wall of the yeast *Saccharomyces cerevisiae* (Sohn et al. 2000). Dimitroglou et al. (2009) reported modulation of the intestinal microbiota in rainbow trout, *Oncorhynchus mykiss* fed MOS-supplemented diets. Furthermore, lactic acid producing bacteria were found to be higher in the intestinal bacterial community of common carp fed MOS (Momeni-Moghaddam et al. 2015). Inulin, that is also considered as a prebiotic substance, is a polysaccharide produced by many plants. Mahious et al. (2005) reported an increase in intestinal vibrio species in turbot (*Psetta maxima* L.) fed 2% dietary inulin. Ringø et al. (2006a) showed that switching 15% dextrin with 15% inulin can alter the hindgut microbiota of Arctic charr. Mouriño et al. (2012) evaluated the effect of dietary inulin on the gut microbiota of hybrid surubins (*Pseudoplatystoma* sp.); although inulin did not affect total gut bacteria count, it decreased the population of *Pseudomonas* and increased intestinal LAB. Another prebiotic, XOS is a xylose-based oligomer that is extracted from bamboo shoots, fruits, vegetables, milk and honey (Vázquez et al. 2000). A study on crucian carp (*Carassius auratus gibelio*) reported that protease and amylase activities increased in the intestinal content and hepatopancreas, depending on the dose of XOS (Xu et al. 2009). Arabinoxylo-oligosaccharides (AXOS) is a potential prebiotic that is extracted from cereals (Grootaert et al. 2009, Courtin et al. 2011). Geraylou et al. (2012) reported that AXOS modulates the gut microbiota of Siberian sturgeon (*Acipenser baerii*) by stimulating the growth of LAB and *Clostridium* sp. These examples indicate that prebiotics can modulate the intestinal microbiota of fish.

Prebiotics are sometimes used in combination with probiotics, a nutritional mixture known as synbiotics (Das et al., 2017). In theory, synbiotics could be beneficial to the host by providing both probiotics and their preferred growth substrates. However, there is not much data on the benefits/effects of synbiotics compared to probiotics and prebiotics.

1.6 Gut microbiota of Atlantic salmon

1.6.1 Current knowledge

Atlantic salmon is one of the most widely cultured fishes, with a reported production of 1.23, 0.5, 0.16, 0.124, 0.056 million tons in Norway, Chile, Scotland, Canada and Tasmania, respectively for the year 2016 (FAO 2016). As one of the most important cultured species, the diversity and composition of the gut microbiota of Atlantic salmon has been studied to a certain extent, mostly by employing culture-based techniques (Cahill 1990, Ringø et al. 1995) and semi-quantitative molecular techniques (Hartviksen et al. 2014, Navarrete et al. 2013). Now researchers are employing NGS to acquire in-depth knowledge on the gut microbiota of salmon (Llewellyn et al. 2016, Zarkasi et al. 2016, Rudi et al. 2018, Dehler et al. 2017, Lokesh et al. 2018). Atlantic salmon is an anadromous fish that reproduce in freshwater but spend most of its life in seawater. A study on Atlantic salmon has shown that the freshwater-to-saltwater transition has a significant impact

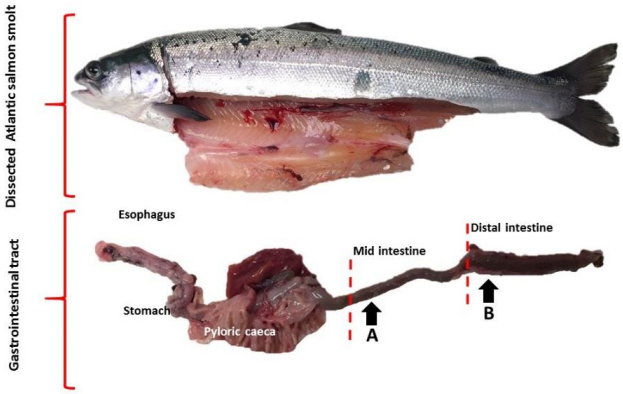

Figure 3. Gastrointestinal tract of Atlantic salmon.

The figure shows esophagus, stomach, pyloric caeca, mid intestine and distal intestine. Arrows show the sampled mid intestine (A) and the distal intestine (B) regions.

on skin microbiota (Lokesh and Kiron 2016). Moreover, the overall microbiota compositions of this fish in freshwater and saltwater are different (Rudi et al., 2018). In the salmon gut microbiota, Proteobacteria, Firmicutes, Tenericutes, and Bacteroidetes are the most often reported phyla (Dehler et al., 2017), suggesting that members of these four phyla make the "core gut microbiota"; Proteobacteria being the predominant one. Gajardo et al. (2016)

showed that bacterial populations of the different regions (proximal, mid and distal) of the intestine vary significantly and even the communities between digesta and mucosa are different. They also reported Proteobacteria and Firmicutes as the most abundant phyla in the digesta, the former almost completely dominating the mucosa. The GIT showing the different intestinal regions of Atlantic salmon is illustrated in Figure 3.

1.6.2 Knowledge gaps

Atlantic salmon is carnivorous, and years ago, farmed salmon was fed diets based on fishmeal. In a move away from the use of fishmeal-based diets, the aquafeed industry made efforts to find fishmeal replacers. The present-day salmon diets contain less fishmeal, but more plant-derived ingredients and functional additives. The effects of these feed components on the gut health and microbiota are gradually being revealed through transcriptomic (Król et al. 2016) and 16S rRNA sequencing (Gajardo et al. 2017) techniques, respectively. Now we know that the intestinal microbiota is undoubtedly an important factor in determining the health status of the host. Therefore, it is ideal to obtain in-depth information on the effect of dietary components on the intestinal microbial communities using high-throughput sequencing techniques. Furthermore, in fish farms, treatment of bacterial disease necessitates the use of antibiotics, although it is known that they can disrupt the stability of the gut microbiota. Probiotics and prebiotics can be considered as cheap and effective alternatives to antibiotics. However, at present, there is a lack of information on the effects of antibiotics or their alternatives on the gut microbiota of the fish. More knowledge of how dietary additives/antimicrobials influence the gut microbiota is essential for better fish health management.

1.7 Methods to assess the gut microbial communities

Almost three decades ago, most studies investigated the intestinal microbial communities using the conventional culture-dependent microbiology methods, which limits the discovery of several uncultivable species due to their specific nutritional, symbiotic and environmental requirements (Su et al. 2012). Over the past decade, our ability to understand the gut-microbiota at a molecular level has increased exponentially with the introduction of non-culture-based approaches. Briefly, they include quantitative real-time PCR (qPCR), finger-

printing methods such as temporal temperature gradient electrophoresis (TTGE) and denaturing gradient gel electrophoresis (DGGE), fluorescent in situ hybridization (FISH), and lately the NGS technology. The rapid and affordable method is now being employed frequently in studies of fish, resulting in substantial improvement in our knowledge of the structure and diversity of fish gut bacterial communities (Zhou et al. 2014, Ringø et al. 2016, Parma et al. 2016). With the development of NGS, the research on fish gut microbiota has expanded dramatically over the past few years. Under the NGS platforms, two approaches have been extensively practiced to understand the gut microbial composition; 16S rRNA gene profiling and shotgun metagenomic sequencing.

Universal 16S rRNA gene-based amplicon sequencing is by far the most commonly used method for assessing the gut bacterial communities because it is cost and time efficient. This approach relies on sequencing of the 16S ribosomal RNA (rRNA) gene, the genetic marker to study the bacterial phylogeny and taxonomy.

The bacterial 16S ribosomal RNA (rRNA) genes generally contain nine “hypervariable regions” (V1 to V9) that help understand the diversity of different bacteria (Van de Peer et al. 1996). However, not all hypervariable regions exhibit the same degree of sequence diversity. Therefore, use of one single hypervariable region will not be enough to identify all bacteria in a sample (Chakravorty et al. 2007). Each hypervariable region of the 16S rRNA gene is flanked by a conserved sequence (Baker et al. 2003). Conserved sequences serve as “anchors” for designing “universal” PCR primers that can amplify these hypervariable regions from a large pool of different bacterial species (Baker et al. 2003). Since the entire 16S rRNA gene region cannot be sequenced using NGS platforms, a short region or tag must be amplified and sequenced (Pollock et al. 2018). Selection of hypervariable regions for sequencing as well as the primer design is essential for this type of sequencing as these factors might contribute to differences in the results. To perform 16S rRNA gene sequencing, PCR primers are designed to target the chosen conserved regions and to amplify the chosen hypervariable regions. The amplicons generated by amplification are then separated through agarose gel electrophoresis; purified from the gel, quantified using qPCR, and finally sequenced on a high-throughput sequencing platform.

Recent sequencing technological methods come with their pros and cons. Pros of 16S rRNA gene amplicon sequencing technique are that it can achieve the necessary sequencing depth

to identify different bacterial species, can make use of the existing large and comprehensive databases as well as several bioinformatics pipelines for the downstream analysis. However, 16S rRNA sequencing also has its cons, which include the introduction of PCR amplification bias and low confidence of taxonomic assignment at the species level.

On the other hand, the whole genome or shotgun metagenomics sequencing, an alternative to 16S rRNA gene sequencing, surveys the entire genomes of all the organisms present in the sample. The pros of this technology are that it can efficiently delineate gene functions of the sequenced microbiome, help in discovering host-microbiota interactions and offer an increased taxonomic resolution. Furthermore, unlike the 16S rRNA gene amplicon sequencing, it is less prone to PCR amplification bias. However, this technology is expensive, requires deep sequencing and is computationally demanding. It should be noted that differences in sample handling, DNA extraction methods, or computational analysis methods can strongly bias the results obtained using either of these sequencing techniques.

2. Objectives

Although the gut microbiota of Atlantic salmon has been investigated in the past, knowledge about the impact of dietary components on the gut microbial communities is minimal. Most of the studies have reported the effect of diets on the intestinal structure and function, and growth performance of fishes. Furthermore, only few studies have employed high-throughput sequencing to characterize dietary component-induced alterations in the intestinal microbial communities of fish.

Therefore, the overall objective of this PhD project was to study the alterations in the intestinal microbial communities of fish fed preventive or therapeutic agents relevant to aquaculture.

Specific objectives include:

1. To understand the effects of antibiotic feeding on the gut microbial communities of Atlantic salmon (**Paper I**).
2. To investigate the effects of dietary lactic acid bacteria on the intestinal microbiota of Atlantic salmon (**Paper II**).
3. To determine changes in the intestinal bacterial community of Atlantic salmon in response to a potential prebiotic (**Paper III**).

The studies were based on the hypothesis that different dietary components can alter the structure of the intestinal microbiota of Atlantic salmon.

3. General discussion

Intensive aquaculture is often faced with challenges such as disease outbreaks, caused by the transmission of several agents including bacteria. Although vaccination has helped to contain several of the bacterial diseases in Norway, amphenicols- and quinolones-containing medicated feeds are still offered to farmed fish under veterinary guidance. Such antibiotic exposure can have detrimental effects on the gut microbes of the host fish, necessitating alternative therapies. These alternative strategies include offering the fish dietary supplements like probiotics and prebiotics. Studies have shown that dietary changes can modify the gut microbial communities of the host and this modification can, in turn, affect the health status of the host. However, there is not much information on the impacts of dietary components on the intestinal microbiota of salmonids, especially those that were gathered using the NGS technology. Therefore, we investigated the diversity, composition and microbe-microbe association of the gut microbiota of Atlantic salmon under the influence of two antibiotics (**Paper I**), and two lactic acid probiotics (**Paper II**). Furthermore, we studied the effect of macroalga-derived oligosaccharides (candidate prebiotics) on the intestinal microbiota profile of Atlantic salmon (**Paper III**). In addition, the bacterial diversity of tank water and biofilm samples were also explored to understand if the surrounding environmental bacteria affected the intestinal bacterial population of the fish. In general, the bacterial communities of the environmental samples were different from the respective fish-associated bacterial communities. Similarly to the present study, other studies have reported differences between host and environmental bacteria (Lyons et al. 2017a, Lyons et al. 2017b). In the following sections, most of the discussion focuses on the intestinal mucus bacterial community, which is more representative of the resident microorganisms of the host.

3.1 Dominant and core bacteria in the intestine of Atlantic salmon

Our comprehensive characterization of the intestinal microbiota of Atlantic salmon revealed the intestinal bacterial diversity, composition and community structure. Regardless of the different feeding studies, in all control samples, Proteobacteria and Tenericutes (especially genus *Mycoplasma*) were the predominant bacterial phyla (**Papers I**, **II** and **III**). Consistent with our findings, few other high-throughput sequencing studies have also

reported the dominance of Proteobacteria and Tenericutes in the gut microbiota of Atlantic salmon (Gajardo et al. 2016, Lokesh et al. 2018, Llewellyn et al. 2016, Dehler et al. 2017). Moreover, Proteobacteria is also a common dominant phylum in the intestine of rainbow trout (Kim et al. 2007), coho salmon (Romero and Navarrete 2006), paddlefish (*Polyodon spathala*), bighead carp (*Aristichthys nobilis*) (Li et al. 2014b), juvenile farmed pikeperch (*Sander lucioperca*) (Dulski et al. 2018), and grass carp (*Ctenopharyngodon idella*) (Ni et al. 2014). Tenericutes are also found dominant in the intestine of wild largemouth bronze gudgeon (*Coreius guichenoti*) and the gut of Chinese mitten crabs (*Eriocheir sinensis*) (Li et al. 2016, Zhang et al. 2016a). Furthermore, we reported that Proteobacteria and Tenericutes also make the core intestinal microbiota of the fish (**Paper I**, **II** and **III**), and the observations of Dehler et al. (2017) and Webster et al. (2018) corroborate this finding. In addition to Proteobacteria and Tenericutes, Spirochaetes were also identified as the common dominant phylum and the common intestinal core microbiota in salmon (**Paper II** and **III**). Similar to our findings, previous studies have also reported the dominance of Spirochaetes in the intestinal microbiota of post-smolts (Gajardo et al. 2016, Lokesh et al. 2018). Another study has shown that the phylum Spirochaetes has higher abundance in other carnivorous fish, including mahi-mahi (*Coryphaena hippurus*) and great barracuda (*Sphyraena barracuda*) (Givens et al. 2015). Moreover, in the current studies, Firmicutes and Actinobacteria were also detected as the dominant members of the intestinal microbiota of the fish. Previous studies have indicated Firmicutes as the most commonly found bacterial phylum in the intestine of salmon and other marine and fresh water fishes (Dehler et al. 2017, Gajardo et al. 2016, Izvekova et al. 2007, Wang et al. 2018b). Wang et al. (2018b) also reported that Actinobacteria is a dominant phylum in intestinal samples of healthy salmon.

The stable, dominant and core members of the gut microbiota are of great importance to the host. The diversity and composition of the intestinal microbiota varies from individual to individual and species to species, depending on the environmental factors, including diets. Therefore, it is important to investigate the influence of dietary changes on the intestinal microbial communities.

3.2 Compositional differences in the intestinal microbiota of Atlantic salmon fed dietary supplements and antibiotics

Several studies, including those in fish, have shown that the gut bacterial composition can be altered by diets (David et al. 2014, De Filippo et al. 2010, Bruce et al. 2018, Ringø et al. 2006b). Limited studies have assessed the effect of dietary components on the gut microbiota of salmonids. The following sub-sections are intended to shed light on the impacts of selected feed- delivered components on the gut bacterial composition of Atlantic salmon.

3.2.1 Feed-delivered antibiotics alters the intestinal bacterial composition

Antibiotic feeding can alter the host-associated intestinal microorganisms. In the present study, changes in the intestinal mucus microbiota of Atlantic salmon were investigated after feeding them with two antibiotics, namely florfenicol and oxolinic acid (**Paper I**). The antibiotics used in this study are broad-spectrum antimicrobials that are effective against a wide range of bacteria, for example, the pathogens *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio salmonicida* (Fellesskatalogen 2002a, Fellesskatalogen 2002b). Our findings show that consumption of antibiotics shifts the microbiota by altering the composition and abundance of the dominant bacterial phyla viz. Tenericutes, Proteobacteria, Firmicutes, Spirochaetes, Actinobacteria, Thermotogae, and Bacteroidetes in intestinal mucus of the fish.

Moreover, we observed that antibiotic intake increases the overall diversity of the intestinal bacteria. Florfenicol and oxolinic acid feeding caused a general increase in abundance of the phyla Proteobacteria and Bacteroidetes, in both intestinal content and mucus. Similar to our results, studies on florfenicol-fed channel catfish and mice have also reported an increase in abundance of Proteobacteria and Bacteroidetes (Li et al. 2017, Wang et al. 2019). Most members of Proteobacteria had higher abundance in the antibiotic-fed groups. However, there were also members of Proteobacteria that had low abundance in the antibiotic-fed fish. Interestingly, the family Vibrionaceae was found to have higher abundance in the oxolinic acid-fed fish. Members of the genus *Vibrio* are known as opportunistic pathogens in fish (Austin and Austin 2007, Schmidt et al. 2014). Schmidt et al. (2017) also reported a higher abundance of *Vibrio* in the microbiota of black molly (*Poecilia sphenops*) exposed to

streptomycin. Vibrios are thought to be “r-strategists”, which are capable of fast colonization in a disturbed microbial community.

Furthermore, the relative abundance of Spirochaetes was differentially affected by the two antibiotics; florfenicol decreased their abundance while oxolinic acid caused an increase in their abundance in the fish intestine. Antibiotic-specific alterations were observed in two families of Spirochaetes–Spirochaetaceae, and Leptospiraceae. The genus *Leptospira* (within the family Leptospiraceae) is known to cause diseases in humans and animals (Picardeau 2014). Mgode et al. (2014) reported a high prevalence of *Leptospira* in catfish and tilapia species. However, the functional importance and pathogenicity of these bacteria in Atlantic salmon needs to be elucidated. The abundance of Actinobacteria was higher only in the distal intestine mucus of the florfenicol-fed fish compared to the control fish. On the other hand, oxolinic acid did not exert any influence on the phylum.

Within Firmicutes, the abundances of two families; Bacillaceae and Lactobacillaceae were mostly increased by antibiotic-feeding. Bacillaceae was found to be a member of the core microbiota in the mid intestine. Members belonging to Bacillaceae includes both pathogenic and non-pathogenic species (Schmidt et al. 2011). In addition, antibiotic-feeding caused a general decrease in abundance of Tenericutes (in both content and mucus), the dominant phyla in the intestine of Atlantic salmon (Abid et al. 2013, Llewellyn et al. 2016). Within Tenericutes, the abundance of the family Mycoplasmataceae was decreased, and these bacteria also belong to the core gut microbiota of the fish. Furthermore, the abundance of the dominant phylum Thermotoage (family Fervidobacteriaceae) was found to be increased in the gut microbiota of antibiotic-fed fish. However, it should be noted that their abundance was lower compared to other dominant phyla in the intestinal mucus. Lokesh et al. (2018) have reported the presence of Thermotoage in the salmon intestine.

Taken together, feed-delivered antibiotics altered the composition and abundance of the dominant and core intestinal microbiota of Atlantic salmon.

3.2.2 Probiotic supplement facilitated the dominance of *Lactobacillus* in the intestine

Probiotics can influence the intestinal microbial composition and diversity (Scott et al. 2015) through suppressing the growth of other microorganisms and competing for their receptors

and binding sites (Spinler et al. 2008, O'Shea et al. 2012). Strains of LAB belonging to the genera *Lactobacillus* are commonly used as probiotics (Salminen et al. 2010). *Lactobacilli* are a group of gram-positive bacteria that utilize non-digestible carbohydrate through fermentation, to produce organic acids as end products (Bernardeau et al. 2006, Watson et al. 2013). These bacteria maintain the intestinal health by producing lactic acid that can be utilized by SCFAs producing microorganisms; SCFAs like butyrate have anti-inflammatory effects on the host cells and is also used as energy source by the intestinal epithelial cells (Louis et al. 2014). Offering diets supplemented with beneficial bacteria such as LAB is an alternative approach to control diseases in farmed fish (Martinez Cruz et al. 2012, Fečkaninová et al. 2017). In the present study, we investigated changes in the distal intestinal content and mucus microbiota of Atlantic salmon after feeding them with dietary supplements of two *Lactobacillus* spp., named RII and RIII (**Paper II**).

LAB feeding not only resulted in an increase in diversity of the intestinal mucus bacteria, but also promoted the abundance and dominance of the genus *Lactobacillus.* In this thesis, though the functional significance of *Lactobacillus* is not explored, two species of the genus, *L. paraplantarum* (LP; in RII fed salmon), and *L. fermentum* (LF; in RIII fed salmon) were abundant.

Previous studies have reported that *Lactobacillus* is part of the normal intestinal bacterial community of fish (Ringø et al. 1995, Spanggaard et al. 2000, Ringø and Olsen 1999) and it even enables the fish to overcome infection as shown in zebrafish, *Danio rerio* (He et al. 2017a). In silkworm, LP 11-1 was found to stimulate the innate immune system, which was suggested to be the reason for the acquired tolerance of the worms to *Pseudomonas aeruginosa* infection (Nishida et al. 2017). LF is found to improve fish immune response in Nile tilapia (*Oreochromis niloticus*) (Nwanna and Bamidele 2014). Adenike and Olalekan (2009) reported that a combination of LF (LbFF4 strain) and LP (LbOG1 strain) can evoke in vitro antibacterial activities against fish pathogens in African catfish *Clarias gariepinus.* In addition to stimulation of *Lactobacillus*, in the current study LAB feeding promoted the abundance and dominance of other members of the phylum Firmicutes. *Enterococcus cecorum*, was found to be dominant in the content of salmon offered diets with RII, while Clostridiales had higher abundance in the mucus of salmon fed RIII. Intestinal *Enterococcus* spp. isolated from rainbow trout exhibited antimicrobial activity against fish pathogens (Araújo et al. 2015). Commensal

Clostridiales are known to modulate gut homeostasis and participate in immune activation to maintain gut health in higher vertebrates (Lopetuso et al. 2013).

LAB also significantly altered the gut bacteria of the fish by favoring the abundance of certain members of Tenericutes (genus *Mycoplasma*). There was a general decrease in Spirochaetes (*Brevinema andersonii*) in the intestinal content and mucus. *Mycoplasma* was also found to be a member of the core microbiota and they have consistently been isolated from salmon intestine (Holben et al. 2002, Zarkasi et al. 2016). The presence of *B. andersonii* has been reported in the intestinal microbiota of flatfish, *Solea senegalensis* (Tapia-Paniagua et al. 2010). This species is known to digest lignocellulose and fix nitrogen in termite guts (Kudo 2009); however, its functional importance in salmon needs to be elucidated. Moreover, surprisingly LAB-feeding in the present study largely reduced the abundance of many members of intestinal Proteobacteria like *Photobacterium phosphoreum*, *Novosphingobium sediminicola*, *Phyllobacterium myrsinacearum,* and *Ralstonia pickettii*.

In summary, the higher abundance of intestinal *Lactobacillus* members and the altered bacterial composition confirms that dietary supplementation with LAB can alter the intestinal microbial composition of Atlantic salmon.

3.2.3 Prebiotic feeding shifts the intestinal bacterial composition

Prebiotics are dietary supplements that are gradually gaining ground in aquaculture. However, their effects on the intestinal microbiota of farmed salmonids have not been thoroughly investigated. Alginate oligosaccharide (AlgOS), derived from the macroalga *Laminaria* sp., is suggested as a candidate prebiotic agent; it favours the beneficial intestinal microbes, promoting host health (Wang et al. 2006). The changes in the gut microbiota of Atlantic salmon under the influence of AlgOS were investigated; the fish were offered feeds with low AlgOS inclusion (0.5g/100g- AlgOS-L), high inclusion of AlgOS (2.5g/100g - AlgOS-H) or without AlgOS (Control) (**Paper III**).

AlgOS-H supplementation caused a reduction in the bacterial diversity in the distal intestine of the fish. Similar to our results, other studies have also reported a reduction in bacterial diversity; galacto-oligosaccharides (GOS) and inulin reduced the bacterial diversity in mouse fecal samples (Cheng et al. 2017), and feeding mice with pectic oligosaccharides decreased

the cecal microbial diversity and richness (Bindels et al. 2015). However, contrasting observations are also seen in other articles; for instance, the intestinal bacterial diversity was increased in gilthead sea bream (*Sparus aurata*) and rats fed oligosaccharide supplementation (Dimitroglou et al. 2010, Ou et al. 2016). At the same time, a prebiotic blend of FOS, GOS, inulin, and anthocyanins did not alter the gut microbial diversity of mice (Chen et al. 2017). AlgOS supplementation in the present study reduced the abundance of certain Firmicutes and Bacteroidetes in Atlantic salmon intestine. Previous studies have reported that oligosaccharide can reduce the abundance of Firmicutes in the gut microbiota of humans and mice (Vigsnaes et al. 2011, Petersen et al. 2010). Furthermore, a reduction in Firmicutes and an increase in Bacteriodetes was reported in obese mice upon FOS administration (Everard et al. 2011). In the present report, AlgOS at low level facilitated the growth of certain members of Proteobacteria, Spirochaetes and Actinobacteria in the intestinal content and mucus of the fish. Within Proteobacteria, the class Gammaproteobacteria had high representation, followed by Alphaproteobacteria, Betaproteobacteria, and Deltaproteobacteria. *P. phosphoreum* and *Aliivibrio logei* belonging to the class Gammaproteobacteria were significantly more abundant in the content of the AlgOS-fed fish. *P. phosphoreum* is a known gut symbiont of marine fish which is capable of chitin digestion and uses luciferase to reoxidize reduced coenzymes and other molecules for metabolism (Nealson and Hastings 1979). A decrease in the abundance of *P. phosphoreum* in the distal intestine of Atlantic salmon fed plant-based diet was shown by Desai et al. (2012).

B. andersonii belonging to the phylum Spirochaetes, a core bacterial member of the distal intestine of the fish, was dominant in AlgOS-fed group; significantly higher in the content. Actinobacteria were significantly abundant in the mucus of AlgOS-L fish; represented by *Microbacterium ginsengiterrae*. However, their functional role in the fish intestine needs to be elucidated.

Overall, our findings show that dietary supplementation of the *Laminaria* sp.-derived AlgOS causes a shift in intestinal microbial diversity and composition of Atlantic salmon.

3.2.4 Feed-additive linked modulation of salmon intestinal microbiota

Diet is a significant factor that governs the changes in the intestinal environment, which in turn drive the diversity and composition of the intestinal microbiota. In this section, the results

from the 3 studies are collated and presented based on the communities of the intestinal content and mucus.

Interestingly, in the probiotic-fed fish, the relative abundances of many members of Proteobacteria were lower (**Paper II**). Conversely, antibiotic and prebiotic (especially 0.5% AlgOS) feeding caused a general increase in the abundance of Proteobacteria in the intestinal content and mucus (Paper I, III; only in mucus in the antibiotic study). Prebiotic feeding increased the abundance of *P. phosphoreum* and *A. logei* (Proteobacteria), in the intestinal content **(Paper III)**. On the other hand, probiotic feeding lowered the abundance of *P. phosphoreum* in both intestinal content and mucus, and *A. logei* was lowered in the mucus (**Paper II**). *Photobacterium* and *Aliivibrio* are closely related; these light-emitting sister genera have bioluminescent symbiotic association with some marine animals like fish and squids (Urbanczyk et al. 2011). Although these genera were detected in Atlantic salmon, their functions are yet to be elucidated.

Furthermore, *A. parvum*, *B. jicamae*, and *M. fujisawaense* were found to be abundant in the intestinal mucus of fish fed low level of the prebiotic (**Paper III**), while in fish fed probiotics the abundance of the three taxa were less abundant in mucus and content compared to the control fish **(Paper II)**. *A. parvum* is known as a nitrate-dependent Fe(II)-oxidizing bacteria (Zhang et al. 2016b), while many bacteria belonging to the genus *Bradyrhizobium* are known to fix nitrogen (Ramírez-Bahena et al. 2015). Species belonging to Methylobacterium are methylotrophs, and are described as agents of contamination and opportunistic infections in humans (Lai et al. 2011). However, currently, no functional information is available for *B. jicamae* and *M. fujisawaense* in Atlantic salmon. Moreover, in the mucus of prebiotic-fed (low level) fish, the abundances of *P. myrsinacearum and U. oligocarboniphilm* were increased **(Paper III)**, whereas the two probiotic altered these taxa in the content (RII increased and RIII decreased); **(Paper II)**. *P. myrsinacearum* was detected in grass carp (*Ctenopharyngodon idella*) larvae (Wang et al. 2015). However, no studies to date have reported these species in salmon intestine.

In the fish that did not receive the additives, the genus *Mycoplasma* (Mycoplasmataceae, Tenericutes) was predominant and it was a core member of the intestinal content and mucus. Their abundance was decreased in fish fed antibiotics and prebiotics (except in content of high level fed fish) (**Paper I and III**) while probiotics favored their abundance (**Paper II**).

The relative abundance of Spirochaetes was higher in fish that received oxolinic acid feed. On the other hand, florfenicol reduced their abundance in the intestinal mucus of fish (**Paper I**). Within Spirochaetes, *B. andersonii* was found to be abundant in the intestinal mucus of probiotic-fed fish and in the content of prebiotic-fed fish (**Paper II and III**). Other studies have also reported high abundance of Spirochaetes in the intestine of Atlantic salmon and mahi-mahi, *Coryphaena hippurus* (Gajardo et al. 2016, Givens et al. 2015).

The abundances of certain members of the phylum Firmicutes were found to be increased in fish receiving antibiotics and probiotics (**Paper I and II**), which is contrary to the reduction observed in prebiotic-fed fish (**Paper III**). Antibiotic feeding mostly increased the abundance of Bacillaceae and Lactobacillaceae (**Paper I**). LAB feeding also promoted the abundance and dominance of members of Lactobacillaceae; RII-feeding stimulated the growth of *L. paraplantarum* while RIII-feeding boosted the abundance of *L. fermentum*. It should be noted that members belonging to Bacillaceae and Lactobacillaceae can be both pathogenic and non-pathogenic (Schmidt et al. 2011). In addition to the stimulation of *Lactobacillus*, LAB feeding promoted the abundance of *E. cecorum* (in intestinal content) and Clostridales (in intestinal mucus) of the fish (**Paper II**). However, AlgOS supplementation reduced the abundance of *Weisella cibaria*. The abundance of this Firmicute, was found to be reduced in the intestinal content of the fish fed antibiotics and prebiotics (**Paper II and III**).

Florfenicol increased the abundance of Micromonosporaceae (Actinobacteria) in the intestine mucus whereas oxolinic acid fed-fish had lower abundance of the bacteria belonging to this family (**Paper I**). AlgOS-L increased the abundance of *M. ginsengiterrae* (**Paper III**). Furthermore, Bacteroidetes was detected as the dominant phylum only in the antibiotic and prebiotic experiments (**Paper I and III**). However, it was not a dominant member in the intestinal microbiota of salmon fed probiotics (**Paper II**). Although, in general they were found in higher abundance in fish on antibiotics, in fish on prebiotics their abundance was decreased in the mucus and content compared to the control groups (**Paper I and III**). Previous studies have reported Bacteroidetes to be core and dominant in the salmon intestine (Wang et al. 2018b, Dehler et al. 2017).

This knowledge about the differential modulation of specific bacteria can be considered for the development of effective intestinal microbial community manipulation strategies. Furthermore, the details help to understand the impact of the different feed-delivered

components such as antibiotics, probiotics and prebiotics on the intestinal microbiota of Atlantic salmon.

3.3 Feed additives affect the intestinal microbe-microbe association and stability

Inferring interactions among different microbes within a community is crucial to understand how they adapt to an environment and interact with the host (Gao et al. 2018). In the intestine, these diverse microbes interact with each other to obtain nutrients required for their colonization and proliferation, and these interactions occur by developing complex microbe-microbe associations. Such microbial associations are relevant to establish the functional stability of intestinal microbial community (Gao et al. 2018). Various dietary factors can affect this stability; they may maintain/improve or disturb the interactions among the intestinal microbial species by altering the abundance of one species which may shift the relative abundances of other microbial members (Sun and Chang 2014). In the studies presented here, single-domain bacterial (SDB) network graphs were generated using SPIEC-EASI framework (Kurtz et al. 2015) to describe the topology of the network association of the intestinal bacterial communities in the fish groups offered antibiotic or probiotic-supplemented diet (**Paper I, II**). The significantly abundant and/or relevant OTUs were labeled on the SDB network based on their membership in different modules. A microbial association network consists of a set of vertices (commonly called as nodes) and edges that connect them. The degree of a node is the number of microbial connections it has with the other nodes in the network. The number of shortest paths that pass through the nodes in the network is called betweenness and the extent of the selectively connected labeled pair of nodes is called assortativity coefficient (Kolaczyk and Csrdi 2014). The co-occurrence networks of only the antibiotic and probiotic fed fish were investigated to uncover the probable biological interactions occurring within the intestinal microbes.

Paper I: The inferred SDB network of the intestinal mucus bacteria of the fish offered florfenicol-incorporated feed had higher overall connectivity, betweenness and hubs with less node degree. On the other hand, inferred SDB network of the fish offered oxolinic acid-containing feed had lower overall connectivity, betweenness, and hubs with more node degree. The higher overall linking of the microbes in the florfenicol group indicates greater interactions among the gut bacteria. The microbial communities that cooperate are

functionally dependent and therefore are accountable for providing stability to the microbial community. Coyte et al. (2015) have suggested that an increased microbial diversity along with higher share of cooperative microbial interactions can disturb the microbial stability. Higher cooperation among the microbes can trigger over-representation of the most stable communities, which can initiate a runaway effect that can disintegrate the competing microbial population (McNally and Brown 2016). The lower predicted connectivity of the microbes in the oxolinic group indicates less interactions among the intestinal bacteria. It has been shown that antibiotic exposure can cause microbial network fragility in murine models (Ruiz et al. 2017). Furthermore, the networks of distal intestine microbes of the antibiotic-fed fish had dyads and triads representing mostly Proteobacteria; they were not connected to the main network component, suggesting that antibiotic feeding reduced the association between certain members of Proteobacteria and other intestinal microbes. We observed that, although most of the labeled OTUs belonged to different modules in the distal intestinal bacterial network of the antibiotic-fed group, one OTU belonging to Rhizobiaceae was connected to other significantly different OTUs. Members of family Rhizobiaceae are known for their ability to establish a beneficial interaction with the host (in plants) and contribute to the process of biological nitrogen fixation (Carareto et al. 2014). In the mid intestine mucus of antibiotic-fed groups, the labeled, significantly different OTUs were connected to the main network but belonged to different modules (the exception being one OTU belonging to Proteobacteria in the oxolinic acid-fed group), suggesting that antibiotic feeding can affect the microbe-microbe interactions. Our results also suggest that exposures to different antibiotic do not effect microbial interactions similarly.

Paper II: The inferred SDB network for distal intestine mucus of RII-fed fish showed lower overall connectivity and hubs with more node degree, indicating less interactions among the intestinal bacteria. Furthermore, the dyads in the bacterial networks of LAB-fed fish were dissimilar. Only one dyad was similar, namely the one that was constructed with 2 OTUs of *Mycoplasma*. This result suggests that intestinal mucus *Mycoplasma* in fish offered LAB was not associated with other gut bacterial communities. Furthermore, this genus had higher abundance in the RII-fed fish and lower abundance in the RIII-fed fish. In the SDB network of the control fish, Clostridiales and Rhodobacteraceae were present in the same module, suggesting their functional dependence. A previous study has reported that Clostridiales

indirectly participates in nitrogen cycling through nitrate respiration by providing fermentation substrates like, acetate, formate, or hydrogen to Rhodobacteraceae-like denitrifiers (Kraft et al. 2014). In the inferred SDB network of mucus of RIII-fed fish, OTUs belonging to *L. paraplantarum*, *Clostridium aestuarii*, and Clostridiales were found to be in one module, suggesting that LAB feeding altered the bacterial association. Overall, these findings imply that the the intestinal microbe-microbe associations can be altered by feeding antibiotics and probiotics to Atlantic salmon. However, alterations of their membership after such feeding should be further investigated through culture-based studies.

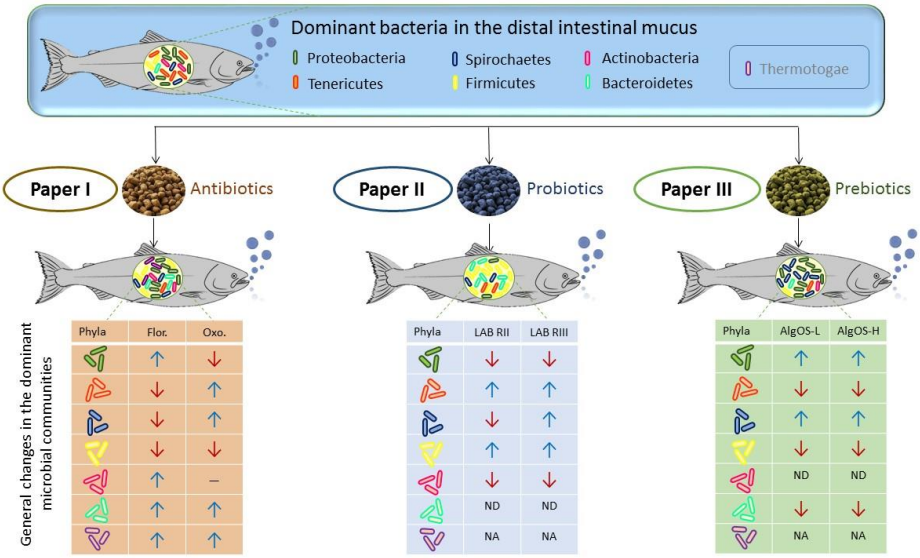

Figure 4. Summary of the main findings of this thesis.

Feed additives affected the dominant intestinal microbial community. Results indicate differential alterations of the phyla by the feed additives. ND indicates; taxa not dominant and NA indicates; taxa not present.

4. Conclusions and future perspectives

This study provides insights into the influence of different dietary supplements on the diversity and composition of the intestinal microbiota of Atlantic salmon, characterized using a state-of-the-art sequencing approach. We demonstrated the impact of two antibiotics, two lactic acid probiotics and a candidate prebiotic oligosaccharide from a macroalga on the intestinal bacterial profile of the fish. Furthermore, microbe-microbe interactions within the intestinal microbiota of fish were also assessed. The highlights of this PhD project are the following:

- In the intestinal bacterial microbiota of farmed Atlantic salmon, Proteobacteria and Tenericutes are the predominant and core phyla, followed by Spirochaetes, Firmicutes, Actinobacteria, and Bacteroidetes.
- The compositional profiles of the intestinal communities of the assessed fish groups indicated the dominance of Tenericutes, largely due to the abundance of the genus *Mycoplasma*.
- Antibiotics altered the composition and abundance of the dominant and core bacterial phyla. The medicated feeds caused a general increase in abundance of Proteobacteria, Actinobacteria, Firmicutes and Spirochaetes, considering both content and mucus.
- LAB provided through feeds reduced the abundances of many members of Proteobacteria in the intestine of salmon. However, it promoted the dominance of Firmicutes (*Lactobacillus*), and the phyla Tenericutes (Mycoplasma).
- The predicted co-occurrence networks of the intestinal bacteria indicated that both antibiotics and probiotics affect the microbe-microbe interactions differentially.
- The prebiotic, oligo-alginate caused a shift in the diversity and composition, leading to the dominance of Proteobacteria and Spirochaetes in the intestinal microbiota of the fish.

Similar to mammals, the intestinal microbial communities can influence key physiological functions of fishes, indicating the relevance of the microbes in health maintenance. In aquaculture, the animals are offered feeds containing recommended levels of macro- and

micro-nutrients. In addition, immune-modulatory additives such as prebiotics and probiotics help the fish to maintain a good health status. The correlation between intestinal microbiota and host health emphasizes the need to understand the effect of dietary additives on the fish intestinal microbiota composition. Undeniably, the NGS technology will help in such investigations, providing comprehensive information about the microbial taxonomy. Complex interactions among the intestinal microbes and between host and intestinal microbes are crucial for maintaining host health. The present thesis is one of the few studies that has predicted the intestinal microbe-microbe interactions in fish. Therefore, future investigations should explore the fundamental factors underlying microbe-microbe and host-microbe interaction in the GIT of fish. Moreover, researchers should culture the specific microbes (microbial culturomics) of interest in a community to gain insights about their functions and their effect on other microbes as well on the host. Many studies have found changing patterns of the intestinal bacterial composition at the phylum level. However, studying bacteria only at this primary level may not provide sufficient insights; for example, to discover their links to certain bacterial diseases. For that reason, future investigators must adopt methods like shotgun metagenomics that can also help uncover the rare bacterial communities also.

Furthermore, there is an emerging need to test the efficacy of feed additives to alter the beneficial intestinal microbes in fish. Although aquaculture research is now focusing on NGS platforms for such investigations, convincing evidence to support the link between additive-stimulation of beneficial microbe growth and fish health, and nutrition should be gathered by conducting in-depth studies. In addition, new investigations should address the immunological response to the intestinal bacteria responding to the additive. Another line of research should consider the dose-dependent response of antibiotics on the fish intestinal microbiota. The benefits of probiotics on the host and its gut microbiota are already known, but not many studies have reported their side-effects (if any); this is yet another area to be explored. Prebiotics derived from microalgae and macroalgae are known to impart health benefits to humans; similar effects in fish should be demonstrated.

Predicting the taxonomy based on the selected segments of the 16S rRNA gene, such as the V3-V4 region has limitations. A short sequence when mapped to several bacterial sequences in the reference database can reduce the accuracy of taxonomy predictions. We did not

include a mock community control to quantify sequencing errors. However, spike-in standards were employed to minimize such errors.

Overall, adopting culturomic and shotgun metagenomic approaches that can integrate the information on the effects of feed additives on the intestinal community profile of the fish will broaden our knowledge on the relevance of the dietary approaches considered in this thesis.

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Paper I

Antibiotic-induced perturbations are manifested in the dominant intestinal bacterial phyla of Atlantic salmon

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Running title: Antibiotics alter intestinal microbiota of salmon

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Abstract

The intestinal microbiota of certain farmed fish is often exposed to antimicrobial substances, such as antibiotics, that are used to prevent and treat bacterial diseases. Antibiotic treatment, which is intended to kill or inhibit the growth of harmful microorganisms, can rapidly alter the intestinal microbial diversity and composition, with potential effects on the host health. In this study, we have elucidated the effects of two antibiotics, florfenicol and oxolinic acid on the distal and mid intestinal microbial communities of Atlantic salmon (*Salmo salar*). We employed high-throughput 16S rRNA gene amplicon sequencing to investigate the bacterial communities in the intestinal mucus of Atlantic salmon fed diets with and without antibiotics. Our results show that antibiotic exposure shifts the intestinal microbial profile differentially. Beta diversity analysis revealed significant differences between the bacterial compositions of the control and antibiotic-fed groups. Antibiotic-feeding altered the composition and abundance of the dominant bacterial phyla viz. Proteobacteria, Actinobacteria, Firmicutes, Spirochaetes, Bacteroidetes, Tenericutes, and Thermotogae. Furthermore, the bacterial association network analysis revealed that the co-occurrence pattern of bacteria of the three study groups were different. We conclude that both florfenicol and oxolinic acid can modulate the composition and interaction of the intestinal microbiota of Atlantic salmon.

Introduction

Antibiotics either kill pathogenic bacteria or inhibit their growth. Although antibiotic administration is intended to help the host fight infections, they can have a detrimental effect on the commensal gut microbiota of the host. The gut microbiota is an ecological community of commensal, symbiotic and pathogenic microorganisms (1, 2) that inhabit the gastrointestinal tract (GIT). The microbial assemblage that colonizes the GIT includes many bacterial species as well as other microorganisms such as fungi, viruses, and archaea (3). These microbes have co-evolved over thousands of years to establish a complex and mutually beneficial relationship with the host (4). In healthy humans, the gut bacterial population that are the most dense and extremely diverse in the large intestine of the GIT (5) offers various functions; many of which provide health benefits, including maturation of immune system (6), immune homeostasis and health maintenance (7). Other functions of commensal bacteria that have significant consequences on the health include biosynthesis of microbial amino-acids (8), fermentation of nondigestible dietary carbohydrates into absorbable bioactive metabolites (9, 10), vitamin synthesis (11), and pathogen displacement (12).

Antibiotic-induced perturbations in the established gut microbial community may result in dysbiosis that could culminate in the ill-health of the host. The extent of the detrimental effects of antibiotics on the commensal organisms depends on the specific antibiotic used, the dose/duration, its mode of action and the degree of resistance by the gut microbial community (13). An imbalance in the gut microbial composition can affect the interplay between them and the host resulting in immune-mediated diseases (14). Several studies have confirmed that antibiotic exposure rapidly alters the gut microbiome composition (15), causing an imbalance in their stability.

The global use of antibiotics–in human medicine, animal agriculture and aquaculture–continues to escalate. Aquaculture is one of the fastest growing food-producing sectors, and the widely adopted intensive farming technique is associated with infectious diseases. Therefore, in aquaculture antibiotics are administered, as required, for short periods of time (16). Excessive use of antibiotics in aquaculture farms in many countries has caused problems and concerns, such as antimicrobial resistance and food safety risks. Among the employed antimicrobials, florfenicol (FFC) is by far the most commonly and frequently used antibacterial agent in Atlantic salmon farms (17). Oxolinic acid (OA) is another antibiotic that salmon are occasionally exposed to (18). However, its use has decreased compared to FFC (17). FFC is an amphenicol and members under this group are broad-spectrum (i.e., they are effective against both pathogenic and symbiotic bacteria) bacteriostatic antibiotics that slow the bacterial growth mainly by inhibiting protein synthesis (19). OA is a first generation quinolone (20). Members of quinolones are broad-spectrum bactericidal antibiotics that are capable of killing infectious bacteria (21) by affecting their DNA metabolism through inhibiting the activities of two bacterial enzymes, DNA gyrase and topoisomerase IV, which leads to DNA fragmentation (22, 23). All quinolones can exert both bactericidal (24), and bacterostatic effects; when bacteriostatic, it targets the DNA replication process (25). Depending on the dose, most antimicrobials can exhibit bactericidal and bacteriostatic properties. A recent study has linked higher dose of FFC and occurrence of antibiotic resistance bacteria in the gut microbiota of

farmed Atlantic salmon (26). However, such correlation based on OA feeding has not yet been described.

The effects of antimicrobials are not adequately addressed through necessary scientific research, not to mention their impact on the intestinal microbial composition of Atlantic salmon, especially when feeds are employed as the antibiotic delivery vehicles. In this study, we examined the effects of FFC and OA on the intestinal microbiota of Atlantic salmon. In addition, we describe the differences in the topology of co-occurrence networks associated with the intestinal bacteria of Atlantic salmon offered feeds with or without the two antibiotics.

Material and methods

Ethics Statement

The present study was approved by the Norwegian Animal Research Authority, FDU (Forsøksdyrutvalget ID-7898). Fish handling and sampling methods were in agreement with the regulations of animal use in experiments described in LOVDATA. The fish were given enough time to acclimatize to the rearing facility. Furthermore, we adhered to our standard biosecurity and safety procedures at the Research Station of Nord University (Norway).

Experimental fish, rearing conditions and antibiotic dosing

Atlantic salmon of initial average weight 321.9 ± 36.2 g were maintained in 800 L tanks in a flow-through sea water system. A 12-day feeding trial was conducted at the Research Station, Nord University, Bodø, Norway. Three groups of fish received commercial feeds coated with (florfenicol (FFC) – F; oxolinic acid (OA) – O) or without antibiotics (Control – C). The dose of FFC and OA per fish was 2g/kg and 5g/kg, respectively as given in Felleskatalogen (26, 27). The two antibiotics were administered as per the recommendation of The European Agency for the Evaluation of Medicinal Products (28, 29). The recommended feed ration was delivered using automatic feeders (Arvo-Teck, Huutokoski, Finland). The water flow rate, temperature, salinity and O_2 levels in the tanks were 800 L/h, 6.7-7.1°C, 32 ppt, >85% saturation measured at the outlet, respectively. The fish were maintained under a 24 h light regime.

Sampling strategy

We sampled the mucus from the distal intestine (DI) and mid intestine (MI) of the experimental fish at the end of the feeding regime. For this, first, the fish were euthanized using 160 mg/L of MS222 tricaine methanesulfonate (Argent Chemical Laboratories, Redmond, WA, USA). After that, the body surface of the fish was cleaned using 70% ethanol. The fish were then dissected asceptically to remove the GIT from the abdominal cavity. The DI and MI regions were carefully separated from the GIT, and the content was removed using sterile forceps. The intestinal mucus was collected (n = 9) using sterile glass slides into cryotubes and stored at -80°C. In addition to the fish samples, we collected environmental samples: biofilm samples scraped from the walls of the rearing tanks (n = 3). The tank biofilm samples were also stored at -80°C.

The sample abbreviations used are: i) fish samples− control distal intestine mucus (CDM), FFC distal intestine mucus (FDM), OA distal intestine mucus (ODM), control mid intestine mucus

(CMM), FFC mid intestine mucus (FMM), OA mid intestine mucus (OMM), ii) environmental samples− control tank biofilm (CB), FFC tank biofilm (FB), OA tank biofilm (OB).

Bacterial DNA isolation, PCR amplification, 16S rRNA gene amplicon library preparation and sequencing

To study the microbial composition, total genomic DNA was isolated from the mucus and biofilm samples using the Quick- DNA™ Fecal/Soil Microbe 96 kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol. The quality of the isolated DNA was checked on 1.2% (w/v) agarose gel and quantified using Qubit 3.0 fluorometer (Life Technologies, Carlsbad, USA).

To describe the antibiotic-induced perturbations in the intestinal microbiota of the fish, we amplified the V3-V4 region of the bacterial 16S rRNA gene employing a dual-index sequencing strategy (30). The 16S rRNA gene region of the DNA was amplified in triplicates using PCR; each PCR reaction (25 µl) volume contained 12.5 µl of Kapa HiFi Hot Start PCR Ready Mix (KAPA Biosystems, Woburn, USA), 1.5 µl of each forward and reverse primer (at a final concentration of 100 nM), 3.5 µl of DNAse and nuclease-free water (Merck, Darmstadt, Germany) and 6 µl of DNA template and/ or 6 µl of negative PCR control. The thermocycling conditions included initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 98°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 45 s, and the final extension performed at 72°C for 2 min. The resulting amplicon triplicates were pooled in equal amounts, visualized on 1.2% (w/v) agarose gel stained with SYBER® Safe, and the amplicon size was compared to a 1kb DNA ladder (Thermo Fisher Scientific, Rockford, IL, USA). The negative PCR control did not indicate any positive amplification. Amplicons (~550bp) were further purified using the ZR-96 Zymoclean™ Gel DNA Recovery Kit (Zymo Research) and eluted in 15 µl of elution buffer. The eluted amplicon library was quantified by qPCR using the KAPA Library Quantification Kit (KAPA Biosystems). After quantification, each amplicon library was normalized to an equimolar concentration (3 nM) and validated on the TapeStation (Agilent Biosystems, Santa Clara, USA), prior to sequencing. The normalized amplicon library pool was further diluted to 12 pM, spiked with equimolar 10% PhiX control and then paired-end sequencing was performed using the 600 cycle v3 sequencing kit on the Illumina MiSeq sequencer (Illumina, San Diego, CA, United States).

Bioinformatic analysis of the 16S rRNA gene sequence data

Sequence data quality check

As the first step in the 16S rRNA gene sequence data analysis, the quality of the raw reads was checked using FastQC (31) and the reads with Phred quality score (Q) $\leq$ 15 were discarded. Only the forward reads (R1) corresponding to the V3 region of 16S rRNA gene were employed for subsequent analyses because they were of better quality than the reverse reads (R2) corresponding to V4 region.

Sequence data processing

The bioinformatic pipeline UPARSE (USEARCH version 9.2.64) by Edgar (32) was used for the sequence data processing. For this, the raw FastQ files that were input into the UPARSE pipeline were truncated to remove the low-quality base pairs at the 3'-end and for quality-

filtration. Furthermore, using the UCHIME algorithm (33) chimeric sequences were removed. The quality-filtered sequences were clustered at 97% sequence similarity level to generate the operational taxonomic units (OTUs). For taxonomy annotation, the 16S rRNA Ribosomal Database Project training set with species names v16 was employed and the taxonomic ranks were assigned to the OTUs using the SINTAX algorithm (34), using a bootstrap cutoff value of 0.5. The OTUs with a confidence score of <1 at the domain level were removed. Furthermore, OTUs belonging to the phyla Cyanobacteria and Chlorophyta were removed. After the OTU table construction, the counts were rarefied to the lowest number of sequences per sample to get an even sampling depth to facilitate comparisons between the treatment groups. The rarified OTU count data was divided into 3 sets based on the sample type, namely the DI mucus, MI mucus, and tank biofilm samples. The downstream analyses were performed separately on these 3 sets.

Furthermore, only 9 fish per group were considered for the downstream analyses to ensure that the samples across the different tissues were from the same fish. In total, 63 samples were used for the downstream analyses, including the tank biofilm samples.

Accession number

The raw 16S rRNA gene sequence data from this study has been deposited in the European Nucleotide Archive (ENA) under the accession number xxxx (in the process of submission).

Sequence data analysis to understand the gut microbial diversity and composition

For gut microbial diversity and composition analysis, we employed customized R codes in RStudio v3.5.0 (R Development Core Team, 2018). The functions of the R packages; 'iNEXT' v2.0.12 (35), 'phyloseq' v1.22.3 (36) and 'ggplot2' v2.2.1 (37) were used to make the rarefaction curves for the species richness, to calculate and visualize diversity indices and to prepare the abundance plots. Furthermore, another R package called 'microbiome' v1.0.2 (38) was used to make core and rare microbiota (relative abundance of core taxa) plots. The alpha diversities - Shannon diversity (effective number of common OTUs) and Simpson diversity (effective number of most abundant OTUs) were calculated based on the formula suggested by Jost (39). Beta diversity was examined by conducting double principal coordinate analysis (DPCoA, for fish and biofilm samples) (40).

Statistical analyses of the sequence data

R studio v3.5.0 was used to perform the statistical analysis of the sequencing data. To detect significant differences in alpha diversity we employed Kruskal-Wallis test followed by Dunn's test. To check the assumption of heterogeneity in dispersions we employed Betadisper; after that Adonis (PERMANOVA) followed by pairwise comparisons (999 permutations) was used to understand the significant dissimilarities of the communities and the differences were considered as statistically significant at $p < 0.05$ and statistical trends at $p \leq 0.15$. The same statistical analyses procedures were also used to compare tank biofilm samples with the intestinal samples from the control and treatment groups. Furthermore, to detect the differentially abundant OTUs in the treatment groups we employed 'ANCOM' v1.1-3 R package (41). To find the relevant OTUs that caused the differences in the intestinal bacteria of the three fish groups we employed 'Boruta' v5.3.0 R package (42).

Microbial association graph construction and network topology inference

We used association network analysis to explore the associations between the OTUs. To generate the single-domain bacterial association network, we used 'SPIEC-EASI' v1.0.2 R package (SParse InversE Covariance Estimation for Ecological Association Inference), as described in Gupta et al (43). The latest version of SPEIC-EASI allows analysis with fewer OTUs than we have employed before (44); the co-occurrence microbial networks were constructed using the top 90 OTUs (for DI) and top 150 OTUs (for MI). To construct the co-occurrence microbial networks, we employed the functions of the R package 'igraph' v1.2.1 and customized ggplot2 commands. We compared the topology of the networks of the DI and MI mucus samples separately by analysing the node degrees and betweenness of the control and antibiotic-fed groups using Kruskal-Wallis test followed by Dunn's test.

Results

Sequence data

We analyzed the 16S rRNA V3 amplicon sequences of the intestinal bacterial communities of 54 samples. Twenty-seven each were of intestinal mucus samples of DI and MI, and the remaining 9 were of the tank biofilm samples. We obtained a total of 25,673,984 high-quality reads that were clustered into 1,380 OTUs at 97% identity threshold. The majority of the reads were rarified based on sample-size; the saturation point being 10 044. Rarefied data was employed to assess most of the underlying microbial diversity (Supplementary Figures 1A, B and C).

The differences in the DI and MI mucus bacterial communities of the antibiotic-fed fish compared to the control fish are explained based on the below mentioned diversity metrics, taxonomic composition and relative abundances of the bacterial taxa. We also present the significant and relevant bacterial communities and the topology of the networks of the intestinal bacterial communities in the three fish groups.

Changes in the microbial diversity of the intestinal mucus and environmental microbiota

Antibiotic feeding increased the species richness and diversity of the bacterial community in the DI and MI of the fish. The species richness was found to be higher in the DI of antibiotic-fed groups ($p = 0.0001$ for FDM vs. CDM and $p = 0.105$ for CDM vs. ODM; Figure 1A). In MI, the species richness was higher only in the F-fed group ($p = 0.048$ for FMM vs. OMM; Figure 2A). We observed significant differences in the effective number of common and dominant OTUs in the DI of the antibiotic-fed groups (DI mucus: $p = 0.002$ for FDM vs.CDM and $p = 0.001$ for ODM vs. FDM, Figure 1B; $p = 0.020$ for FDM vs. CDM and $p = 0.001$ for ODM vs. FDM; Figure 1C). Comparison of Faith's phylogenetic diversity (PD) of the DI ($p = 0.0001$ for FDM vs. CDM; Figure 1D) and MI ($p = 0.044$ for OMM vs. FMM; Figure 2D) revealed differences. PCoA based on the weighted unifrac distance matrix revealed that the beta diversity of the bacterial communities was different; the differences between F- and O-fed

groups were statistically significant (DI: Figure 1E: F statistic = 2.277, R^2 = 0.159, p = 0.028; MI: Figure 2E: F statistic = 5.64, R^2 = 0.32, p = 0.011).
The beta diversity of the bacterial communities of the biofilm samples were also analysed. The bacterial communities of the tank biofilm of the three study groups were not different (Supplementary Figure 2A, F statistic = 0.76, R^2 = 0.20, p = 0.538). On the other hand, the bacterial communities in the biofilm were significantly different from those of the fish (Supplementary Figures 2B-G).

Changes in the intestinal mucus bacterial composition, influenced by antibiotics

Bacteria belonging to 21 phyla were present in the DI and MI (Figures 3A, 4A). Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Spirochaetes, and Tenericutes were found to be dominant in the three study groups. However, Thermotogae was also found to be a dominant phylum in the MI of the three study groups (Figure 3B, 4B).

DI mucus
Phylum-level: FFC feeding caused an increase in abundance of Actinobacteria, Proteobacteria, and Bacteroidetes, but decreased the abundance of Tenericutes, Spirochaetes and Firmicutes compared to the control group. Proteobacteria were found to be more abundant than the rest (Figure 3A). OA feeding caused a slight decrease in the abundance of Actinobacteria, Proteobacteria, and Firmicutes but an increase in abundance of Tenericutes and Spirochaetes compared to the control group.

Family-level: The families Micromonosporaceae (Actinobacteria); Colwelliaceae, Comamonadaceae, Hyphomicrobiaceae, Methylobacteriaceae, Moraxellaceae, Phyllobacteriaceae, Propionibacteriaceae, Pseudomonadaceae, Rhizobiaceae, Nitrobacteraceae, Burkholderiaceae, Caulobacteraceae, and Vibrionaceae (Proteobacteria); Chitinophagaceae (Bacteriodetes); Ruminococcaceae and Bacillaceae (Firmicutes), Mycoplasmtaceae (Tenericutes) and lastly Spirochaetaceae (Spirochaetes) were found to be the dominant ones (Figure 3C). The family Mycoplasmataceae and the families belonging to Proteobacteria were found to be dominant than the rest in the three study groups. The abundance of Comamonadaceae, Hyphomicrobiaceae, Moraxellaceae, Phyllobacteriaceae, Nitrobacteraceae, Ruminococcaceae were found to be decreased, while that of Vibrionaceae increased in the antibiotic-fed fish compared to the control fish. The abundances of Mycoplasmataceae and Spirochaetaceae were found to be decreased in the F-fed group compared to the control.

MI mucus
Phylum-level: The abundances of all the dominant phyla except Tenericutes were increased in the F-fed group compared to the control (Figure 4A, B). Tenericutes were found to be abundant than the other phyla in the control and O-fed group (Figure 4A, B). The abundance of Thermotogae in OA-fed fish was lower and those of Actinobacteria, Bacteroidetes, Firmicutes and Spirochaetes were higher compared to the control fish. The changes in the abundance of most bacterial phyla in both DI and MI of the antibiotic-fed groups compared to the control group is shown in Table 1.

Family-level: Bacteria belonging to 19 families were present in the DI and 17 families were present in the MI (Figures 3C, 4C). The families Caulobacteraceae, Chitinophagaceae, Alcaligenaceae, Comamonadaceae, Colwelliaceae, Methylobacteriacae, Moraxellaceae, Propionibacteriaceae, and Oxalobacteraceae (Proteobacteria); Clostridiaceae, Bacillaceae and Lactobacillaceae (Firmicutes); Micromonosporaceae (Actinobacteria); Leptospiraceae, and Spirochaetaceae (Spirochaetes); Mycoplasmataceae (Tenericutes) and lastly Fervidobacteriaceae (Thermotogae) were found to be the dominant ones (Figure 4C). The abundance of Methylobacteriacae and Mycoplasmataceae decreased in the antibiotic-fed groups compared to the control group. In contrast, the abundance of Comamonadaceae, Spirochaetaceae, and Moraxellaceae increased in the antibiotic-fed groups compared to the control group. The changes in the abundance of most bacterial families in both DI and MI of the antibiotic-fed groups compared to the control group is shown in Table 1.

Core bacterial communities of the intestinal mucus microbiota

In the present study, the core microbiota was identified as the members of the bacterial communities that were shared among 99% of the samples. The common core taxa–at prevalence (relative population frequency) of 99% and compositional-abundance detection threshold of 20% –are shown in Figures 5 and 6. In the DI, only few dominant bacterial families, namely Nitrobacteraceae, Mycoplasmataceae, and Methylobacteriaceae were detected as the core members. Along with these dominant families, another family Fervidobacteriaceae was a member of the shared taxa in the DI (Figure 5). In the MI, the dominant bacterial families in the three study groups, namely Mycoplasmataceae, Comamonadaceae, Bacillaceae, Moraxellaceae, and Caulobacteraceae were among the core members. In addition, Sphingomonadaceae, Mycobacteriaceae and Pseudomonadaceae were also the core members of the MI in the three study groups (Figure 6).

The DPCoA indicated differences in the core members of the antibiotic-fed and the control group (DI mucus: F-statistic = 2.13 , R^2 = 0.15 , p = 0.10 ; MI mucus: F-statistic = 3.42, R^2 = 0.22, p = 0.04, Supplementary Figures 3A, 3B).

Significantly abundant and relevant taxa of the intestinal mucus microbiota

ANCOM analysis detected 7 significantly abundant OTUs (compared to those in the control fish) in the DI, which included Cytophageaceae, Burkholderiaceae, Rhizobiaceae, 2 OTUs belonging to Sphingomonadaceae and 2 OTU's belonging to Flavobacteriaceae. ANCOM analysis did not detect any OTU that had significantly different abundances in the MI of the 3 groups.

Boruta analysis detected 32 and 4 relevant OTUs in the DI and MI, respectively. The changes in abundance of most of the relevant OTUs in the DI are shown in Table 1.

Co-occurrence network description of OTUs

The DI mucus bacteria

The single-domain bacterial (SDB) network, associated with the DI of the three study groups, is comprised of many small components (Supplementary Figure 4A). The significantly abundant OTUs were labeled based on their membership in different modules (Figures 7A, B, and C). We observed that the number of dyads, triads, quadruplets, and quintuplets in bacterial

networks had no connection with the main connected network; their connection patterns were different for all the three study groups (Supplementary Figure 4A). The average node degrees were 1.49 (SD: 2.17), 1.56 (SD: 1.02), 1.36 (SD: 1.68) for the control, F- and O-fed fish, respectively. Similarly, the values for betweenness were 14.8 (SD: 46.1), 30.3 (SD: 69.4), 9.03 (SD: 25.5) for the control, F- and O-fed fish, respectively. The average path lengths of the SDB network for the three study groups (control, F-fed, and O-fed) were 3.14, 5.07, and 2.99. The average node degrees and betweenness of the three groups were found to be different (for node degree: p = 0.12 for F-fed vs. Control group and p = 0.06 for O- vs. F-fed group; for betweenness: p = 0.03 for F-fed vs. Control group and p = 0.03 for O- vs. F-fed group). The degree of assortativity (assortativity coefficient c_a) of the network associated with the three groups were -0.27, 0.07 and -0.23 respectively. The degree distribution of the microbial network (for all OTUs) of the three study groups shown in Supplementary Figure 5A revealed that there are two distinct groups of nodes in the control and O-fed groups, while there are four distinct groups of nodes in the F-fed group. The degree distribution also shows that there are some highly connected hub nodes for the bacterial network of the control and O-fed group and hubs of the control group have more node degrees.

The MI mucus bacteria

The SDB network derived from the MI of the antibiotic-treated groups was comprised of one giant connected component (Figures 8A, B and C). The SDB of the control group had small components. Similar to the observation of the DI bacterial network, the dyads, triads, quadruplets, and quintuplets in the MI bacterial networks of three groups were also different (Supplementary Figure 4B). The average node degrees were 1.85 (SD: 1.49), 2.76 (SD: 1.17), 3.03 (SD: 2.31) for the control, F- and O-fed fish, respectively. Similarly, the values for betweenness were 247 (SD: 520), 404 (SD: 375), 393 (SD: 599) for the control, F- and O-fed fish, respectively. The average path length of the SDB network for the three study groups (control, F-fed, and O-fed) was 8.86, 6.64, and 6.57, respectively. Dunn's test identified significant differences between the antibiotic-fed groups and control group (for average node degree: $p < 0.001$ for both control vs. F-fed group and control vs. O-fed group; for betweenness: $p < .001$ for both control vs. F-fed group and control vs. O-fed group, p = 0.02 for F-fed vs. O-fed group). The degree of assortativity (assortativity coefficient c_a) of the phylum-level network of the three groups were -0.17, 0.04 and -0.13 respectively. The histogram showing the node degree distribution of the microbial networks (for all OTUs) of the three study groups (Supplementary Figure 5B) revealed that there are two distinct groups of nodes in the control and O-fed group, while there are four distinct groups of nodes in the F-fed group. Visualization of this network also shows that hubs of the control and O-fed group have more node degrees.

The main results of this study are summarised in Figure 9.

Discussion and conclusion

Antibiotics are antimicrobial agents that are employed to treat infections. Intake of antibiotics can selectively deplete the gut microbial populations of the host, depending on their mode of action (45). Furthermore, they will affect not only the targeted microbes but also the host's entire microbial community (46). In the present study, we employed amplicon sequencing of

highly conserved 16S rRNA gene sequences to investigate changes in the intestinal microbiota of Atlantic salmon after feeding them with two antibiotics, namely florfenicol and oxolinic acid. We analysed and compared the bacterial population in the distal and mid intestine separately; to determine the spatial distribution of the microbial community associated with the mucus. We focused on the intestinal mucus of the host because it consists of a unique microbial niche with distinct communities that have a special functional role in the host-microbial relationship (47). The microbial niche of the intestinal mucus layer provides partial protection against several pathobionts and opportunistic microbes, the presence of which can cause mucosal infections (48).

Exposure of intestinal microbes to antibiotics can alter their diversity and composition. We have confirmed this fact–bacteria belonging to different families were altered in fish that received antibiotics. Furthermore, the co-occurrence networks indicated differential bacterial associations in the control and antibiotic-fed fish. In addition, we confirmed that the tank biofilm bacterial communities might not have influenced the intestinal bacterial profile, as reported earlier (43).

Antibiotic feeding lifted the richness and diversity of the intestinal microbes

The observed increase in the alpha diversity in the present research was not consistent with the significant decrease in the diversity reported by previous antibiotic studies with mice, humans, and fish (49-51). However, a recent study has indicated an increase in the bacterial diversity (richness and Shannon diversity index) in fecal samples of minks that were collected 2 days after oral administration of amoxicillin (52). According to the intermediate disturbance hypothesis theory, the bacterial population maximizes its diversity at intermediate rates of disturbance (53). Intermediate levels of antibiotics are associated with increase in the diversity of bacterial colony size phenotype (54). This disturbance - diversity relationship depends on the colonizing (favored by r-selection) and competitive (favored by k-selection) ability of the bacterial population under different rates/levels of disturbances. At intermediate levels of disturbance, coexistence of microorganisms that can thrive in different environments causes peaks in diversity resulting in a unimodal disturbance - diversity relationship (53). The medicated feeds had different levels of antibiotics, and we did not observe significant changes in diversity in O-fed fish (in MI) similar to that noted for F-fed fish. By linking this finding and the intermediate disturbance hypothesis, we suggest that an intermediate level of disturbance was induced in F-fed fish to cause the co-existence of both r- and k-strategists.

Antibiotic feeding altered the composition of the intestinal mucus microbial consortia

Administration of antimicrobial agents can dramatically disturb the ecological balance between the host and associated microorganisms. The mode of action of the bacteriostatic antibiotics is via suppressing bacterial growth mostly through inhibiting the process of protein synthesis and interfering with bacterial replication (25, 55). Moreover, broad-spectrum antimicrobials such as those employed in the present study are effective against a wide range of commensal and pathogenic bacteria. The mode of action of the antibiotic has a bearing on the extent to which the gut microbiota composition and functions are modulated in humans (55). Our findings demonstrate that consumption of antibiotics shifted the intestinal microbiota composition in

salmon. The composition and abundance of the dominant bacterial phyla viz. Proteobacteria, Actinobacteria, Firmicutes, Spirochaetes, Bacteroidetes, Tenericutes, and Thermotogae were altered in the distal and mid intestine of antibiotic-fed fish compared to control fish (Figures 3B, 4B and Table 1). The bacterial families that were influenced due to antibiotic feeding are briefly discussed in the following paragraphs.

Antibiotic-feeding caused a general increase in abundance of the phylum Proteobacteria in intestinal mucus (Table 1, Figure 3A, 4A). Furthermore, the significantly abundant families that were detected in the DI belong to Proteobacteria and Bacteroidetes. Similar to our observation, studies on F-fed channel catfish and mice have also reported an increase in abundance of Proteobacteria and Bacteroidetes (49, 51). However, the phylum Proteobacteria was low in abundance in the DI of O-fed group compared to control group (Table 1). Members of this phylum are abundant in many marine and freshwater fishes (56), and are also known to dominate the gut microbiota of Atlantic salmon (57-59). Previous studies have shown that Proteobacteria contributes to the digestive process in fish (60). Members of this phylum are also involved in the stress response regulatory system and in metabolic pathway modules that participate in carbon and nitrogen fixation (61). While most members of Proteobacteria had higher abundance in the antibiotic-fed groups, there were also members that had low abundance in the antibiotic-fed fish (Table 1). Burkholderiaceae, Rhizobiaceae, Sphingomonadaceae were significantly abundant in the DI of F-fed fish. Although Vibrionaceae was not a significantly abundant OTU, its abundance was higher in the O-fed fish. The members of the genus *Vibrio* are known as opportunistic pathogens in fish (62, 63), and these r-strategists were higher in the microbiota of black molly (*Poecilia sphenops*) exposed to streptomycin (64).

While two families within Actinobacteria had higher abundance only in the DI of the F-fed fish compared to the control fish, the abundances of two families within Firmicutes were mostly increased by antibiotic-feeding. One of these families, Bacillaceae was detected as a member of the core microbiota in the MI (Figure 6). Members belonging to Bacillaceae includes both free-living and pathogenic species (65) for example, *Bacillus mycoides* are considered pathogenic to some fish species (66).

Two members of Spirochaetes –Spirochaetaceae and Leptospiraceae– displayed antibiotic-specific alterations (Figure 3C, 4C). Within the family Leptospiraceae, *Leptospira* is known to cause Leptospirosis in humans and animals (67). The high prevalence of *Leptospira* in catfish and tilapia species has been previously reported (68). The finding on differential abundance patterns of families belonging to Spirochaetes merits further investigation. The functional importance and pathogenicity of these bacteria in salmon need to be elucidated.

It is interesting to find a general decrease in abundance of Mycoplasmataceae (Tenericutes), the dominant family in Atlantic salmon intestine (43, 59, 69, 70), in the antibiotic-fed groups compared to the control group. It should be noted that Mycoplasmataceae was also a core member of intestinal microbiota of salmon. Further, the phylum Thermotoage (family Fervidobacteriaceae) was found to be a dominant member only in the MI of the fish. We observed a general increase in the abundance of Fervidobacteriaceae in the antibiotic-fed groups. The presence of Thermotoage in the salmon intestine has been previously reported (58).

Antibiotics affected the intestinal mucus microbial association and stability

Inferring interactions among different microbes within a community is vital to understand how the microbes adapt, develop, and interact with the host (43). The diverse microbes residing in the intestine interact with other microbes to obtain nutrients required for their colonization and proliferation, and these interactions occur by developing complex ecological networks i.e. microbe-microbe associations. Such microbial networks help to establish intestinal microbial compositional stability (71). Exposure to antibiotics can collapse this stability and thereby disturb the interactions among microbial species. We inferred single-domain networks using the SPEIC-EASI framework.

The inferred SDB network of the DI and MI bacteria of F-fed fish had higher overall connectivity, betweenness and hubs with less node degree (Supplementary Figure 5A, 5B). On the other hand, inferred SDB network for the O-fed fish had lower overall connectivity, betweenness and hubs with more node degree. The higher selective linking and higher average node degree of the F-fed group indicate more interactions among the gut bacteria. Cooperative microbial communities are functionally dependent and therefore are responsible for providing stability to the microbial community. An increase in microbial diversity along with higher proportion of cooperative microbial interactions can disturb the stability (72). Higher cooperation among the microbes leads to over-representation of the most stable communities, which in turn can cause a runaway effect that can disintegrate the competing microbial population (73).

Presence of independent dyads and triads in the DI mucus bacterial networks of the antibiotic-fed fish suggests that antibiotic exposure reduces the association between members of the most abundant phylum, namely Proteobacteria and other intestinal microbes. In the DI of the antibiotic-fed fish, most of the labeled OTUs belonged to different modules. However, Rhizobiaceae was connected to other significantly different OTUs, and members of this family are known for their ability to establish a beneficial interaction with the host (plants) and participate in the process of biological nitrogen fixation (74). In the MI, the labeled OTUs in the F-fed group were connected to the main network. However, they belonged to different modules. In the O-fed group, the labeled OTUs were connected to the main network and belonged to different modules, except one OTU that belongs to the phylum Proteobacteria (family Halomonadaceae), which was in a dyad. This suggests that both FFC and OA can differentially affect the microbe-microbe interactions (Supplementary Figures 4A, B). Overall, these findings imply that the taxa belonging to the same module can be functionally dependent, but the alteration of their membership in the predicted network after antibiotic feeding has to be further investigated.

In conclusion, antibiotic exposure increased the bacterial diversity of the distal intestine and shifted the intestinal bacterial community composition in Atlantic salmon. Florfenicol feeding caused the intestinal microbial communities to be more diverse compared to the other study groups. Antibiotic-feeding altered the composition and abundance of the dominant bacterial phyla viz. Proteobacteria, Actinobacteria, Firmicutes, Spirochaetes, Bacteroidetes, Tenericutes, and Thermotogae. Certain families that were low in abundance in control fish became abundant in fish that consumed medicated feed. Furthermore, the two antibiotics even disturbed the core

microbiota of the fish. The co-occurrence networks of the intestinal bacteria indicated that the antibiotics affect the microbe-microbe interactions differentially. Though intriguing, the results improve our understanding of the structure, diversity and composition of the Atlantic salmon intestinal microbiota, following antibiotic intervention.

Author contributions

VK and JF conceived and supervised the study. VK and SG designed and conducted the feeding experiment. SG collected the samples, performed the laboratory work and the bioinformatics analysis. SG and VK analyzed and interpreted the experimental data. SG wrote the manuscript with the guidance of VK. All authors read, revised and approved the manuscript.

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Competing interest

The authors declare that they have no competing interests.

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683 Table 1. Overview of the change in abundance of the bacterial taxa in the intestine of Atlantic
684 salmon that were offered medicated feeds.

Sample type	**Distal intestine mucus**		**Mid intestine mucus**	
Groups / Taxa	F-fed group	O-fed group	F-fed group	O-fed group
Acidobacteria	↑	–	↑	↑
Actinobacteria	↑	–	↑	–
Aquificae	↑	–	↑	–
Armatimonadetes	↑	↑	↑	↑
Bacteroidetes	↑	↑	↑	↑
Chloroflexi	↑	↑	↑	↓
Deinococcus-Thermus	↑	↑	↓	↓
Fusobacteria	↑	↓	↑	–
Marinimicrobia	↑	–	↓	↓
Parcubacteria	↑	–	↑	↑
Firmicutes	↓	↓	↑	↑
Proteobacteria	↑	↓	↑	↑
Spirochaetes	↓	↑	↓	↑
Tenericutes	↓	↑	↓	↓
Thermodesulfobacteria	↑	↓	↑	↓
Thermotogae	↑	↑	↑	↓
Planctomycetes	–	–	↑	↑
Synergistetes	–	–	↑	–
Deferribacteres	–	–	–	↓
Verrucomicrobia	↑	–	↑	↓
Bacillaceae	↑	↓	↑	↑
Burkholderiaceae	↑	↑	↓	↑
Caulobacteraceae	↑	↓	↑	↓
Chitinophagaceae	↑	–	↑	–

Colwelliaceae	↑	↓	↑	↓
Hyphomicrobiaceae	↓	↓	↓	↑
Micromonosporaceae	↑	↓	↑	↓
Mycoplasmataceae	↓	↑	↓	↓
Nitrobacteraceae	↓	↓	↑	↑
Phyllobacteriaceae	↓	↓	↓	↑
Proponibacteriaceae	↑	↓	↑	↑
Pseudomonadaceae	↑	–	↑	↑
Rhizobiaceae	↑	↑	↓	↑
Ruminococcaceae	↓	↓	↑	↑
Vibrionaceae	↑	↑	↑	↑
Alcaligenaceae	↑	↓	↑	–
Clostridiaceae	–	↓	↑	↑
Comamonadaceae	↓	↓	↓	↑
Fervidobacteriaceae	↑	↑	↑	↓
Lactobacillaceae	↑	↑	↑	↑
Leptospiraceae	↑	↑	↑	↓
Methylobacteriaceae	↑	↓	↓	↓
Moraxellaceae	↓	↓	↑	↑
Oxalobacteraceae	↑	–	↑	↑
Flavobacteriaceae	↑	↑	↑	↑
Sphingomonadaceae	↑	↑	↑	↑
Sphingobacteriaceae	↑	↑	↑	↓
Cytophagaceae	↑	↑	↑	↓
Spirochaetaceae	↓	↑	↓	↑

685 Arrows indicate changes in abundance (blue arrow: increase, red arrow: decrease, black lines:
686 no change)
687

Figure legends

Figure 1: Diversity of the bacterial communities present in the distal intestine mucus of Atlantic salmon. Boxplots show the species richness (A), Shannon index (B), Simpson index (C) and Faith's phylogenetic diversity (D). Principal coordinate analysis plot (E) shows the differences in composition of the bacterial communities. Different letters indicate statistically significant differences ($P < 0.05$) between the fish groups. The codes for the mucus samples are as follows: control group, CDM; florfenicol-fed group, FDM; oxolinic acid-fed group, ODM.

Figure 2: Diversity of the bacterial communities present in the mid intestine mucus of Atlantic salmon. Boxplots show the species richness (A), Shannon index (B), Simpson index (C) and Faith's phylogenetic diversity (D). Principal coordinate analysis plot (E) shows the differences in composition of the bacterial communities. Different letters indicate statistically significant differences ($P < 0.05$) between the fish groups. The codes for the mucus samples are as follows: control group, CMM; florfenicol-fed group, FMM; oxolinic acid-fed group, OMM.

Figure 3: Barplots showing the relative abundance of all the bacterial phyla (A), dominant phyla (B), and dominant family (C) in the distal intestine mucus of Atlantic salmon from the three fish groups. The height of each bar segment represents the abundance of individual operational taxonomic units (OTUs) stacked in order from greatest to least and separated by a thin black borderline. Color codes for Proteobacteria – shades of green, Spirochaetes – dark blue, Firmicutes – yellow, Actinobacteria – orchid, and Tenericutes – dark orange.

Figure 4: Barplots showing the relative abundance of all the bacterial phyla (A), dominant phyla (B), and dominant family (C) in the mid intestine mucus of Atlantic salmon from the three fish groups. The height of each bar segment represents the abundance of individual operational taxonomic units (OTUs) stacked in order from greatest to least, and separated by a thin black borderline. Color codes for Proteobacteria – shades of green, Spirochaetes – dark blue, Firmicutes – yellow, Actinobacteria – orchid, and Tenericutes – dark orange.

Figure 5: Relative abundance of the core bacterial family in the distal intestine mucus of Atlantic salmon from the three fish groups. Color codes: Shades of green – families of Proteobacteria and dark orange –families of Tenericutes.

Figure 6: Relative abundance of the core bacterial family in the mid intestine mucus of the three fish groups. Color codes: Shades of green – families of Proteobacteria, yellow – families of Firmicutes and dark orange – families of Tenericutes.

Figure 7: Network graphs showing the significantly abundant OTUs of Atlantic salmon distal intestine mucus in different modules of the network. Bacterial networks of the Control (A), Florfenicol-fed (B), and Oxolinic acid-fed (C) fish. Nodes represent OTUs and specific colors of the modules reveal the membership of the significantly abundant OTUs.

Figure 8: Network graphs showing the significantly abundant OTUs of Atlantic salmon mid intestine mucus in different modules of the network. Bacterial networks of the Control (A), Florfenicol-fed (B), and Oxolinic acid-fed (C) fish. Nodes represent OTUs and specific colors of the modules reveal the membership of the significantly abundant OTUs.

Figure 9: Summary of the main findings in the study. DI, distal intestine; MI, mid intestine; C, control group; F-fed and O-fed, antibiotic-fed groups.

Supplementary Figure legends

Supplementary Figure 1: Sample-size-based rarefaction curves for the reads obtained from Atlantic salmon intestinal mucus - distal (A), mid (B), and tank biofilm (C) samples. The shaded portion around each line represents the 95% confidence interval. Color codes for the distal intestine samples: green lines- control group, red lines- florfenicol-fed group, blue lines- oxolinic acid-fed group. Color codes for the mid intestine samples: grey lines- control group, orange lines- florfenicol-fed group, yellow lines- oxolinic acid-fed group. Color codes for the tank biofilm samples: orange lines- control group, green lines- florfenicol-fed group, purple lines- oxolinic acid-fed group.

Supplementary Figure 2: Double principal coordinate analysis plots showing the beta diversity of the bacterial communities of Atlantic salmon intestine and biofilm. Tank biofilm bacteria (A), control group distal intestine and tank biofilm bacteria (B): F-statistic = 4.035, R^2 = 0.211, P = 0.01; F group distal intestine and tank biofilm bacteria (C): F-statistic = 2.375, R^2 = 0.136, P = 0.07; O group distal intestine and tank biofilm bacteria (D): F-statistic = 5.006, R^2 = 0.250, P = 0.002; control group mid intestine and tank biofilm bacteria (E): F-statistic = 16.291, R^2 = 0.520, P = 0.003; F group mid intestine and tank biofilm bacteria (F): F-statistic = 2.934, R^2 = 0.163, P = 0.051; O group mid intestine and tank biofilm bacteria (G): F-statistic = 3.910, R^2 = 0.206, P = 0.03.

Supplementary Figure 3: Double principal coordinate analysis plots showing the differences in the composition of the core bacterial communities in the intestine mucus of Atlantic salmon. Distal intestine (A): F-statistic = 2.061, R^2 = 0.120, P = 0.082 and mid intestine (B): F-statistic = 2.061, R^2 = 0.120, P = 0.082 samples of the antibiotic-fed and control-fed groups.

Supplementary Figure 4: Single-domain network graph of the bacteria in the intestine mucus of Atlantic salmon. Distal intestine (A) and mid intestine (B) of the three fish groups. Phyla in different nodes are color-coded. The three panels represent the three feed groups: Control (A), F-fed (B), O-fed (C).

Supplementary Figure 5: Histograms showing the degree distribution of the bacterial networks associated with the intestine of Atlantic salmon. Distal intestine (A) and mid intestine (B).

Figure 1.

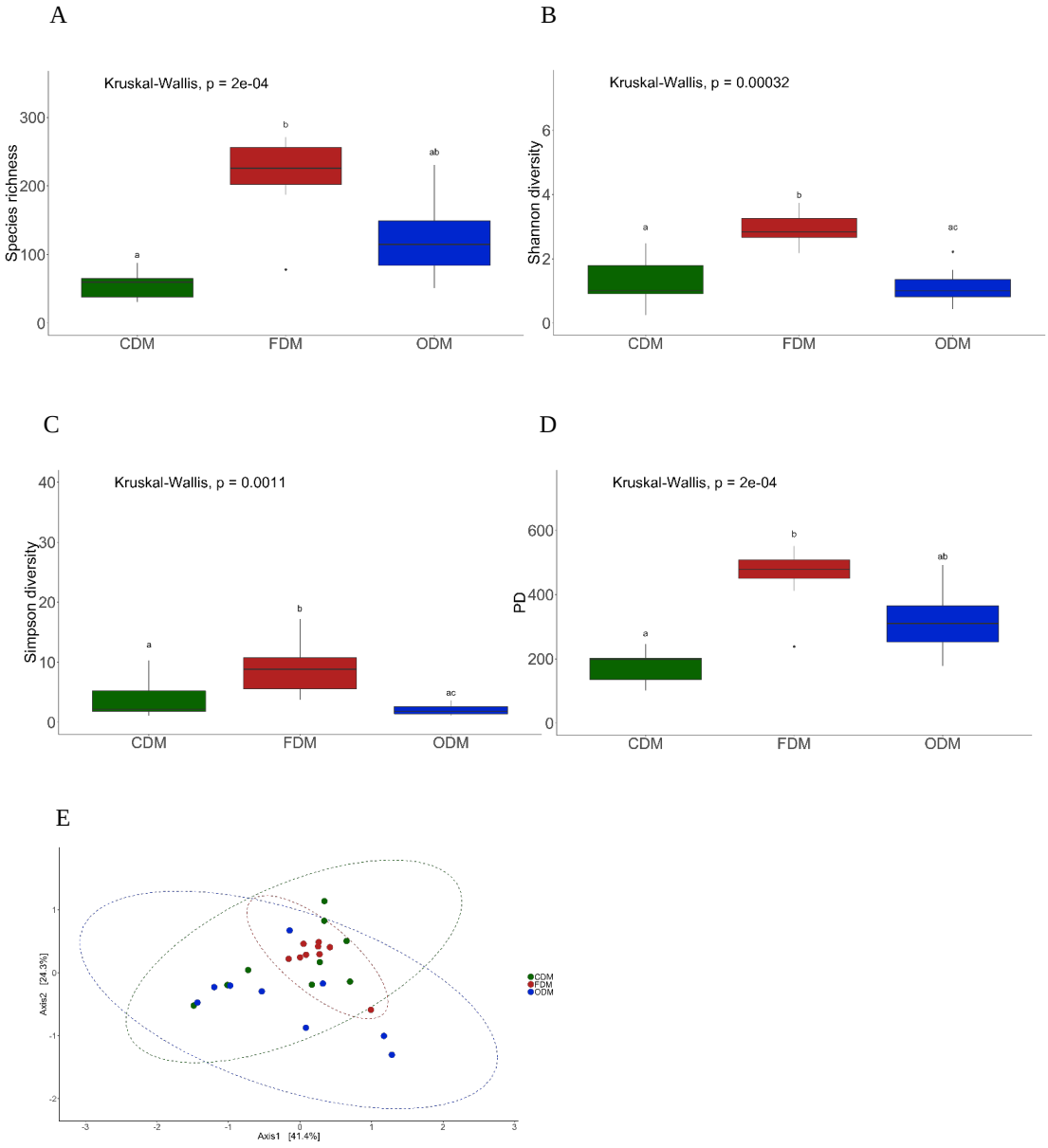
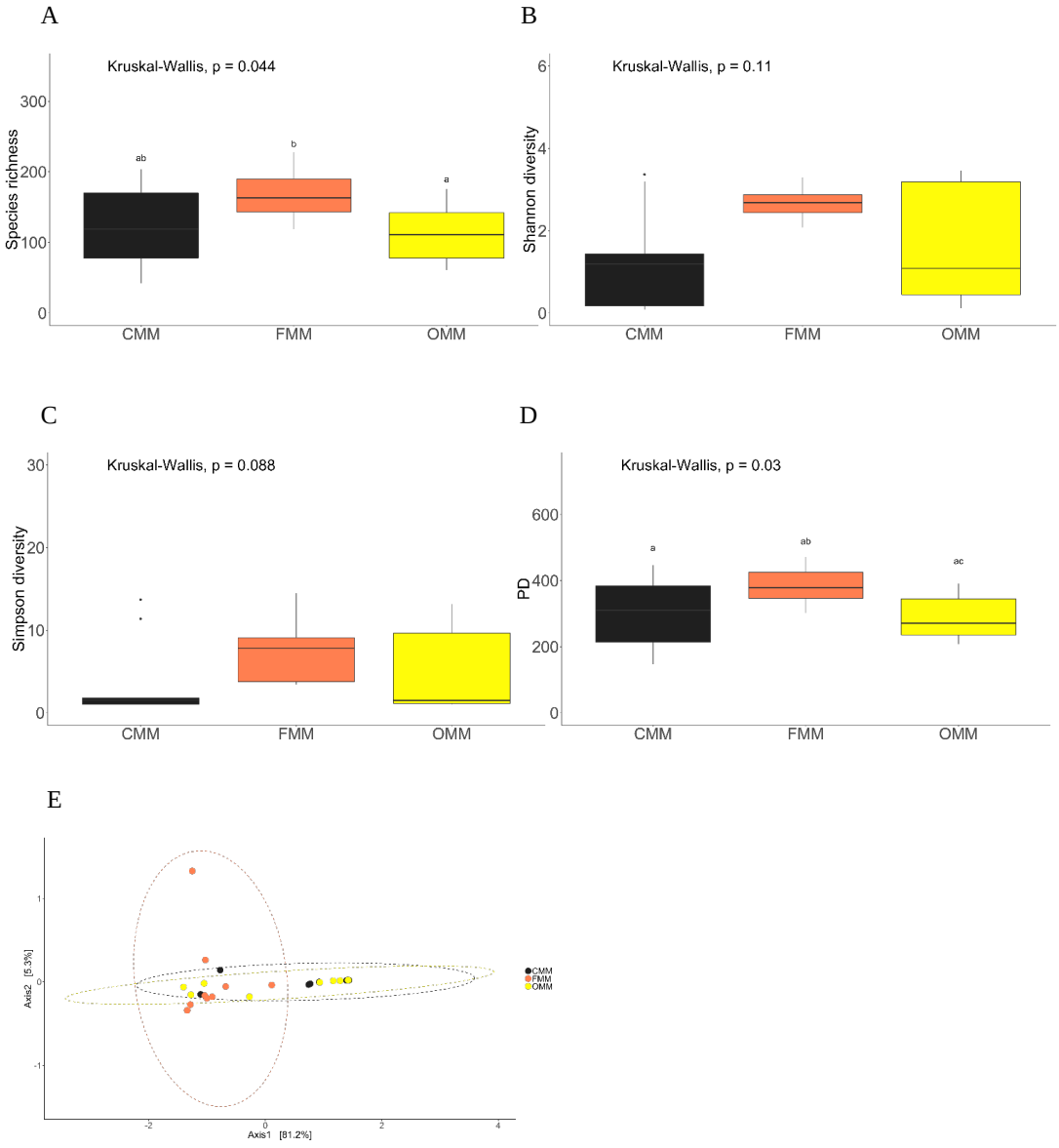

A
Kruskal-Wallis, p = 2e-04
Species richness
300
200
100
0
a
b
ab
CDM
FDM
ODM
B
Kruskal-Wallis, p = 0.00032
Shannon diversity
6
4
2
0
a
b
ac
CDM
FDM
ODM
C
Kruskal-Wallis, p = 0.0011
Simpson diversity
40
30
20
10
0
a
b
ac
CDM
FDM
ODM
D
Kruskal-Wallis, p = 2e-04
PD
600
400
200
0
a
b
ab
CDM
FDM
ODM
E
Axis2 [24.3%]
Axis1 [41.4%]
CDM
FDM
ODM

Figure 2.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Species richness
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ab
b
a
CMM
FMM
OMM
B
Kruskal-Wallis, p = 0.11
Shannon diversity
6
4
2
0
CMM
FMM
OMM
C
Kruskal-Wallis, p = 0.088
Simpson diversity
30
20
10
0
CMM
FMM
OMM
D
Kruskal-Wallis, p = 0.03
PD
600
400
200
0
a
ab
ac
CMM
FMM
OMM
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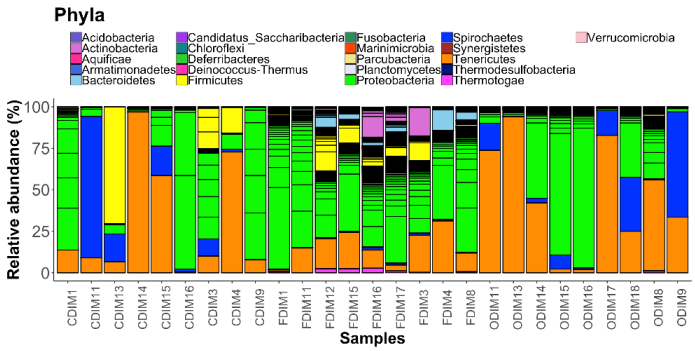
Axis2 [5.3%]
Axis1 [81.2%]
CMM
FMM
OMM

Figure 3.

A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Acidobacteria
Actinobacteria
Aquificae
Armatimonadetes
Bacteroidetes
Candidatus_Saccharibacteria
Chloroflexi
Deferribacteres
Deinococcus-Thermus
Firmicutes
Fusobacteria
Marinimicrobia
Parcubacteria
Planctomycetes
Proteobacteria
Spirochaetes
Synergistetes
Tenericutes
Thermodesulfobacteria
Thermotogae
Verrucomicrobia
Relative abundance (%)
100
75
50
25
0
CDIM1
CDIM11
CDIM13
CDIM14
CDIM15
CDIM16
CDIM3
CDIM4
CDIM9
FDIM1
FDIM11
FDIM12
FDIM15
FDIM16
FDIM17
FDIM3
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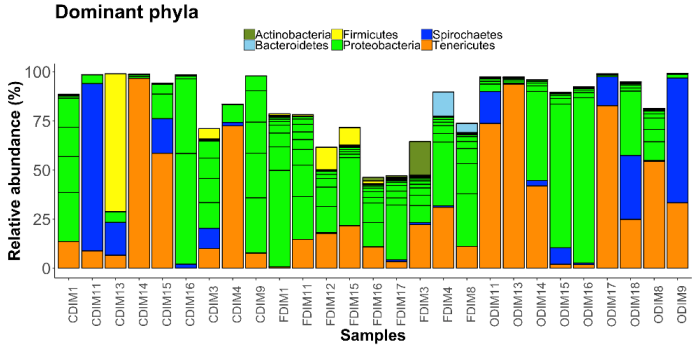
ODIM17
ODIM18
ODIM8
ODIM9
Samples

B


Dominant phyla
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Spirochaetes
Tenericutes
Relative abundance (%)
100
75
50
25
0
CDIM1
CDIM11
CDIM13
CDIM14
CDIM15
CDIM16
CDIM3
CDIM4
CDIM9
FDIM1
FDIM11
FDIM12
FDIM15
FDIM16
FDIM17
FDIM3
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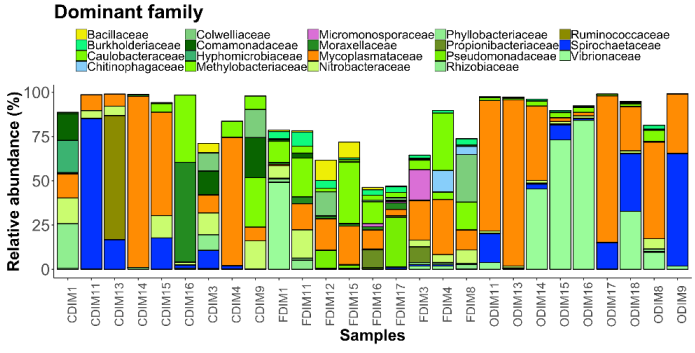
ODIM17
ODIM18
ODIM8
ODIM9
Samples

C


Dominant family
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Burkholderiaceae
Caulobacteraceae
Chitinophagaceae
Colwelliaceae
Comamonadaceae
Hyphomicrobiaceae
Methylobacteriaceae
Micromonosporaceae
Moraxellaceae
Mycoplasmataceae
Nitrobacteraceae
Phyllobacteriaceae
Propionibacteriaceae
Pseudomonadaceae
Rhizobiaceae
Ruminococcaceae
Spirochaetaceae
Vibrionaceae
Relative abundance (%)
100
75
50
25
0
CDIM1
CDIM11
CDIM13
CDIM14
CDIM15
CDIM16
CDIM3
CDIM4
CDIM9
FDIM1
FDIM11
FDIM12
FDIM15
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FDIM17
FDIM3
FDIM4
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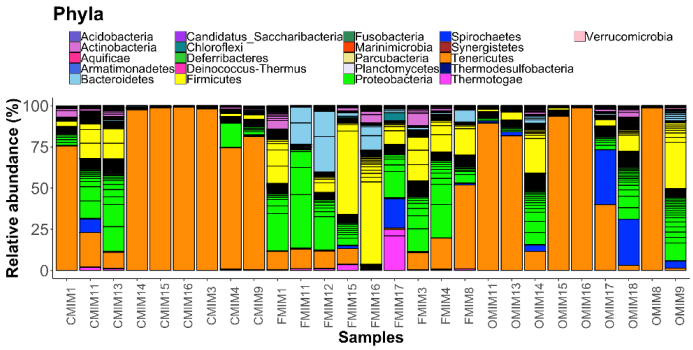
ODIM9
Samples

Figure 4.

A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Aquificae
Armatimonadetes
Bacteroidetes
Candidatus_Saccharibacteria
Chloroflexi
Deferribacteres
Deinococcus-Thermus
Firmicutes
Fusobacteria
Marinimicrobia
Parcubacteria
Planctomycetes
Proteobacteria
Spirochaetes
Synergistetes
Tenericutes
Thermodesulfobacteria
Thermotogae
Verrucomicrobia
Relative abundance (%)
100
75
50
25
0
CMIM1
CMIM11
CMIM13
CMIM14
CMIM15
CMIM16
CMIM3
CMIM4
CMIM9
FMIM1
FMIM11
FMIM12
FMIM15
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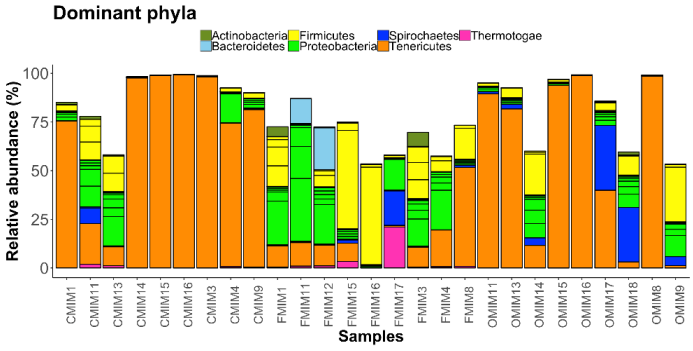
Samples

B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Bacteroidetes
Firmicutes
Proteobacteria
Spirochaetes
Tenericutes
Thermotogae
Relative abundance (%)
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75
50
25
0
CMIM1
CMIM11
CMIM13
CMIM14
CMIM15
CMIM16
CMIM3
CMIM4
CMIM9
FMIM1
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FMIM3
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OMIM8
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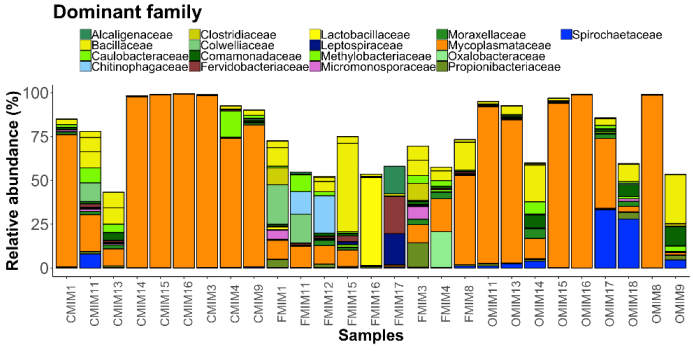
Samples

C


Dominant family
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Caulobacteraceae
Chitinophagaceae
Clostridiaceae
Colwelliaceae
Comamonadaceae
Fervidobacteriaceae
Lactobacillaceae
Leptospiraceae
Methylobacteriaceae
Micromonosporaceae
Moraxellaceae
Mycoplasmataceae
Oxalobacteraceae
Propionibacteriaceae
Spirochaetaceae
Relative abundance (%)
100
75
50
25
0
CMIM1
CMIM11
CMIM13
CMIM14
CMIM15
CMIM16
CMIM3
CMIM4
CMIM9
FMIM1
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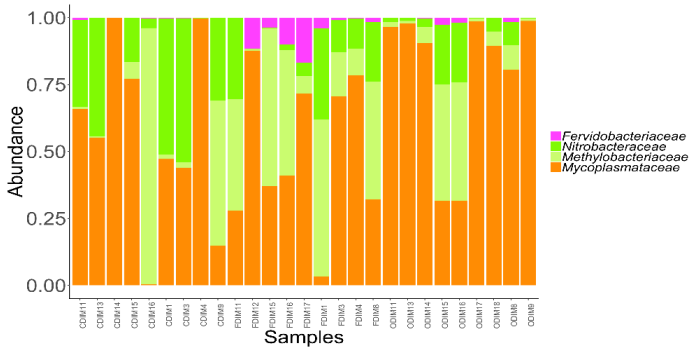
Samples

Figure 5.



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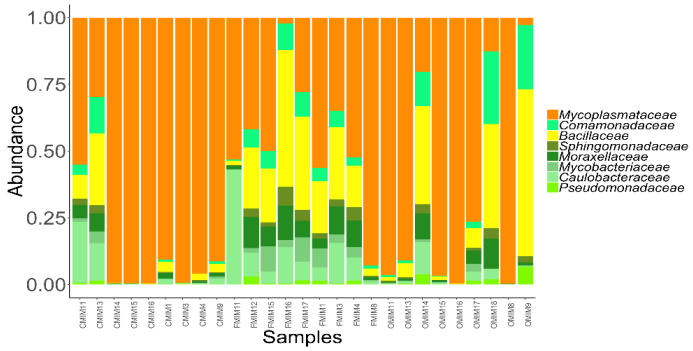

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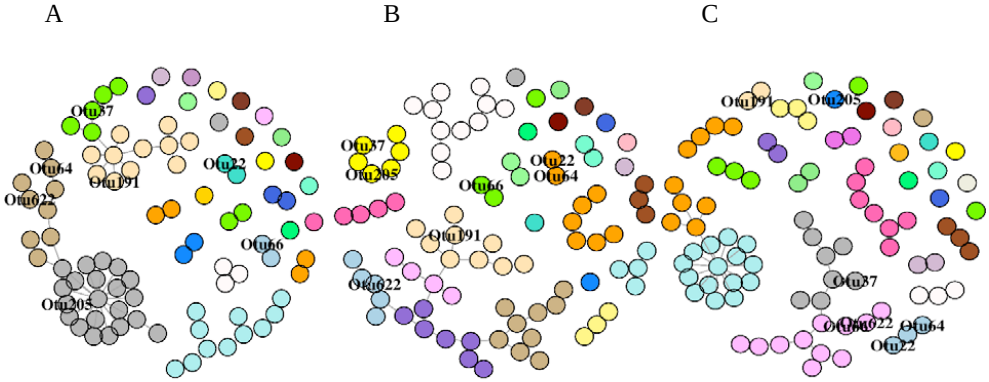

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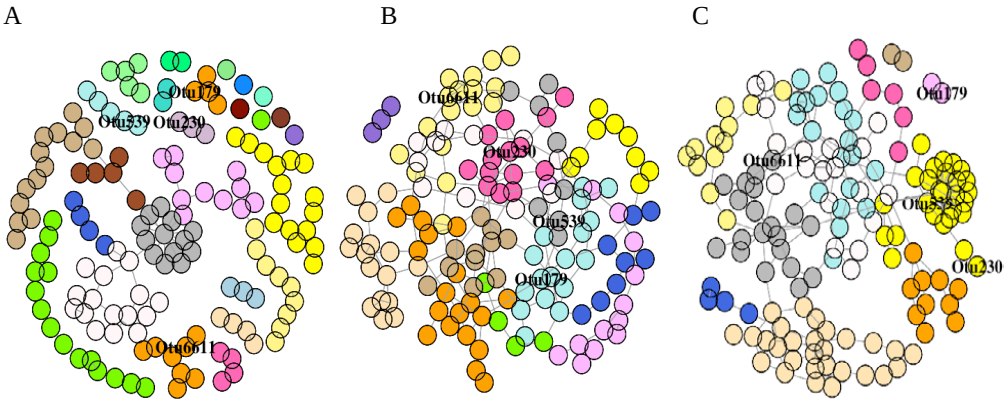

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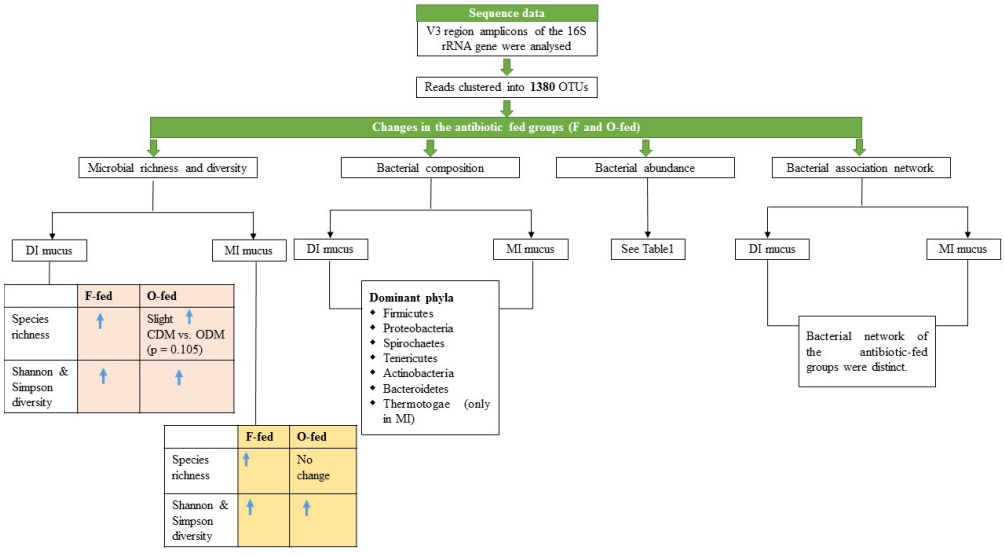

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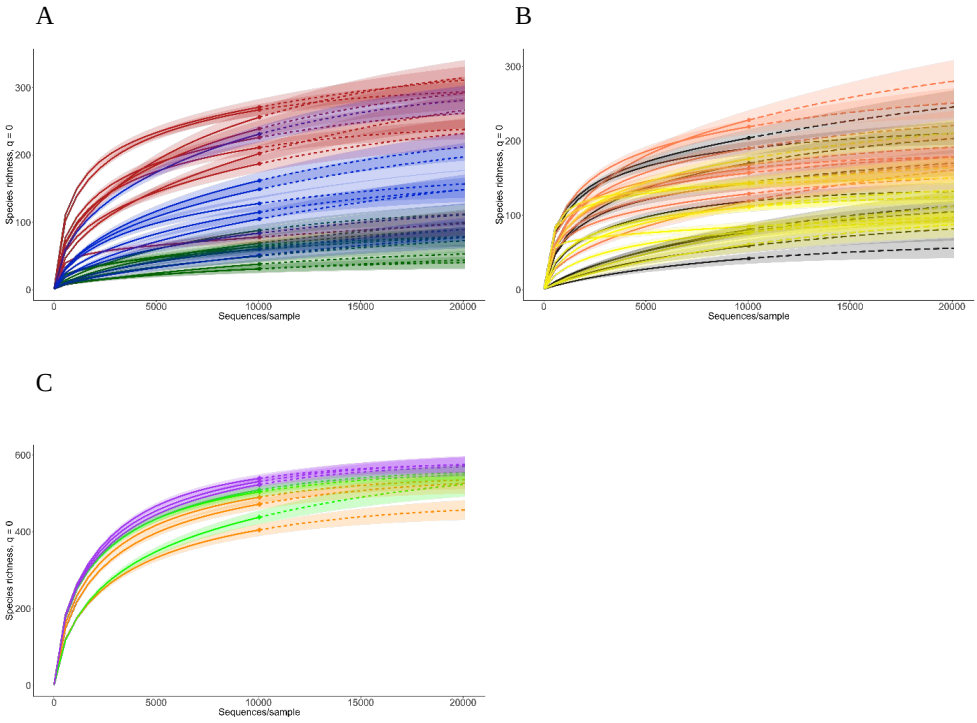

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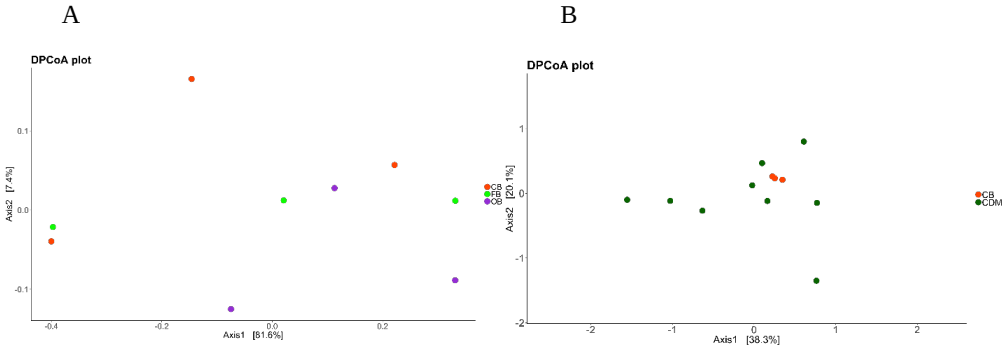

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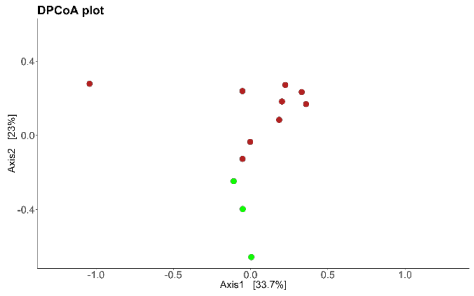
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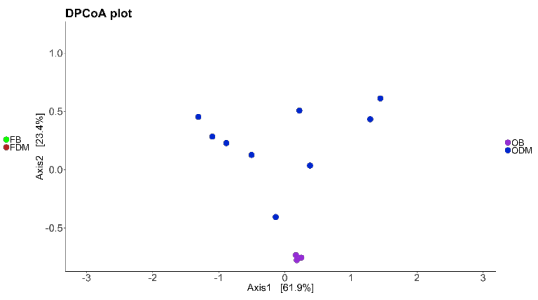
FB
FDM

D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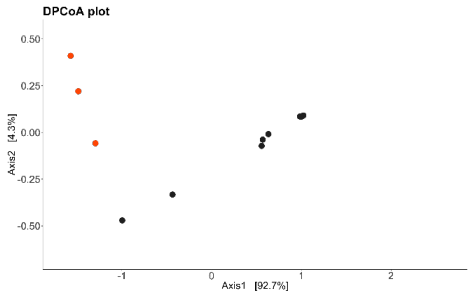
ODM

E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CR
CMM

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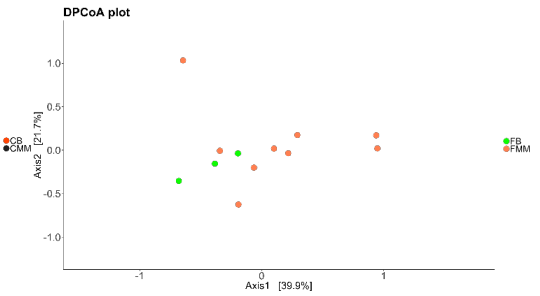
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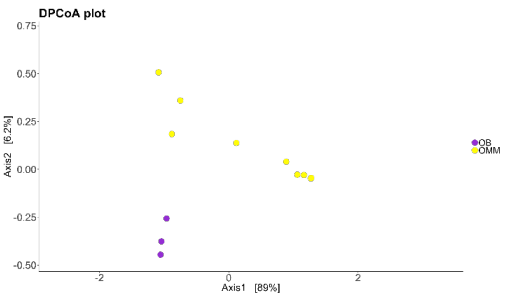
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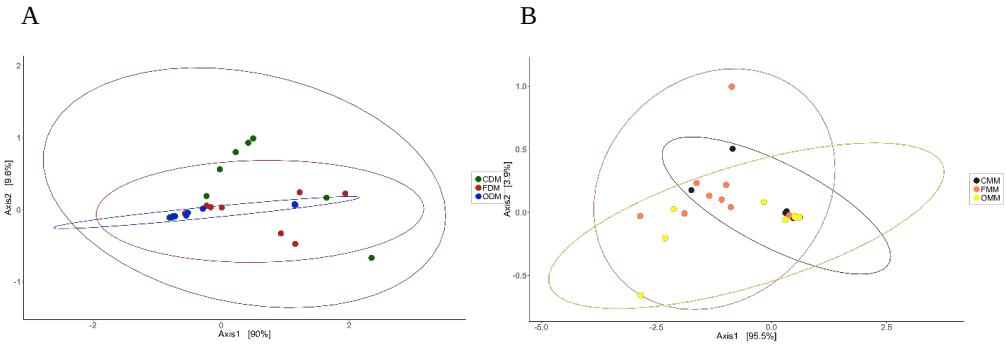
Supplementary Figure 3.



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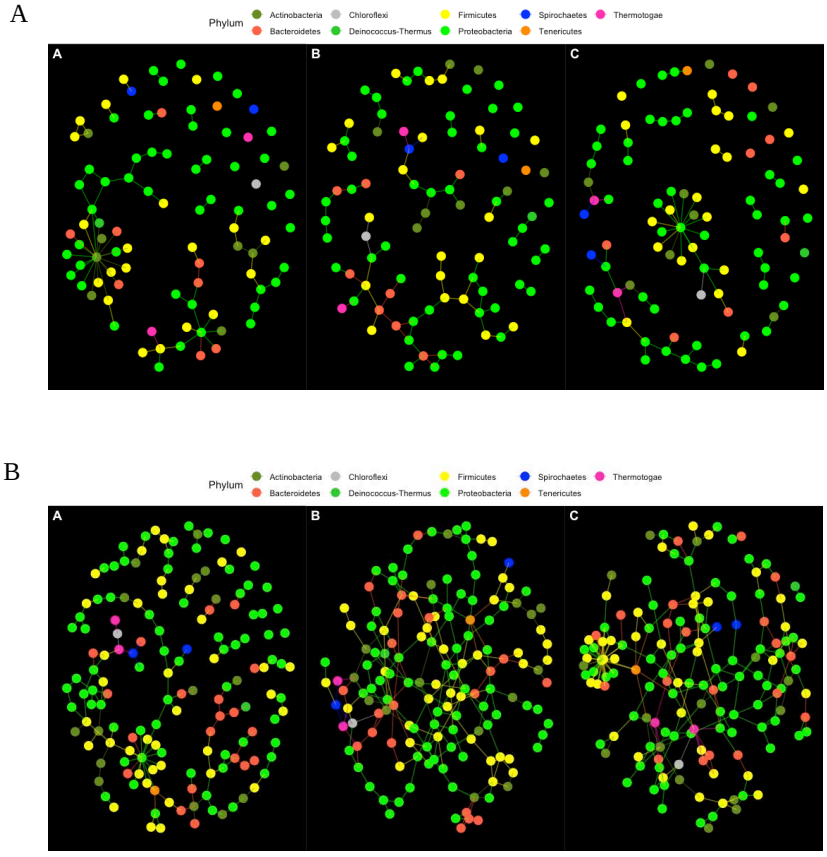

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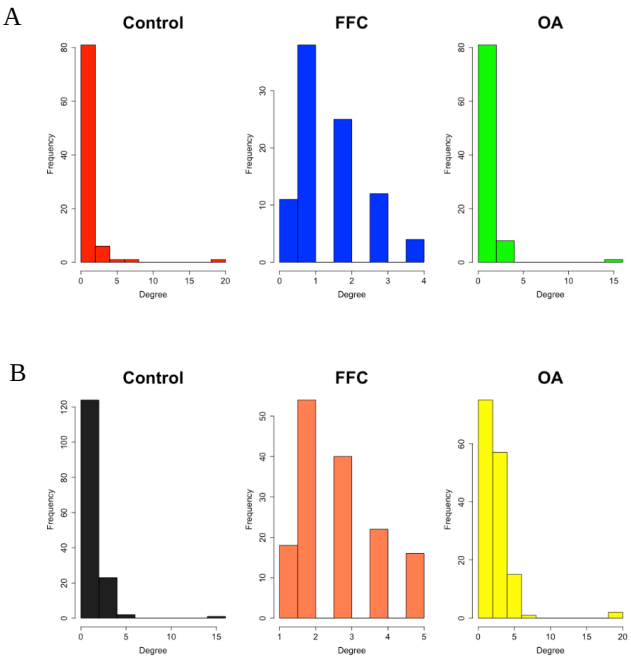

Paper II

frontiers in Microbiology

ORIGINAL RESEARCH
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Lactobacillus Dominate in the Intestine of Atlantic Salmon Fed Dietary Probiotics

*Shruti Gupta[1], Adriána Fečkaninová[2], Jep Lokesh[1], Jana Koščová[3], Mette Sørensen[1], Jorge Fernandes[1] and Viswanath Kiron[1]**

[1] Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway, [2] Department of Food Hygiene and Technology, University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia, [3] Department of Microbiology and Immunology, University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia

Probiotics, the live microbial strains incorporated as dietary supplements, are known to provide health benefits to the host. These live microbes manipulate the gut microbial community by suppressing the growth of certain intestinal microbes while enhancing the establishment of some others. Lactic acid bacteria (LAB) have been widely studied as probiotics; in this study we have elucidated the effects of two fish-derived LAB types (RII and RIII) on the distal intestinal microbial communities of Atlantic salmon (*Salmo salar*). We employed high-throughput 16S rRNA gene amplicon sequencing to investigate the bacterial communities in the distal intestinal content and mucus of Atlantic salmon fed diets coated with the LABs or that did not have microbes included in it. Our results show that the supplementation of the microbes shifts the intestinal microbial profile differentially. LAB supplementation did not cause any significant alterations in the alpha diversity of the intestinal content bacteria but RIII feeding increased the bacterial diversity in the intestinal mucus of the fish. Beta diversity analysis revealed significant differences between the bacterial compositions of the control and LAB-fed groups. *Lactobacillus* was the dominant genus in LAB-fed fish. A few members of the phyla Tenericutes, Proteobacteria, Actinobacteria, and Spirochaetes were also found to be abundant in the LAB-fed groups. Furthermore, the bacterial association network analysis showed that the co-occurrence pattern of bacteria of the three study groups were different. Dietary probiotics can modulate the composition and interaction of the intestinal microbiota of Atlantic salmon.

Keywords: fish, *Salmo salar*, feed additive, probiotics, intestinal bacteria, *Lactobacillus*, microbiota, amplicon sequencing

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INTRODUCTION

The ecological community of microorganisms that reside (Marchesi and Ravel, 2015) in the gastrointestinal tract (GIT) of an organism is referred to as the gut microbiota (Lozupone et al., 2012). The GIT of a healthy human harbors a dense (Kelsen and Wu, 2012; Marchesi et al., 2016) and diverse population (Lozupone et al., 2012) of commensal microorganisms, which offer many benefits to the host, including immune homeostasis and health maintenance (Sommer and Bäckhed, 2013). These commensal gut bacteria are also known to aid in amino-acid production

(Lin et al., 2017), nutrient metabolism and absorption (Morowitz et al., 2011; Semova et al., 2012), vitamin and bioactive metabolite' synthesis (Cummings and Macfarlane, 1997; LeBlanc et al., 2013), and pathogen displacement (Kamada et al., 2013). An imbalance in the gastrointestinal microbial composition can lead to immune-mediated diseases (Petersen and Round, 2014). A healthy gut bacterial assembly is essential for the well-being of the host organisms including fish, the microbiome of which is shaped by environment- and host-related factors (Wong and Rawls, 2012; Eichmiller et al., 2016; Lokesh et al., 2018).

Probiotics are "living bacteria," and when they are administered as supplements in the right amount they can confer health benefits to humans (FAO and WHO, 2006), by targeting, among others intestinal health through stimulation of intestinal epithelial cell proliferation and differentiation, fortification of intestinal barrier and immunomodulation (Gareau et al., 2010; Thomas and Versalovic, 2010; Hemarajata and Versalovic, 2013). Probiotics also have both direct and indirect effects on the intestinal microbial composition and diversity, and global host metabolic functions (Scott et al., 2015). These live bacteria produce antimicrobial compounds that suppress the growth of other microorganisms and compete for their receptors and binding sites (Spinler et al., 2008; O'Shea et al., 2012); thus altering the gut microbiota (Collado et al., 2007). Members of the genera *Lactobacillus* and *Bifidobacterium* are the most commonly used probiotic organisms for humans (O'Toole and Cooney, 2008).

Lactic acid bacteria (LAB) maintain intestinal health by producing lactic acid that can be utilized by short-chain fatty acids (SCFAs)-producing microorganisms. SCFAs (particularly acetate, propionate and butyrate) contribute to host health maintenance; for example, butyrate is used as energy source by the intestinal epithelial cells and also have anti-inflammatory effects on the host cells (Louis et al., 2014). LAB that is generally found in the GIT of endothermic animals have been extensively investigated and their benefits have been reviewed by many researchers (Pavan et al., 2003; Masood et al., 2011; Yang et al., 2015; Karamese et al., 2016). The importance of fish gut-dwelling LAB in aquaculture has been described in other reviews (Ringø and Gatesoupe, 1998; Gatesoupe, 2008). *Lactobacillus* that colonize the intestinal regions of fish are able to evoke immune responses and impart protection against diseases (He et al., 2017).

Feeding diets supplemented with beneficial bacteria such as LAB is being considered as an alternative approach to control diseases in farmed fish (Martínez Cruz et al., 2012; Fečkaninová et al., 2017; Rodriguez-Nogales et al., 2017). Not many studies in fish have employed high-throughput sequencing techniques to understand the changes in bacterial communities following LAB feeding. In this study, we examined the ability of *Lactobacillus* to modulate the distal intestinal microbiota of Atlantic salmon, a farmed salmonid fish. In addition, we describe the differences in the topology of co-occurrence networks associated with the intestinal bacteria of Atlantic salmon offered feeds with and without *Lactobacillus*.

MATERIALS AND METHODS

Ethics Statements

This study was approved by the Norwegian Animal Research Authority, FDU (Forsøksdyrutvalget ID-7898). Fish handling and sampling procedures were in compliance with the description in LOVDATA. The rearing water was treated with UV rays to remove substances that could be harmful to the fish. Optimum values for water salinity, oxygen and nitrogen concentration were maintained in the rearing tanks. The temperature of the fish rearing hall was kept stable during the entire feeding experiment.

Test Probiotics, Feed Type, and Design

Two species of *Lactobacillus* (RII and RIII) that were previously isolated from the intestinal content of farmed healthy juveniles of rainbow trout (commercial fish farm–Rybárstvo PoŽehy s.r.o., Slovak Republic) were employed in this study. Antimicrobial susceptibility of the microorganisms was assessed based on the "Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance" provided by the European Food Safety Authority. Sensitivity or intrinsic resistance of the isolated organisms to a recommended set of antibiotics make them safe for use as probiotics in aquaculture. Both RII and RIII showed antagonistic activity against salmonid pathogens *Aeromonas salmonicida* subsp. *salmonicida* CCM 1307 and *Yersinia ruckeri* CCM 6093 (Fečkaninová, 2017). Furthermore, high level of tolerance to different pH, bile, temperature, and high growth properties of the two species were confirmed through *in vitro* studies (Fečkaninová, 2017). The test probiotics were coated on commercial salmon feeds. Briefly, a pure culture of probiotic bacteria that were grown on de Man, Rogosa and Sharpe agar (MRS) plates (HiMedia, Mumbai, India) for 48 h were inoculated into 1,000 ml of MRS broth and incubated for 18 h at 37°C. The culture was centrifuged at 4,500 rpm for 20 min at 4°C in a cooling centrifuge (Universal 320 R, Hettich, Germany). The resulting cell pellets were washed twice and resuspended in 30 ml of 0.9% (w/v) sterile saline. The feed (batches of 1,800 g) was thoroughly coated with the bacterial suspensions (Spirit Supreme, Skretting AS, Norway) using a vacuum coater (Rotating Vacuum Coater F-6-RVC, Forberg International AS, Norway). The bacterial counts on feeds were $\sim 10^8$ cells.g^{-1} (RII/RIII), as determined by spread plating on MRS agar plates and incubating for 48 h at 37°C. The control feeds were coated with 0.9% of sterile saline alone. The coated feeds were stored at 4°C until they were offered to Atlantic salmon.

Experimental Fish, Feeding Regime, and Environmental Parameters

Atlantic salmon of average weight 522 ± 68 g were maintained in 800 L tanks in a flow-through seawater system, earlier described in Sørensen et al. (2017). A 20-day feeding trial was conducted at the research station, Nord University, Bodø, Norway. Three groups of fish ($n = 45$ fish/tank; 3 replicate tanks per group) were offered feeds with (RII $\sim 10^8$ cells.g^{-1}-RII; RIII $\sim 10^8$ cells.g^{-1}-RIII) or without probiotics (Control—C). The fish were fed *ad*

libitum; the feeds were dispensed two times a day, between 08.00–09.00 and 14.00–15.00, using automatic feeders (Arvo-Teck, Huutokoski, Finland). The water flow rate, temperature, salinity and O_2 levels in the tanks were 800 L/h, 6.7–7.1°C, 33 ppt, >85% saturation measured at the outlet, respectively. A photoperiod of 24:0 LD was maintained throughout the feeding trial.

Collection of the Intestinal, Tank Biofilm, and Rearing Water Samples

First, the fish were euthanized using 160 mg/L of MS222 tricaine methanesulfonate (Argent Chemical Laboratories, Redmond, WA, USA). Thereafter, the body surface of the fish was swiped with 70% ethanol. The fish were then dissected to aseptically remove the GIT from the abdominal cavity. The distal intestinal (DI) region was separated from the GIT and the content and surface mucus samples from the DI were collected ($n = 18$ for each group; 6 fish/tank) using sterile forceps and sterile glass slides, respectively. In addition to these fish samples, we collected environmental samples: water from the main inlet to the rearing hall (inlet water, $n = 1$), water from the rearing tanks ($n = 3$) and biofilm from the walls of the rearing tanks ($n = 3$). From the 3 tanks of each group, one liter of rearing water was filtered using 0.2 μm pore-size filters (Pall Corporation, Hampshire, United Kingdom) and the filter paper was stored at −80°C. The biofilm samples were scraped from the walls of the 3 tanks of each group. The fish and biofilm samples were collected in cryotubes, snap-frozen in liquid nitrogen and stored at −80°C.

The sample abbreviations reported in this article are: (i) fish samples–Control distal intestine content (CDC), RII distal intestine content (RIIDC), RIII distal intestine content (RIIIDC), Control distal intestine mucus (CDM), RII distal intestine mucus (RIIDM), RIII distal intestine mucus (RIIIDM); (ii) environmental samples– Control tank water (CW), RII tank water (RIIW), RIII tank water (RIIIW), inlet water (IW), Control tank biofilm (CB), RII tank biofilm (RIIB), RIII tank biofilm (RIIIB).

DNA Extraction and PCR Amplification of Bacterial 16S rRNA Gene for Illumina MiSeq Amplicon Sequencing

Genomic DNA was extracted from the content, mucus and biofilm samples using the Quick-DNA™ Fecal/Soil Microbe 96 kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol. Metagenomic DNA Isolation kit for water (Epicenter Biotechnologies, Madison, WI, USA) was employed to extract the genomic DNA from the water samples. The quality of the extracted DNA was checked on 1.2% (w/v) agarose gel. Qubit 3.0 fluorometer (Life Technologies, Carlsbad, USA) was employed to quantify the concentration of DNA.

To describe the changes in the intestinal bacteria under the influence of LAB, we amplified the V3–V4 region of the bacterial 16S rRNA gene employing a dual-index sequencing strategy described by Kozich et al. (2013). The PCR reactions were carried out in triplicates, each reaction (25 μl) volume contained 12.5 μl of Kapa HiFi Hot Start PCR Ready Mix (KAPA Biosystems, Woburn, USA), 1.5 μl of each forward and reverse primer (at a final concentration of 100 nM), 3.5 μl of DNAse and nuclease free water (Merck, Darmstadt, Germany) and 6 μl of DNA template and/ or 6 μl of negative PCR control. The thermocycling conditions included initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 98°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 45 s, and the final extension performed at 72°C for 2 min. After performing the PCR, the resulting amplicon triplicates were pooled and visualized on 1.2% (w/v) agarose gel stained with SYBR® Safe (Thermo Fisher Scientific, Rockford IL, USA), and the amplicon size was compared to a 1 kb DNA ladder (Thermo Fisher Scientific, Inc.). No amplification was observed in the negative PCR control. Only the amplicons (∼550 bp) with clear visible bands were selected, purified using the ZR-96 Zymoclean™ Gel DNA Recovery Kit (Zymo Research) and eluted in 15 μl of elution buffer. The eluted amplicon library (sequencing library) was quantified by qPCR using the KAPA Library Quantification Kit (KAPA Biosystems). After quantification, each amplicon library was normalized to an equimolar concentration (3 nM) and validated on the TapeStation (Agilent Biosystems, Santa Clara, USA), prior to sequencing. The normalized library pool was further diluted to 12 pM, spiked with equimolar 10% Phix control and then paired-end sequencing was performed using the 600 cycle v3 sequencing kit on the Illumina MiSeq Desktop sequencer (Illumina, San Diego, CA, United States) in 2 runs with inter-run calibrators to reduce eventual differences between sequencing runs.

16S rRNA Gene Sequence Data Processing

Sequence data quality check, processing and analyses: The sequence quality of the raw reads generated from the Illumina MiSeq machine was checked using FastQC (Andrews, 2010). The forward reads (R1) corresponding to V3 region were employed for subsequent analyses because they were of better quality than the reverse reads (R2) corresponding to V4 region [Phred quality score (Q) ≤ 15]. Sequence processing was performed using the UPARSE (USEARCH version 9.2.64) software by Edgar (2013); this step included quality filtering and operational taxonomic units OTU clustering. FastQ files were used as the input file for the UPARSE pipeline. The raw reads were truncated to 240 bp and quality-filtered. The reads were truncated to remove the low-quality base pairs at the 3′-end and to make all samples of same sequence length. Furthermore, chimeric sequences were removed using the UCHIME algorithm (Edgar et al., 2011). The quality-filtered sequences were clustered into OTUs at 97% sequence similarity level. For taxonomy prediction, we employed the 16S rRNA Ribosomal Database Project (RDP) training set with species names v16. This RDP training set was used as a reference database because the large 16S databases like SILVA, Greengenes, or the full RDP database may give unreliable annotations of short 16S rRNA tags (Edgar, 2018). Taxonomic ranks were assigned to the OTUs using the SINTAX algorithm (Edgar, 2016) using a bootstrap cutoff value of 0.5. Afterwards, OTUs with a confidence score <1 at the domain level and the OTUs belonging to the phyla Cyanobacteria and Chlorophyta were removed to exclude the plant-related sequences from the microbiota analysis. After

constructing the OTU table, the counts were rarefied to the lowest number of sequences per sample to get an even sampling depth to facilitate comparisons between the treatment groups. The OTU count data was divided into 4 sets based on the sample type, namely the DI content, DI mucus, tank water and tank biofilm samples. The downstream analyses were performed separately on these 4 sets. Furthermore, to ensure that we employ content and mucus data from the same fish, only 14 fish from each group were considered for the downstream analyses. In total 103 samples were used for the downstream analyses, including the tank water and biofilm samples. The raw 16S rRNA gene sequence data from this study has been deposited in the European Nucleotide Archive (ENA) under the accession number ERP110004.

Analyses of microbial diversity and composition: R codes were executed in RStudio v3.5.0 (RStudio Team, 2016) and the functions of the R packages "iNEXT" v2.0.12 (Hsieh et al., 2016), "phyloseq" v1.22.3 (McMurdie and Holmes, 2013) and "ggplot2" v2.2.1 (Wickham, 2016) were used to make the rarefaction curves for the species richness, to calculate and visualize diversity indices, and to prepare the abundance plots. Another R package called "microbiome" v1.0.2 (Lahti et al., 2017) was used to make core and rare microbiota (relative abundance of core taxa) plots. Alpha diversities were calculated based on the formula suggested by Jost (2006); for Shannon diversity (effective number of common OTUs) and Simpson diversity (effective number of most abundant OTUs). Beta diversity was examined by conducting weighted UniFrac distance metric (for fish samples)-based PCoA and double principal coordinates analysis (DPCoA, for water and biofilm samples) (Fukuyama et al., 2012).

The feeding design, sample processing and sequencing, and analyses are shown in **Figure 1**.

Statistical Analysis of the Bacterial 16S rRNA Gene Amplicon Data

Statistical analysis was also performed in RStudio v3.5.0. Kruskal-Wallis test followed by Dunn's test was employed to detect differences in alpha diversity, and we report statistically significant differences at $p < 0.05$ and statistical trends at $p \leq 0.15$. Betadisper was used to check the assumption of heterogeneity in dispersions; after that Adonis (PERMANOVA) followed by pairwise comparisons was employed (999 permutations) to understand the significant dissimilarities of the communities. "ANCOM" v1.1–3 (Mandal et al., 2015) was used to detect the differentially abundant OTUs in the treatment groups, and "Boruta" v5.3.0 R package (Kursa and Rudnicki, 2010) was employed to find the relevant OTUs that caused the differences in the intestinal bacteria of the three fish groups.

Microbial Network Construction and Comparison of Topology

We used "SPIEC-EASI" v0.1.4 R package (SParse InversE Covariance Estimation for Ecological Association Inference) for generating the single-domain bacterial network. SPIEC-EASI is a statistical method that assumes the underlying microbial interaction networks to be sparse (Kurtz et al., 2015). In this study, we employed the neighborhood selection (MB) method on the sequenced 16S rRNA gene (V3 region) data of both DI content and mucus samples to understand the community organization.

We explored the co-occurrence networks to uncover the probable biological interactions occurring within the microbial communities. We used the top 200 OTUs for network construction, since it is advised to avoid extremely rare OTUs or OTUs with a large number of zeros (Banerjee et al., 2018). The co-occurrence microbial networks were constructed and analyzed using the functions of the R package "igraph" v1.2.1 and customized ggplot2 commands. A network consists of a set of vertices (commonly called as nodes) and set of edges. The degree of a node is the number of connections it has with the other nodes in the network. Betweenness estimates the number of shortest paths that pass through the nodes in the network and assortativity coefficient quantifies the extent of the selectively connected labeled pair of nodes (Kolaczyk and Gábor, 2014). We compared the topology of the networks of the content and mucus samples separately by analyzing the node degrees and betweenness of the control and LAB-fed groups using Kruskal-Wallis test followed by Dunn's test.

RESULTS

We analyzed the V3 region amplicons of the 16S rRNA gene that was sequenced on our high-throughput sequencing platform. A total of 28,747,884 high-quality reads were clustered into 1,823 OTUs at 97% identity threshold. These reads were rarified based on sample-size to 12,855 reads/sample; this allowed us to assess most of the underlying microbial diversity (**Supplementary Figures 1A,B**).

The differences in the DI bacterial communities of the LAB-fed fish compared to the control fish are explained based on the following diversity metrics: overall microbial richness (i.e., counts of individual OTUs), effective number of OTUs (counts of common and dominant OTUs), taxonomic composition, relative abundances of the bacterial taxa. Furthermore, we present the significant and relevant bacterial communities of the DI microbiota. We also describe the topology of the networks of the bacterial communities in the 3 fish groups.

Differences in the Microbial Diversity and Composition of the Intestinal and Environmental Microbiota

LAB feeding did not affect the species richness of the bacterial community in the DI content (**Figure 2A**). However, this was not the case for bacteria in the DI mucus; the species richness was found to be higher in the mucus of the RIII-fed group ($p = 0.004$ for RII vs. RIII and $p = 0.071$ for RIII vs. C) (**Figure 3A**). We observed differences in the effective number of common and dominant OTUs in the mucus of LAB-fed groups, ($p = 0.109$ and $p = 0.146$ for RII vs. RIII; **Figures 2B**, **3B** and **Figures 2C**, **3C**). Comparison of the Faith's phylogenetic diversity (PD) of the DI content did not reveal any significant differences (**Figure 2D**).

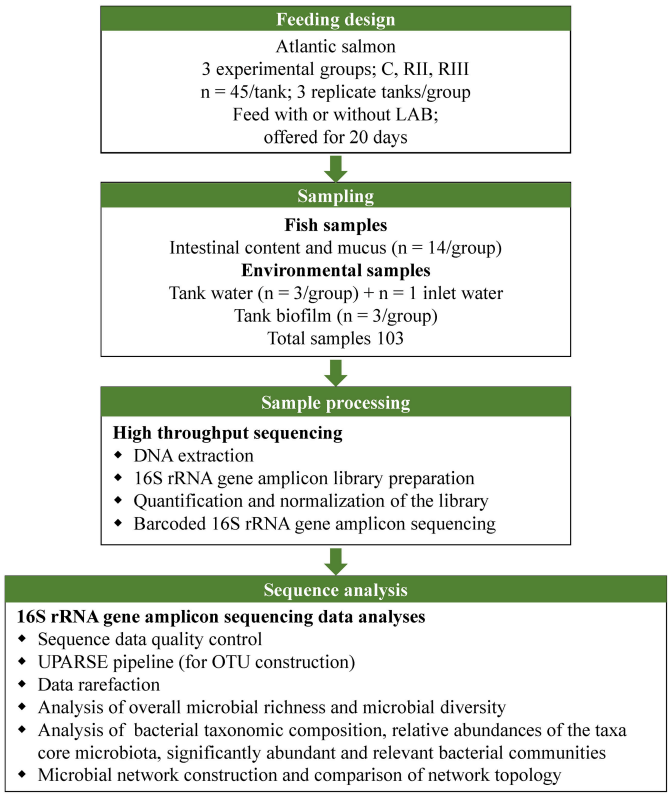

FIGURE 1 | Illustration of the study design, sampling and sequence analyses.

For the DI mucus, differences were observed between the PD associated with the three fish groups ($p = 0.004$ for RII vs. RIII and $p = 0.079$ for RIII vs. C; **Figure 3D**). It is noteworthy that the median alpha diversities of RII lies below the corresponding values of C although we did not detect a trend or statistically significant difference between the feed groups. PCoA based on the weighted UniFrac distance matrix revealed the beta diversity of the bacterial communities; the differences between the control and LAB-fed groups were statistically significant (**Figure 4A**: F statistic = 9.215, $R^2 = 0.320$, $p < 0.001$; and **Figure 4B**: F statistic = 3.114, $R^2 = 0.137$, $p < 0.002$).

The beta diversity of the bacterial communities in the rearing tank water and biofilm samples were also analyzed. The bacterial communities in the water of the 3 study groups were not different (**Supplementary Figure 2A**, F-statistic = 0.753, $R^2 = 0.273$, $p = 0.684$), as was the case with the bacteria in the biofilm (**Supplementary Figure 3A**, F statistic = 0.681, $R^2 = 0.185$, $p = 0.574$). On the other hand, the bacterial communities in the water were significantly different from those of the fish (**Supplementary Figures 2B–G**). Although we did not observe any significant differences between the bacterial communities of the tank biofilm and the intestinal mucus bacteria of the LAB-fed fish (**Supplementary Figures 3B–D,F–G**), the biofilm and mucus bacteria of the control group were different (**Supplementary Figure 3E**, F statistic = 16.29, $R^2 = 0.520$, $p = 0.003$).

Intestinal Bacterial Composition Under the Influence of LAB

Bacteria belonging to 23 phyla were present in the DI content and mucus (**Figures 5A**, **6A**). Firmicutes, Proteobacteria, Spirochaetes, Tenericutes, and Actinobacteria were found to be dominant in the intestine of the three study groups (**Supplementary Figures 4A,C**). Firmicutes were found to be more abundant than the rest, in both the content and mucus of the LAB-fed fish (**Figures 5A,B** and **Figures 6A,B**). The abundance of the phylum Tenericutes (content and mucus) was higher in RII-fed fish, than in the RIII-fed fish group (**Figures 5A,B** and **Figures 6A,B**). Proteobacteria (content and

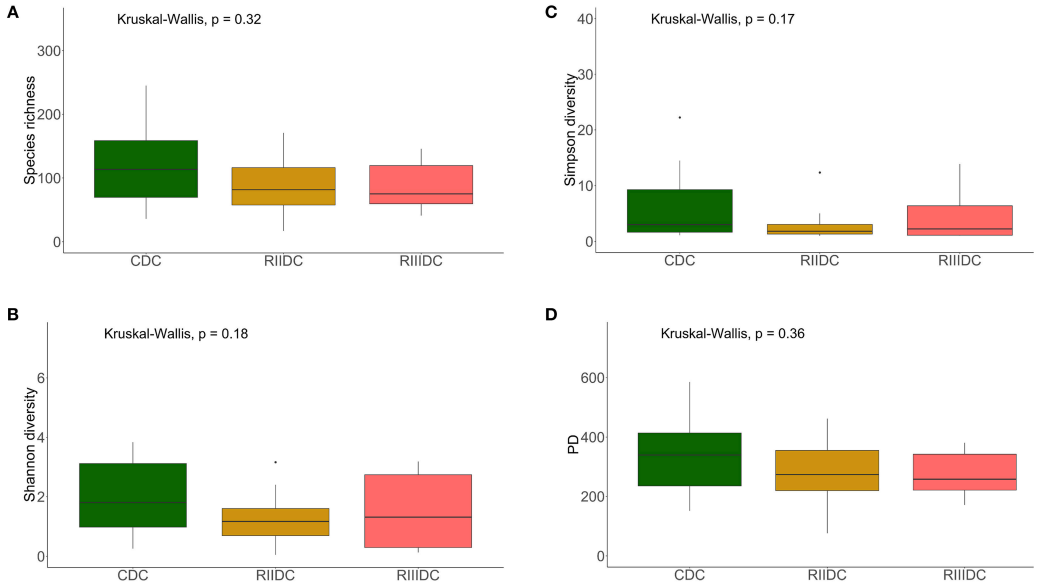

FIGURE 2 | Diversity of the bacterial communities of the intestinal content. Boxplots show the species richness **(A)**, Shannon index **(B)**, Simpson index **(C),** and Faith's phylogenetic diversity **(D)**. The feed group codes are as follows: Control, CDC; RII, RIIDC; RIII, RIIIDC.

mucus) decreased in the LAB-fed groups compared to the control group (**Figures 5A,B**; **Figures 6A,B** and **Table 1**). The abundance of Spirochaetes was higher in the DI mucus of RIII-fed fish and lower in the RII-fed fish (**Figures 6A,B**). The abundant phyla in water is shown in **Supplementary Figure 5A**. The dominant phyla in water were Bacteriodetes and Proteobacteria (**Supplementary Figure 5B**). The changes in the abundance of most bacterial taxa in both DI content and mucus of the LAB-fed groups compared to the control group is shown in **Table 1**.

At the genus level, *Lactobacilli* (*Lactobacillus fermentum* and *Lactobacillus paraplantarum*) were found to be the most dominant bacteria in the content and mucus of LAB-fed fish (**Figure 5B**, and **Supplementary Figures 4B–D**) and Mycoplasma was also found to be dominant in the DI mucus of LAB-fed fish (**Figure 6B**).

Core Bacterial Communities of the Intestinal Microbiota

We identified the core microbiota, i.e., the members of the bacterial communities that were commonly shared among 99% of the samples.The common core taxa–at prevalence (bacterial community population frequency) of 99% and abundance detection threshold of 20%–are shown in **Figures 7A,B**. In the DI content, the abundant genera in the LAB-fed fish, namely *Lactobacillus, Ralstonia* (*L. paraplantarum, R. pickettii)* and *Mycoplasma* were noted to be among the core bacterial members. *Bradyrizhobium, Photobacterium, Phyllobacterium, Brevinema, Methylobacterium* (*B. jicamae*, *P. phosphoreum, P. myrsinacearum, B. andersonii, M. fujisawaense*), and *Sphingomonas* were also the shared core taxa in the content (**Figure 7A**). In the DI mucus, the genera that had higher abundance in the RIII-fed fish viz. *Brevinema* and *Pelomonas* (*B. andersonii*, *P. saccharophila*) were observed among the core bacterial members. *Photobacterium, Ralstonia, Aquabacterium, Bradyrizhobium, Methylobacterium, Phyllobacterium,* (*P. phosphoreum, R. pickettii, A. parvum, B. jicamae*, *M. fujisawaense, P. myrsinacearum*), *Sphingomonas, and Mycoplasma* were also the shared core taxa of the intestinal mucus (**Figure 7B**).

The DPCoA indicated differences in the core members of the LAB-fed and the control group (content: F-statistic: 3.879, $R^2 = 0.165$, $p = 0.004$; mucus: F-statistic: 5.844, $R^2 = 0.219$, $p = 0.001$; **Supplementary Figures 6A,B**).

Significantly Abundant and Relevant Bacterial Taxa of the Intestinal Microbiota

ANCOM analysis detected the significantly abundant bacterial OTU in the DI content, which turned out to be *L. fermentum* in RIII-fed fish (**Table 1**). However, this bacterium was not detected as a significant feature in the DI mucus.

Boruta analysis gave 9 and 8 relevant OTUs in the intestinal content and mucus, respectively. In the DI content, *L. fermentum, L. paraplantarum, Streptococcus sobrinus, Corynebacterium simulans, Lactococcus plantarum, W. cibaria, C. amphilecti,* and bacterial taxa belonging to *Streptococcus* and *Xanthomonodales*

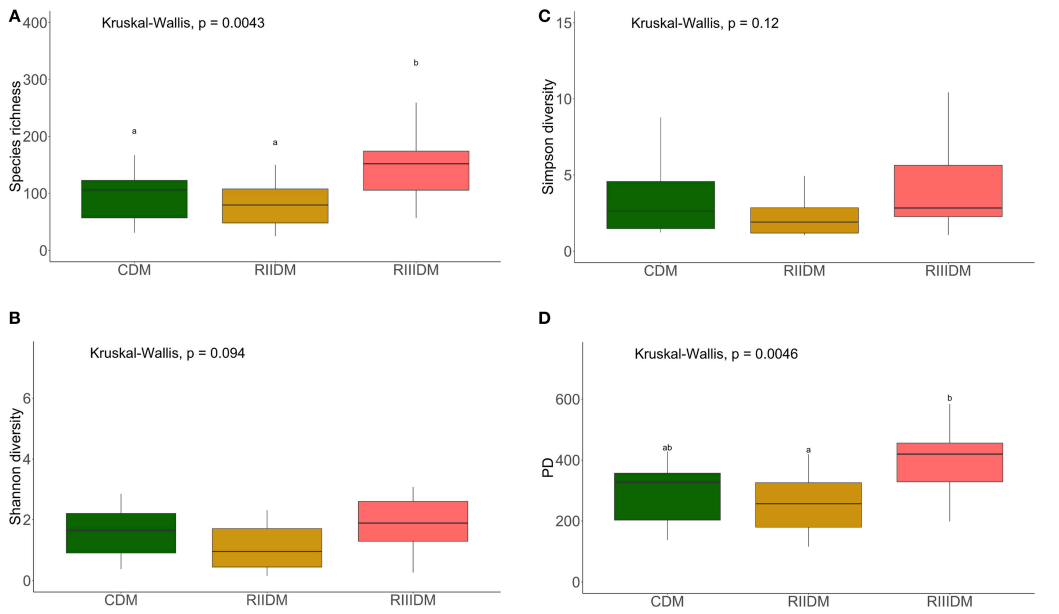

FIGURE 3 | Diversity of the bacterial communities of the intestinal mucus. Boxplots show the species richness **(A)**, Shannon index **(B)**, Simpson index, **(C)** and Faith's phylogenetic diversity **(D)**. Different letters indicate statistically significant differences ($P < 0.05$) between the study groups. The feed group codes are as follows: Control, CDM; RII, RIIDM; RIII, RIIIDM.

were the relevant bacteria. *L. paraplantarum* was found to be abundant in the RII-fed group, whereas *L. fermentum* and *Xanthomonodales* were found to be abundant in the RIII-fed group. *S. sobrinus, C. simulans, L. plantarum, W. cibaria, C. amphilecti* were reduced in abundance in the LAB-fed groups. In the mucus, *Lewinella antarctica, L. paraplantarum, L. fermentum, Salinisphaera, Colwellia aestuarii* and bacteria belonging to-*Gammaproteobacteria, Rhodobacteraceae,* and *Clostridiales* were found to be the relevant bacterial taxa (most of them were abundant in the mucus of the RIII-fed fish–**Table 1**).

Association Network of OTUs

The DI Content Bacteria

The single-domain bacterial (SDB) network derived from the DI content of the 3 groups comprised of one giant connected component (**Supplementary Figure 7**). The significantly abundant and relevant OTUs were labeled based on their membership in different modules (**Figures 8A–C**). The connectivity pattern of the significantly abundant and relevant OTUs in the phylum-level co-occurrence network is shown in **Supplementary Figures 9 A–C**. The average node degrees were 4.27 (SD: 3.44), 3.71 (SD: 1.52), 4.06 (SD: 2.48) for the control, RII- and RIII-fed fish, respectively. Similarly, the values for betweenness were 370 (SD: 369), 396 (SD: 351), 388 (SD- 391). The average node degrees and betweenness of the three groups were not significantly different. The degree of assortativity (assortativity coefficient c_a) of the phylum-level network associated with the three groups (control, RII- and RIII-fed fish) were 0.09, 0.19, and 0.10, respectively. The significantly abundant and relevant OTUs belonged to different phyla and modules (**Figures 8A–C** and **Supplementary Figures 9 A–C**). The degree distribution of the microbial network (for all OTUs) of the study groups (**Supplementary Figure 11A**) revealed that there are many highly connected hub nodes for the bacterial network of the RII-fed fish and the hubs of the control group have more node degrees.

The DI Mucus Bacteria

The SDB network derived from the DI mucus of the control, RII, and RIII groups comprised of one giant connected component (**Supplementary Figure 8**). In the bacterial network of RII-fed fish, we observed a singleton (*C. aestuarii*), a dyad (2 OTUs of *Mycoplasma*), and a triad (*L. paraplantarum*, *W. cibaria*, and *P. piscicola*) with no connection to the main network (**Supplementary Figure 8**). As for the RIII-fed group, there were 3 dyads (Sphingobacteriales + Myxococcales, 2 OTUs of *Mycoplasma*, and Xanthomonadales + Gammaproteobacteria) with no connection to the main network (**Supplementary Figure 8**). The significantly abundant and relevant OTUs were labeled based on their membership in different modules (**Figures 9A–C**).

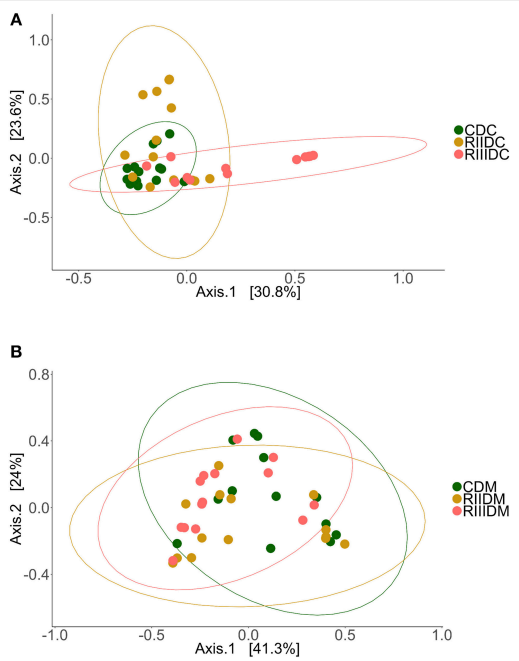

FIGURE 4 | Beta diversity of the intestinal bacterial communities. Principal coordinate analysis plot **(A)** shows the differences in the composition of the bacterial communities in the intestinal content (Control, CDC; RII, RIIDC; RIII, RIIIDC). Principal coordinate analysis plot **(B)** shows the differences in the composition of the bacterial communities in the intestinal mucus (Control, CDM; RII, RIIDM; RIII, RIIIDM).

The connectivity pattern of the significantly abundant and relevant OTUs in the phylum-level co-occurrence network is shown in **Supplementary Figures 10 A–C**. The average node degrees were 4.12 (SD: 2.20), 2.29 (SD: 2.09), 2.74 (SD: 1.19) for the control, RII- and RIII-fed fish, respectively. The values for betweenness of the control, RII- and RIII-fed fish were 505 (SD: 664), 481 (SD: 596), 613 (SD: 766), respectively. Dunn's test identified significant differences between the LAB-fed groups, and between control and RIII-fed fish; for node degree, but not for edge betweenness; $p = 0.0002$, $p = 0.003$ and $p = 0.08$, $p = 0.07$, respectively. The degree of assortativity (assortativity coefficient c_a) of the phylum-level network for the three groups (control, RII- and RIII-fed fish) were −0.01, −0.07, and 0.13, respectively. The degree distribution of the microbial network (for all OTUs) of the three groups is shown in **Supplementary Figure 11B**. The node degree histogram showed that the hubs of the RII-fed groups have higher node degrees than the other groups.

The main results of this study are summarized in **Figure 10**.

DISCUSSION

Probiotics are live microbes that can impart health-benefiting effects on host organisms. For instance, feeding of some species belonging to genera *Lactobacillus* and *Bifidobacterium* can elicit positive effects on host health (Wang et al., 2015; Bagarolli et al., 2017). Probiotics alter the gut microbiota and interact with them to produce several types of metabolites, vitamins, and antimicrobial agents that affect the host physiology (Saulnier et al., 2011; O'Shea et al., 2012; LeBlanc et al., 2017). In the present study, we investigated the intestinal microbiota changes in Atlantic salmon after feeding them with dietary supplements of two *Lactobacillus* spp., named RII and RIII. To understand the differences in the microbial community associated with the content and mucus of the DI, the bacteria in the two samples were analyzed separately because the microbial niche in the DI mucus is distinct compared to the intestinal contents.

Feeding LAB to the fish may facilitate their establishment in the intestine, although significant difference was noted for the abundance of only one of the two LAB species. The feed-delivered organisms also altered the diversity and composition of the DI bacteria differently. RIII supplementation caused a significant increase in the species richness and phylogenetic diversity of the bacterial community in DI mucus. Furthermore, both RII and RIII caused a shift in the community composition; bacteria belonging to different genera were altered in the two feed groups. The co-occurrence networks indicated differential bacterial associations in the control and LAB-fed groups.

Water bacterial communities may have an effect on the microbiota of fish. To clarify this, we compared the microbial community composition in the intestinal and environmental samples. Notwithstanding the fact that different extraction methods can cause small variations in the microbial profile (Wagner Mackenzie et al., 2015) studies have shown that rearing water has a minor effect on the GI microbiota in fish (Giatsis et al., 2015; Uren Webster et al., 2018). Betiku et al. (2018) have demonstrated that recirculating water systems have more diverse microbial composition compared to the flow-through system. However, similar to other reports (Yan et al., 2016, Lokesh et al., 2018, Gupta et al., under review) water bacterial communities might not have affected the intestinal bacterial profile in our study. Also, none of the dominant OTUs of water were detected in the DI of fish, suggesting that host-specific gut microbial species selection is modulated by the host gut habitat and host's genotype (Giatsis et al., 2015).

LAB Increases the Microbial Diversity in the Intestinal Mucus

Corresponding to our observation on the content bacteria, a few previous studies have also shown that LAB supplementation does not alter the intestinal bacterial diversity (Chao1 and Shannon diversities); in humans (Van Zanten et al., 2014) and in mice with colon cancer (Mendes et al., 2018). On the other hand, species richness, Shannon and Simpson diversities, and PD of the bacteria in the DI mucus were higher in the RIII-fed

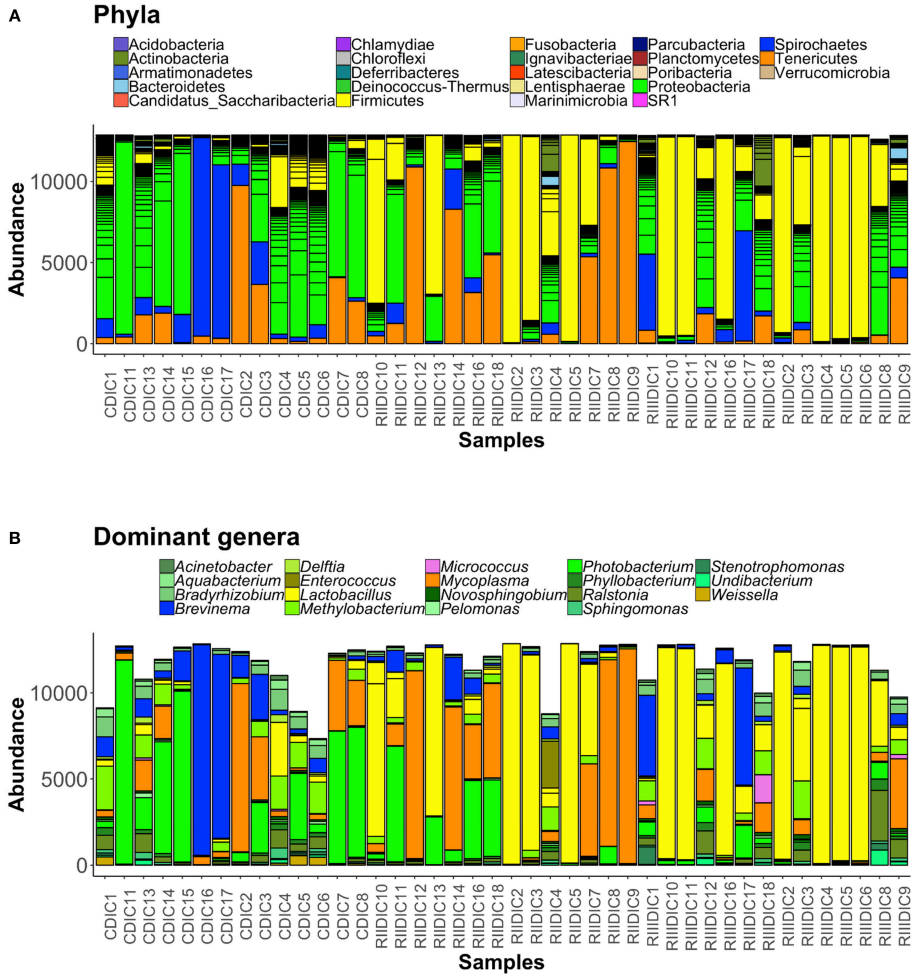

FIGURE 5 | Barplots showing the abundance of all bacterial phyla **(A)**, and dominant genera **(B)**, in the intestinal content. The height of each bar segment represents the abundance of individual operational taxonomic units (OTUs) stacked in order from greatest to smallest, and separated by a thin black border line. Color codes for the dominant genera: Proteobacteria—shades of green, Spirochaetes—dark blue, Firmicutes—shades of yellow, Actinobacteria—orchid, and Tenericutes—dark orange.

fish. In the case of mucus bacteria of RII-fed fish, we noted a slight decrease ($p > 0.05$) in alpha diversity compared to the control fish. Previous studies have shown that *Lactobacillus* can increase the bacterial PD in the gut of mice (Usui et al., 2018) and weaning piglets (Zhao et al., 2016). On the contrary, offering LAB in combination with *Bifidobacterium breve* and *Bifidobacterium longum* did not result in greater bacterial species diversity (Chao1, Shannon index and PD) in mice that received antibiotics (Grazul et al., 2016).

LAB Promotes the Abundance and Dominance of Intestinal *Lactobacillus* and Other Firmicutes

L. paraplantarum (LP) is related to *L. plantarum* (Curk et al., 1996). It was dominant in the RII-fed group and *L. fermentum* (LF) was found dominant in the RIII-fed group. *Lactobacilli* are a group of gram-positive ubiquitous LAB that produce organic acids as end products of their metabolic activity linked to carbohydrate fermentation (Bernardeau et al., 2006). LP

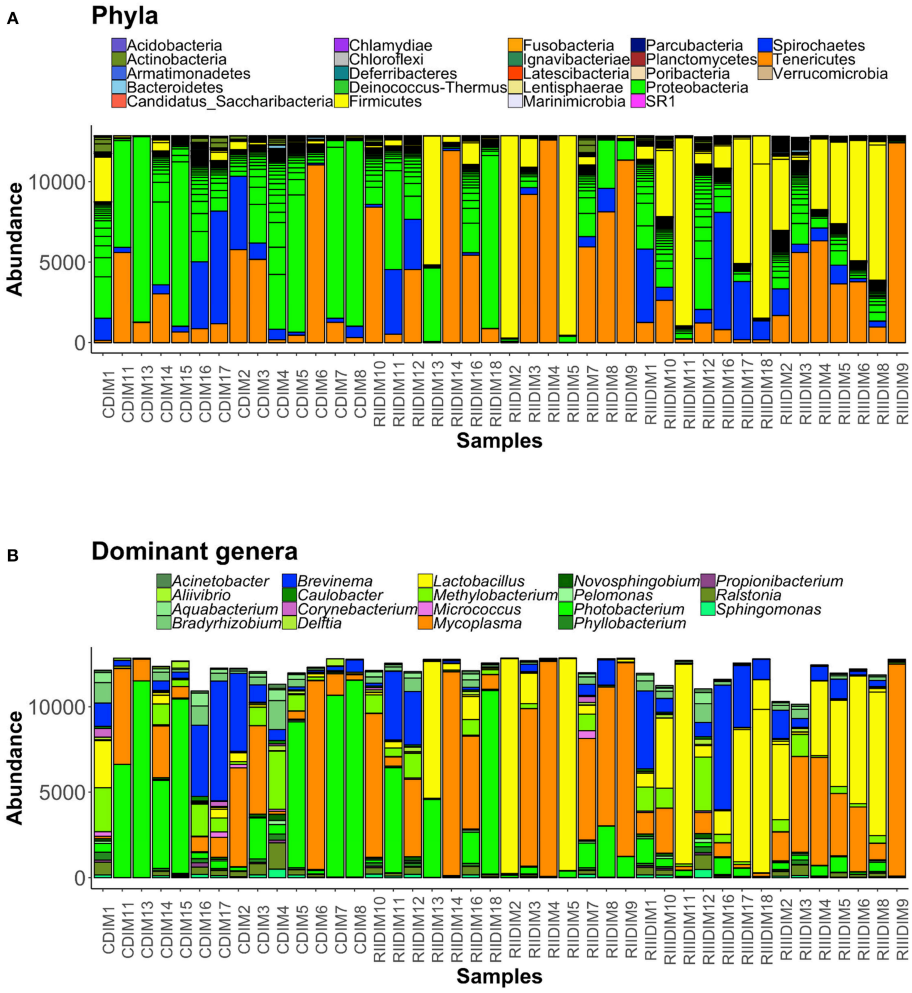

FIGURE 6 | Barplots showing the abundance of all bacterial phyla **(A)**, and dominant genera **(B)** in the intestinal mucus. The height of each bar segment represents the abundance of individual operational taxonomic units (OTUs) stacked in order from highest to smallest, and separated by a thin black border line. Color codes for the dominant genera: Proteobacteria—shades of green, Spirochaetes—dark blue, Firmicutes—shades of yellow, Actinobacteria—shades of orchid, and Tenericutes—dark orange.

is known to produce bacteriocins, which are antimicrobial peptides produced as a defense response (Tulini et al., 2013). A *Lactobacillus* isolate (LP 11-1) stimulated the innate immune system and induced tolerance against the pathogenicity of *Pseudomonas aeruginosa* in silkworm (Nishida et al., 2017). LF has been found to restore the expression of markers associated with the maintenance of intestinal barrier function, and recover the SCFAs- and lactic acid-producing bacterial populations in mouse suffering from colitis (Rodriguez-Nogales et al., 2017).

Lactobacillus is part of the normal intestinal flora of fish (Ringø et al., 1995; Spanggaard et al., 2000; Ringø and Olsen, 2003). In zebrafish, probiotic *Lactobacillus* helps to overcome infection (He et al., 2017). In Nile tilapia (*Oreochromis niloticus*), LF is known to improve fish immune response (Nwanna and Bamidele, 2014). LF (LbFF4 strain) along with *L. plantarum* (LbOG1 strain) exhibit *in vitro* antibacterial activities against fish pathogens in *Clarias gariepinus* (Adenike and Olalekan, 2009). The higher abundance of intestinal *Lactobacillus* members and

TABLE 1 | Changes in abundances of the bacterial taxa by LAB feeding.

Sample type	Intestinal content		Intestinal mucus	
Taxa / groups	RII	RIII	RII	RIII
Acidobacteria	↑	↑	↑	↑
Actinobacteria	↑	↑	↓	↓
Fusobacteria	↓	↑	↓	↓
Deinococcus-Thermus	↑	↑	↓	↑
SR1	↑	–	–	↑
Chloroflexi	↑	↑	NA	NA
Parcubacteria	↓	↓	↑	↑
Planctomycetes	↓	↓	↑	–
Lactobacillus fermentum	↑	↑	↓	↑
Lactobacillus paraplantarum	↑	↓	↑	↑
Colwellia aestuarii	NA	NA	↓	↑
Streptococcus sobrinus	↓	↓	NA	NA
Lewinella antarctica	NA	NA	↑	↑
Lactobacillus plantarum	↓	↓	NA	NA
Acinetobacter radioresistens	↓	↑	↓	↓
Novosphingobium sediminicola	↓	↑	↓	↓
Phyllobacterium myrsinacearum	↓	↑	↓	↓
Ralstonia pickettii	↓	↑	↓	↓
Stenotrophomonas maltophilia	↓	↑	NA	NA
Undibacterium oligocarboniphilm	↓	↑	NA	NA
Micrococcus luteus	↓	↑	↓	↓
Enterococcus cecorum	↑	–	NA	NA
Mycoplasma	↑	↓	NA	NA
Aquabacterium	↓	↓	NA	NA
Bradyrizhobium	↓	↓	↓	↓
Brevinema	↓	↓	NA	NA
Delftia	↓	↓	↓	↑
Methylobacterium	↓	↓	NA	NA
Aquabacterium parvum	NA	NA	↓	↓
Pelomonas	↓	↓	NA	NA
Photobacterium	↓	↓	NA	NA
Sphingomonas	↓	↓	↓	↓
Weissella	↓	↓	NA	NA
Brevinema andersonii	NA	NA	↓	↑
Pelomonas saccharophila	NA	NA	↓	–
Bradyrizhobium jicamae	NA	NA	↓	↓
Methylobacterium fujisawaense	NA	NA	↓	↓
Photobacterium phosphoreum	NA	NA	↓	↓
Aliivibrio logei	NA	NA	↓	↓
Caulobacter segnis	NA	NA	↓	↓
Cornybacterium	↓	↓	↓	↓
Propionibacterium acnes	NA	NA	↓	↓

Arrows indicate changes in abundance (blue arrow: increase, red arrow: decrease, bold black line: no change, NA, taxa not present in a particular group).

the altered bacterial abundance in the LAB-fed fish confirms that LAB feeding can change the intestinal microbial composition.

Enterococcus cecorum, was also found to be dominant in the content of the RII-fed group compared to the control group (**Table 1**). *Enterococcus* spp. isolated from the intestine of rainbow trout (*Oncorhynchus mykiss*) are used as probiotics due to their antimicrobial activity against fish pathogens (Carlos et al., 2015). The functional potential of *E. cecorum* in Atlantic salmon has not yet come to light although one particular strain is known to cause infections in broilers (Herdt et al., 2009).

Clostridiales (belonging to Firmicutes) were higher in the mucus of salmon offered diets with RIII. Commensal Clostridiales are known to promote gut health by modulating gut homeostasis and taking part in immune activation (Lopetuso et al., 2013).

LAB Favors Certain Members of Tenericutes, Spirochaetes, and Actinobacteria

LAB significantly aided in altering the abundance of the genus *Mycoplasma* (Tenericutes) and *B. andersonii* (Spirochaetes) in the mucus, which are the common core members in the DI content of Atlantic salmon (**Figure 7A**). *Mycoplasma* has consistently been isolated from salmon intestine (Holben et al., 2002; Zarkasi et al., 2014) and its presence as a core microbiota suggests that it may be a commensal organism in the intestinal ecosystem. *B. andersonii* has been reported in the intestinal microbiota of flatfish, *Solea senegalensis* (Tapia-Paniagua et al., 2010). Although *B. andersonii* is known to digest lignocellulose and fix nitrogen in termite guts (Kudo, 2009), their functional importance needs to be elucidated. The abundance of the genus *Micrococcus* (*M. luteus*), a member of Actinobacteria, was higher in the DI content of the RIII-fed group (**Table 1**). Though *M. luteus* is known to be a pathogen for rainbow trout (*Salmo trutta L.*) and brown trout (*Oncorhynchus mykiss*) (Pkala et al., 2018) an *in vivo* feeding study has suggested that they can enhance the growth and health of Nile tilapia (Abd El-Rhman et al., 2009).

LAB Largely Decreased the Abundance of Proteobacteria

Proteobacteria is the most abundant phylum in many marine and freshwater fishes (Yan et al., 2016; Lokesh et al., 2018) and it is also known to dominate the gut microbiota of Atlantic salmon (Gajardo et al., 2016; Lokesh et al., 2018). Therefore, it was surprising to find this taxon in low abundance in the LAB-fed and the control fish. A general decrease in the abundance of intestinal Proteobacteria has also been reported in farmed Atlantic salmon that were transferred to seawater (Rudi et al., 2018). Taxa belonging to Proteobacteria are involved in metabolic pathways that participate in carbon and nitrogen fixation and in the stress response regulatory system (Vikram et al., 2016). They are also important in the digestive process in fish (Romero et al., 2014). *P. phosphoreum*, a known gut symbiont of marine fish, helps in chitin digestion and use

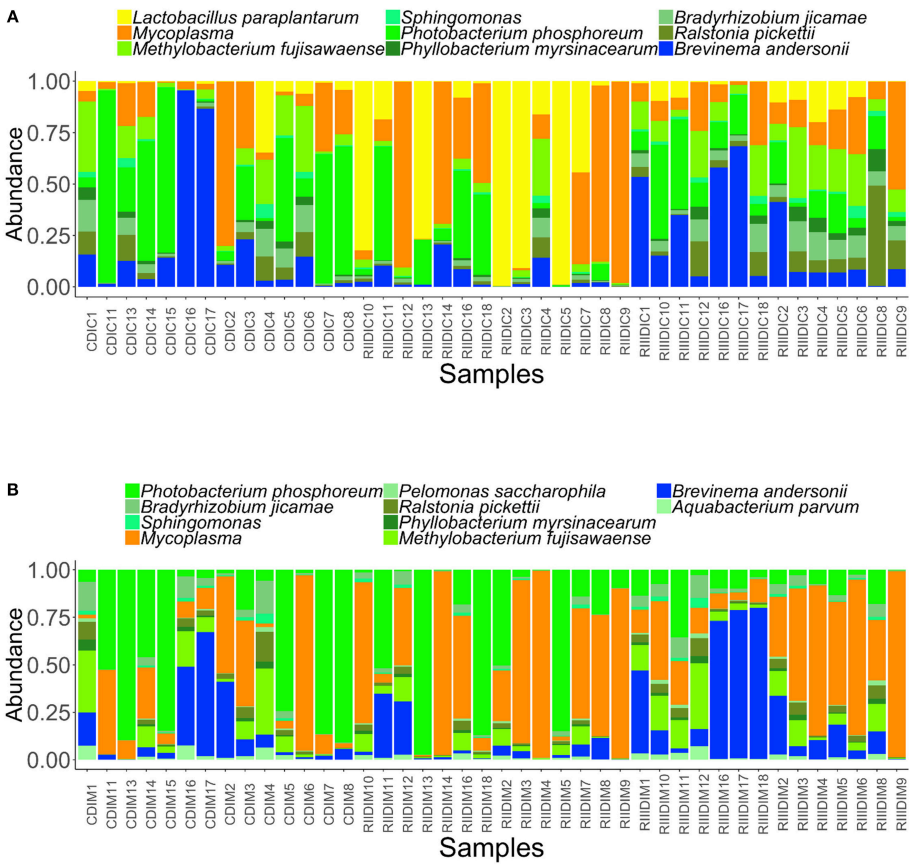

FIGURE 7 | Abundance of the core bacterial taxa in the intestinal content **(A)** and mucus **(B)**. Color codes: Shades of green—Proteobacteria, yellow—Firmicutes, dark blue—Spirochaetes, and dark orange—Tenericutes.

luciferase- reoxidize reduced coenzymes and other molecules for metabolism (Nealson and Hastings, 1979). *N. sediminicola* and *P. myrsinacearum* are known as nitrogen-fixing bacteria (Gonzalez-Bashan et al., 2000; Muangthong et al., 2015). On the other hand, *R. pickettii* formerly known as *Burkholderia pickettii* has genes to biodegrade aromatic hydrocarbons (Ryan et al., 2007). In the current and in our recent (Gupta et al, under review) studies we found that *P. myrsinacearum* and *R. pickettii* are part of the core gut microbiota of Atlantic salmon; *N. sediminicola* was also significantly abundant in the intestinal mucus of the fish fed oligosaccharide. Functions of the aforementioned bacteria are not yet reported in fish.

LAB Affects the Microbial Association

We inferred single-domain networks using the SPEIC-EASI framework, and highlighted the significantly abundant and relevant OTUs in the intestinal microbiota. For DI mucus, the inferred SDB network for RII-fed fish showed lower overall connectivity. The node degree histograms also communicate interesting information about the network; the mucus bacteria of RII-fed group had hubs with more node degree. However, the lower average node degree and lower selective linking of the RII-fed group indicate less interactions among the gut bacteria. Cooperative microbial communities are known to provide microbiome stability because of their functional dependence. Studies have shown that the stability declines with an increase in microbial diversity and proportion of cooperative interactions (Coyte et al., 2015). However, higher cooperating microbial communities can cause a runaway effect that can collapse the competing microbial population due to over-representation of the most stable community (McNally and Brown, 2016).

The dyads in the mucus bacterial networks of LAB-fed fish were different, the exception being the one constructed

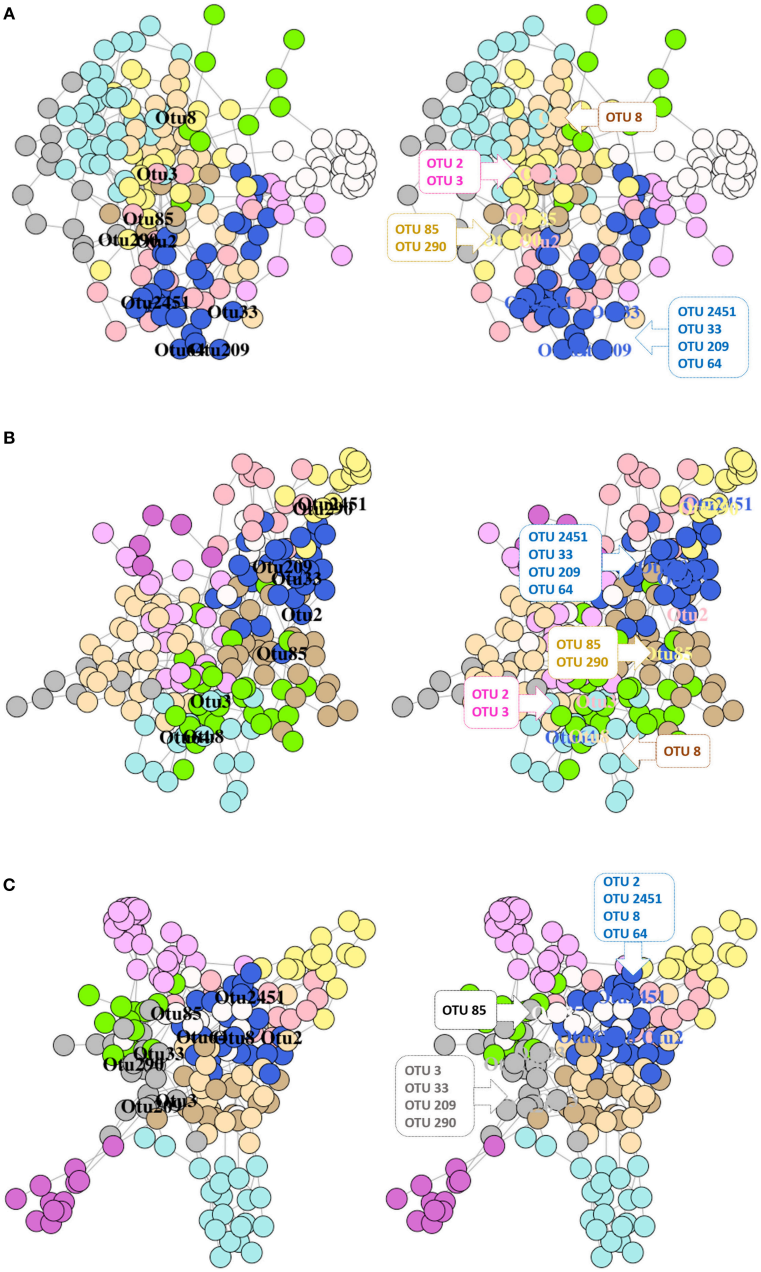

FIGURE 8 | Network graphs showing the significantly abundant and relevant OTUs of the intestinal content in different modules of the network. Bacterial networks of the control **(A)**, RII **(B)**, and RIII **(C)** fish. Nodes represent OTUs and specific colors of the modules reveal the membership of the significantly abundant and relevant OTUs. The left graph shows the location of the OTUs and in the right graph, the significantly abundant and relevant OTUs that belong to the same module are shown in callouts.

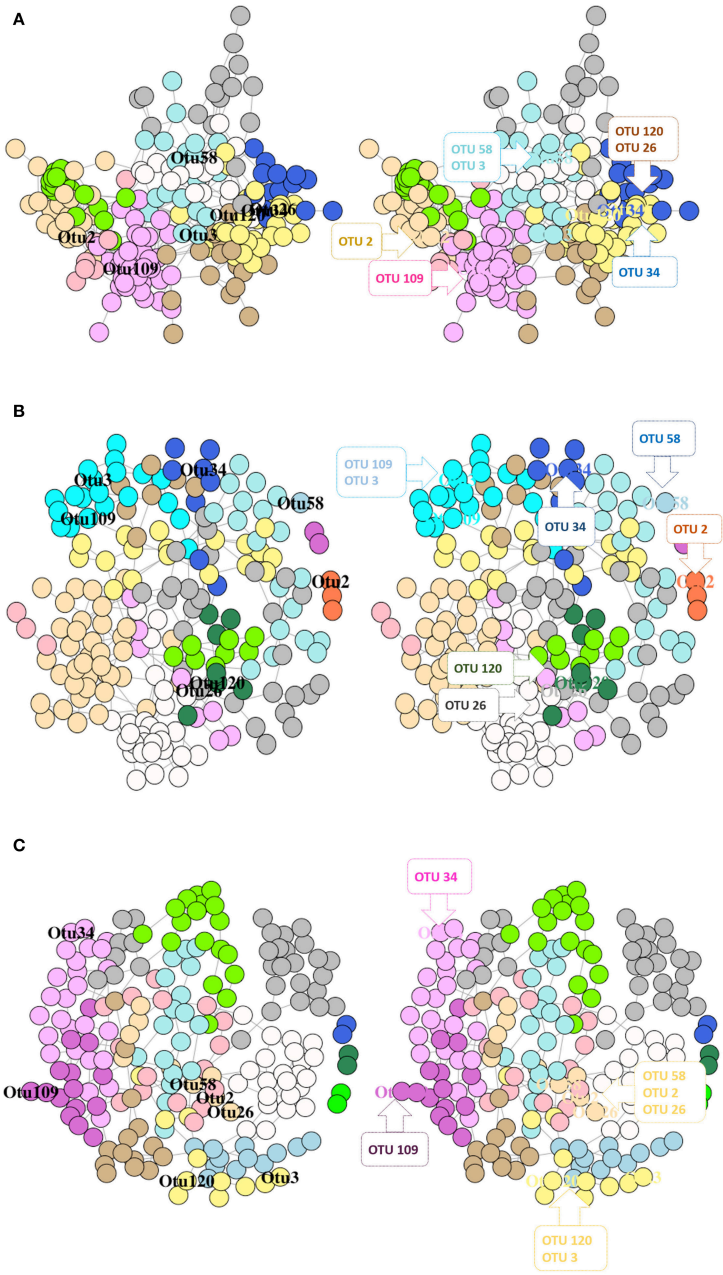

FIGURE 9 | Network graphs showing the significantly abundant and relevant OTUs of DI mucus in different modules based on their membership for control fish **(A)**, RII **(B)**, and RIII fish **(C)**. Nodes represent OTUs and specific colors of the modules reveal the membership of the significantly abundant and relevant OTUs. The left graph shows the location of the OTUs and in the right graph the significantly abundant and relevant OTUs that belong to the same module are shown in callouts.

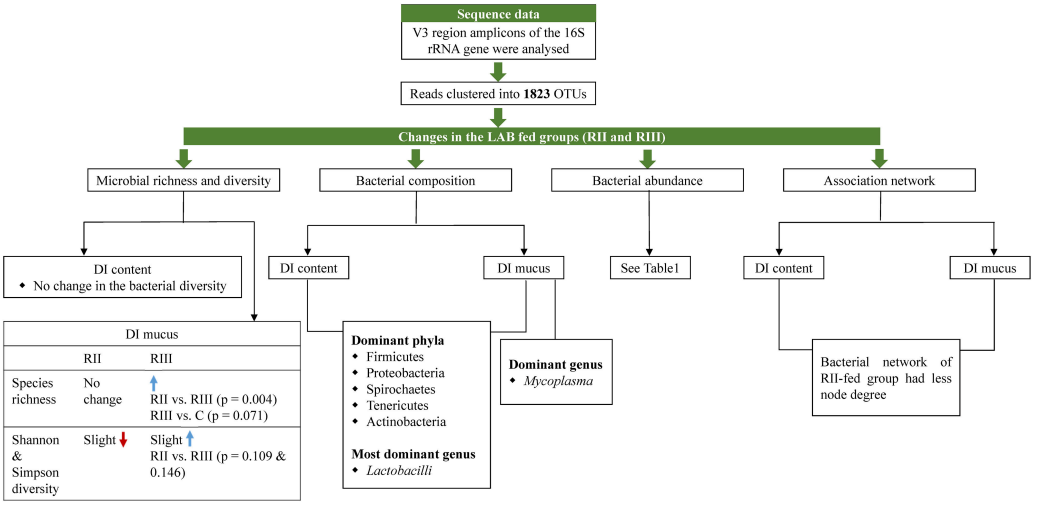

FIGURE 10 | Illustration summarizing the salient observations of the study. DI, distal intestine; C, control group; RII and RIII, LAB-fed groups.

with 2 OTUs of *Mycoplasma* which had higher abundance in the RII-fed fish and lower abundance in the RIII-fed fish. This result could be suggesting that intestinal *Mycoplasma* in the LAB-fed fish was not associated with other gut bacterial communities. In the content of LAB-fed fish, most of the labeled OTUs (except OTU 8) were existing in their respective modules (**Figures 8B, C**). In the mucus of RIII-fed fish OTUs belonging to *C. aestuarii*, *L. paraplantarum* and Clostridiales were found to exist in one module. Clostridiales and Rhodobacteraceae, which had same module membership in the network of the control fish were no longer closely associated after LAB feeding. So was the case with *L. fermentum* and *C. aestuarii*. Members affiliated to Rhodobacteraceae are known for their denitrification properties, and Kraft et al. (2014) have shown that Clostridiales indirectly participates in nitrate respiration by providing fermentation substrates (e.g., acetate, formate, or hydrogen) to Rhodobacteraceae-like denitrifiers. Our findings suggests that the taxa belonging to the same module can be functionally dependent but the alteration of their membership after LAB feeding has to be further investigated.

The mucus bacteria of RIII-fed fish had higher species richness and PD, and the significantly abundant and relevant OTUs belonged to different modules. For the RIII-associated network, 2 OTUs each belonging to two modules (Rhodobacteraceae and *L. fermentum*; *C. aestuarii*, and Clostridiales) had higher abundances compared to the control group. In addition, significantly abundant and relevant bacteria had higher abundance in the RIII-fed fish compared to the control group. This abundance pattern does not indicate negative feedback loops (Coyte et al., 2015). These results of bacterial networks have to be validated through culture-based studies.

CONCLUSION

In summary, LAB feeding promoted the dominance of intestinal *Lactobacillus* (Firmicutes) and certain members of the phyla Tenericutes, Spirochaetes, and Actinobacteria. Although the abundances of many members of Proteobacteria were decreased, the phylum remained dominant in the distal intestine of Atlantic salmon. Dietary supplementation with the two LAB strains shifted the intestinal bacterial community composition. Furthermore, the co-occurrence networks of the intestinal bacteria were also different for the LAB-fed fish. Taken together, our results show that the LAB influences the gut microbiota of Atlantic salmon. This information will help in future studies that explore the microbial interactions between LAB-modulated gut microbiota and the host.

AUTHOR CONTRIBUTIONS

MS and VK procured the funding for the study. VK, MS, JK, AF, and SG designed the study. JK provided the probiotics. AF and SG conducted the feeding experiment. SG performed the 16S rRNA sequencing studies. SG, VK, and JF analyzed the data. SG wrote the manuscript with the guidance of VK. All authors read, revised and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.03247/full#supplementary-material

Supplementary Figure 1 | Sample-size-based rarefaction curves for the reads obtained from the intestinal content **(A)** and mucus **(B)**. The shaded portion around each line represents the 95% confidence interval. Color code for the feed groups: green lines- control, orange lines- RII, pink lines- RIII. Codes for content samples: CDM-control, RIIDC-RII, RIIIDC-RIII. Codes for mucus samples: CDM-control, RIIDM-RII, RIIIDM-RIII.

Supplementary Figure 2 | Double principal coordinate analysis plots showing the beta diversity of the bacterial communities. Tank and inlet water **(A)**, control intestinal content and control tank water: F-statistic = 4.035, $R^2 = 0.211$, $P = 0.01$ **(B)**, RII intestinal content and RII tank water: F-statistic = 2.375, $R^2 = 0.136$, $P = 0.07$ **(C)**, RIII intestinal content and RIII tank water: F-statistic = 5.006, $R^2 = 0.250$, $P = 0.002$ **(D)**, Control intestinal mucus and control tank water: F-statistic = 16.291, $R^2 = 0.520$, $P = 0.003$ **(E)**, RII intestinal mucus and RII tank water: F-statistic = 2.934, $R^2 = 0.163$, $P = 0.051$ **(F)**, RIII intestinal mucus and RIII tank water: F-statistic = 3.910, $R^2 = 0.206$, $P = 0.03$ **(G)**.

Supplementary Figure 3 | Double principal coordinate analysis plots showing the beta diversity of the bacterial communities. Tank biofilm bacteria **(A)**, Control intestinal content and control tank biofilm: F-statistic = 2.061, $R^2 = 0.120$, $P = 0.082$ **(B)**, RII intestinal content and RII tank biofilm: F-statistic = 1.915, $R^2 = 0.113$, $P = 0.015$ **(C)**, RIII intestinal content and RIII tank biofilm: F-statistic = 4.171, $R^2 = 0.217$, $P = 0.043$ **(D)**, Control intestinal mucus and control tank biofilm: F-statistic = 5.807, $R^2 = 0.1279$, $P = 0.002$ **(E)**, RII intestinal mucus and RII tank biofilm: F-statistic = 1.476, $R^2 = 0.09$, $P = 0.146$ **(F)**, RIII intestinal mucus and RIII tank biofilm: F-statistic = 2.078, $R^2 = 0.121$, $P = 0.076$ **(G)**.

Supplementary Figure 4 | Barplots showing the dominant bacterial phyla and species in the intestinal content **(A,B)** and mucus **(C,D)**.

Supplementary Figure 5 | Barplots showing the abundance of the bacterial phyla **(A)**, dominant phyla **(B)** in the tank water. The height of each bar segment represents the abundance of individual operational taxonomic units (OTUs) stacked in order from largest to smallest, and separated by a thin black border line. Color codes: Proteobacteria—green, Bacteroidetes—light blue.

Supplementary Figure 6 | DPCoA showing the differences in the composition of the core members of the intestinal content **(A)** and mucus **(B)** samples of the control and LAB-fed groups.

Supplementary Figure 7 | The single-domain network graph of the bacteria in the intestinal content. Nodes represent different phyla shown in different colors. The three panels represent the three feed groups: Control **(A)**, RII **(B)**, RIII **(C)**.

Supplementary Figure 8 | The single-domain network graph of the bacteria in the intestinal mucus. Nodes represent different phyla shown in different colors. The three panels represent the three feed groups: Control **(A)**, RII **(B)**, RIII **(C)**.

Supplementary Figure 9 | Network association graph showing the connectivity pattern of the significantly abundant and relevant OTUs in the intestinal content of the Control **(A)**, RII **(B)**, and RIII **(C)** groups.

Supplementary Figure 10 | Network association graph showing the connectivity pattern of the significantly abundant and relevant OTUs in the intestinal mucus of the Control **(A)**, RII **(B)**, and RIII **(C)** groups.

Supplementary Figure 11 | Histograms showing the degree distribution of the bacterial networks associated with the intestinal content **(A)** and mucus **(B)**.

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Corrigendum: Lactobacillus Dominate in the Intestine of Atlantic Salmon Fed Dietary Probiotics

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Error in Table

In the original article, there were mistakes in ***Table 1*** as published. **NA was stated as 'not present' in the footnote of Table 1 of the original article. For some of the taxa in the table this was not true. Therefore, we replaced NAs with up or down arrows. Now NA is indicated as dominant taxa.** The corrected ***Table 1*** appears below. The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way.

Table 1. Changes in abundances of the bacterial taxa by LAB feeding.

Sample type	Intestinal content		Intestinal mucus	
Groups / Taxa	RII	RIII	RII	RIII
Acidobacteria	↑	↑	↑	↑
Actinobacteria	↑	↑	↓	↓
Fusobacteria	↓	↑	↓	↓
Deinococcus-Thermus	↑	↑	↓	↑
SR1	↑	–	–	↑
Chloroflexi	↑	↑	–	–
Parcubacteria	↓	↓	↑	↑
Planctomycetes	↓	↓	↑	–
Lactobacillus fermentum	↑	↑	↓	↑
Lactobacillus paraplantarum	↑	↓	↑	↑
Colwellia aestuarii	↑	↓	↓	↑
Streptococcus sobrinus	↓	↓	↓	↑
Lewinella antarctica	↓	↑	↑	↑
Lactobacillus plantarum	↓	↓	↓	↓
Acinetobacter radioresistens	↓	↑	↓	↓
Novosphingobium sediminicola	↓	↑	↓	↓
Phyllobacterium myrsinacearum	↓	↑	↓	↓
Ralstonia pickettii	↓	↑	↓	↓
Stenotrophomonas maltophilia	↓	↑	NA	NA
Undibacterium oligocarboniphilm	↓	↑	NA	NA
Micrococcus luteus	↓	↑	↓	↓
Enterococcus cecorum	↑	–	NA	NA
Mycoplasma	↑	↓	↑	↑
Aquabacterium	↓	↓	↓	↓
Bradyrizhobium	↓	↓	↓	↓
Brevinema	↓	↓	↓	↑
Delftia	↓	↓	↓	↑
Methylobacterium	↓	↓	↓	↓
Aquabacterium parvum	↓	↓	↓	↓
Pelomonas	↓	↓	↓	↑
Photobacterium	↓	↓	↓	↓
Sphingomonas	↓	↓	↓	↓
Weissella	↓	↓	NA	NA

Brevinema andersonii	↓	↓	↓	↑
Pelomonas saccharophila	↓	↓	↓	–
Bradyrizhobium jicamae	↓	↓	↓	↓
Methylobacterium fujisawaense	↓	↓	↓	↓
Photobacterium phosphoreum	↓	↓	↓	↓
Aliivibrio logei	NA	NA	↓	↓
Caulobacter segnis	NA	NA	↓	↓
Cornybacterium	↓	↓	↓	↓
Propionibacterium acnes	NA	NA	↓	↓

Arrows indicate changes in abundance (blue arrow: increase, red arrow: decrease, bold black line: no change, NA: taxa not dominant).

Paper III

Macroalga-derived alginate oligosaccharide alters certain intestinal bacteria of Atlantic salmon

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Abstract

Prebiotics are substrates intended to sculpt gut microbial communities as they are selectively utilized by the microorganisms to exert beneficial health effects on hosts. Macroalga-derived oligosaccharides are candidate prebiotics, and herein, we determined the effects of *Laminaria* sp.-derived alginate oligosaccharide (AlgOS) on the distal intestinal microbiota of Atlantic salmon (*Salmo salar*). Using a high-throughput 16S rRNA gene amplicon sequencing technique, we investigated the microbiota harboured in the intestinal content and mucus of the fish offered feeds supplemented with 0.5 and 2.5% AlgOS. We found that the prebiotic shifts the intestinal microbiota profile; alpha diversity was significantly reduced with 2.5% AlgOS while with 0.5% AlgOS the alteration occurred without impacting the bacterial diversity. Beta diversity analysis indicated the significant differences between control and prebiotic-fed groups. The low supplementation level of AlgOS facilitated the dominance of Proteobacteria and Spirochaetes, few members of which have genes associated with butyrate production. Certain Actinobacteria were also abundant in salmon fed the low level of AlgOS. The results indicate that the low inclusion of AlgOS can plausibly induce a prebiotic effect on the distal intestinal microbiota of Atlantic salmon. These findings can generate further interest in the potential of macroalgae-derived oligosaccharides for food and feed applications.

Introduction

Prebiotics, recently defined by Gibson et al. (2017) are "substrates that are selectively utilized by the host microorganisms conferring a health benefit". They intended to evoke beneficial effects on the host through microbial manipulation and the entailing microbial metabolite production. Studies that employed molecular-based methods have provided evidence on the selective effect of prebiotics; they affect certain (e.g., *Bifidobacterium*, *Anaerostipes*, and *Bilophila*) but not all microorganisms (Vandeputte et al., 2017). Prebiotics such as the non-digestible oligosaccharides are not digested in the gastrointestinal tract (GIT) using host enzymes (Den Besten et al., 2013). The host lacks such enzymes, but certain gut bacteria ferment the carbohydrates into bacterial bioactive metabolites, such as short-chain fatty acids, SCFAs (Flint et al., 2012). One of the main SCFAs, butyrate, benefits the host health by providing energy to colonocytes, maintaining mucosal integrity, and immune homeostasis (O'Keefe, 2016). Despite the evidence on potential benefits of prebiotics, noted in the aforementioned mammalian studies, their effects on the intestinal microbiota of farmed salmonids have not been thoroughly investigated.

Prebiotics are gradually gaining ground in aquaculture, as feed additives that can alter the gut microbiota and positively affect the host metabolism. The most common prebiotics supplemented in aquafeeds include fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), mannanoligosaccharides (MOS) and few others, as reviewed by Ringø et al. (2010). In fish, prebiotics modulate the non-specific immune responses by modifying the gut microbial community, improving mineral uptake and increasing fermentation products (Burr et al., 2005), all of which contribute to improved disease resistance. FOS has been shown to enhance feed efficiency and energy retention in blunt snout bream, *Megalobrama amblycephala* (Wu et al., 2013). scFOS improved specific growth rate and daily feed intake of hybrid tilapia, *Oreochromis aureus* x *O. niloticus* (Hui-Yuan et al., 2007). Dietary MOS modulated the intestinal microbiota and improved the gut morphology of rainbow trout, *Oncorhynchus mykiss* (Dimitroglou et al., 2009). Furthermore, MOS had a positive effect on feed conversion ratio and elevated the lactic acid producing intestinal bacterial community of common carp (Momeni-Moghaddam et al., 2015). Nevertheless, studies examining the ability of prebiotics to alter the intestinal microbial population in salmonids, including Atlantic salmon, are relatively few. The feeds of Atlantic salmon, a high-value farmed fish, contain 70% plant-based ingredients (Ytrestøyl et al., 2015); some of which are known to affect the micromorphology of the distal intestine (Uran et al., 2009) and composition of the intestinal microbiota (Reveco et al., 2014), leading to intestinal diseases. In this context, prebiotics can modulate fish health by guiding the intestinal microbiota towards a healthy state. Therefore, understanding the changes in the gut microbiota of Atlantic salmon under the influence of a candidate prebiotic is important.

Alginate oligosaccharide (AlgOS), a macroalga product, is suggested as a candidate prebiotic agent because it can promote the host health by favouring the beneficial microorganisms in their gut (Wang et al., 2006). The carbohydrate influences the beneficial gut microflora in *Fenneropenaeus indicus*, Indian major shrimp (Kokilam et al., 2016) and modulate the intestinal microbiota of flat fish (*Solea senegalensis*) (Tapia-Paniagua et al., 2010). Low

molecular weight sodium alginate combined with kefir is found to stimulate immunity, disease resistance, and growth performance of Nile tilapia (*Oreochromis niloticus*) (Van Doan et al., 2017). Even though these reports indicate the influence of sodium alginate on the intestinal microbes and fish health, in-depth studies using new techniques can unravel the effect of the compound on the intestinal microbial ecosystem, which is known to have a direct impact on health. Hence, we investigated the effects of AlgOS on the intestinal microbiota of Atlantic salmon.

Materials and methods

Ethics statements

The study was approved by the Norwegian Animal Research Authority, FDU (Forsøksdyrutvalget ID-8002), and the fish handling and sampling procedures were in accordance with the authorized protocols of FDU.

Test product

AlgOS, a candidate prebiotic derived from the macroalga *Laminaria* sp., was obtained from Centre d'Etude et de Valorisation des Algues (CEVA), Pleubian, France. Purified sodium alginate was depolymerized to produce the oligomeric form of sodium alginate.

Experimental fish and feeding

This study of intestinal microbiota of Atlantic salmon (*Salmo salar*) was part of a 9-week feeding trial conducted at the Research station, Nord University, Bodø. The fish (average weight 185.7 g) were maintained in 800 L tanks of a flow-through seawater system. There were 3 study groups (5 replicate tanks/group); fish of a particular group were offered one of the following feeds: low AlgOS inclusion (0.5 g/100g - AlgOS-L) or high AlgOS inclusion (2.5 g/100g - AlgOS-H) or without AlgOS (Control - C). Fish were fed twice daily, between 8:00-9:00 and 14:00-15:00, using automatic feeders (Arvo Teck, Finland). The feed intake was 0.7% BW day^{-1} for all the groups. The water flow rate, temperature and O_2 levels in the tanks were 1000 L/h, 6.8-7.5°C and above 90%, respectively. A photoperiod of 24:0 LD was maintained throughout the feeding trial.

Sampling

At the end of the 9-week feeding period, the fish were sampled after euthanizing them with an overdose (160 mg/L) of MS222 tricaine methanesulfonate (Argent Chemical Laboratories, Redmond, WA, USA). The body surface of the fish was swiped with 70% ethanol before dissection, and the GIT was aseptically removed from the abdominal cavity. The distal intestinal (DI) region was separated from the GIT, the content samples were collected (n = 25) using sterile forceps, and then the surface mucus was collected (n = 25), using a sterile glass slide. The collected samples were transferred to cryotubes, snap frozen in liquid nitrogen and later stored at -80°C.

Samples were also taken from the fish rearing system to gather information of environmental microbiota. The inlet water (n = 1) of the flow-through system, as well as the water from each tank (n = 5), were collected (1 L) and filtered using, 0.2 µm pore-size filter (Pall Corporation, Hampshire, United Kingdom). Furthermore, biofilm samples from the tank walls (n = 5) were scraped and collected in cryotubes. These samples were also flash frozen in liquid nitrogen and stored at -80°C.

DNA extraction, PCR amplification, amplicon library construction and sequencing

Genomic DNA was extracted from all samples (except water filter samples) using the Quick-DNA™ Fecal/Soil Microbe 96 kit (Zymo Research, Irvine, CA, USA). Genomic DNA from the water filter samples was extracted using Metagenomic DNA Isolation kit for water (Epicentre Biotechnologies, Madison, WI, USA), according to the manufacturer's instructions. The quality of the extracted DNA was checked on 1.2% (w/v) agarose gel and the DNA concentration was quantified using the Qubit 3.0 fluorometer (Life Technologies, Carlsbad, USA).

The V3-V4 region of the bacterial 16S rRNA gene was targeted for the PCR reactions, based on the dual-index sequencing strategy described by Kozich et al. (2013). PCR reactions were performed in triplicate; each PCR reaction was carried out in 25 µl reaction volume containing 12.5 µl of Kapa HiFi Hot Start Pcr Ready Mix (KAPA Biosystems, Woburn, USA), 1.5 µl of each forward and reverse primer (at a final concentration of 100 nM), 3.5 µl of DNAse-free water and 6 µl of DNA template. A negative PCR control without DNA template was also included in the run. The thermocycling conditions included initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 98°C for 30 s, annealing at 58°C for 30 s, extension at 72 °C for 45 s, and the final extension performed at 72°C for 2 min. After performing the PCR, the resulting amplicon triplicates were pooled and visualized on 1.2% (w/v) agarose gel. No amplification was observed in the negative PCR control. The amplified products were cut from the gel and purified using the ZR-96 Zymoclean™ Gel DNA Recovery Kit (Zymo Research), following the manufacturer's instructions and eluted in 15 µl elution buffer. The eluted amplicon libraries (sequencing libraries) were quantified using the KAPA Library Quantification Kit (Kapa Biosystems). For amplicon quantification, each library was serially diluted (1:10,000 and 1:20,000), and qPCR was performed on both of the dilutions. The qPCR reaction mixture consisted of KAPA SYBR FAST qPCR master mix containing the primer premix (12 µl), the diluted library or DNA standard (4 µl) and PCR-grade water (4 µl) for negative control. The Cq values corresponding to the different libraries and the values corresponding to the DNA standards were used to calculate the size-corrected dilution factor for each sample. Each amplicon library was subsequently diluted with low TE buffer (Qiagen, Oslo, Norway) to obtain an equimolar concentration (3 nM) before sequencing. The concentration of the normalized amplicon libraries was validated on the TapeStation (Agilent Biosystems, Santa Clara, USA). The normalized library pool was further diluted to 12 pM, spiked with equimolar 10% Phix control and then paired-end sequencing was performed on an Illumina Miseq sequencing machine (Illumina, San Diego, CA, United States) in 2 runs with inter-run calibrators (i.e., few samples of known sequencing depth) to minimize eventual

differences between sequencing runs. FASTQ files from each sample generated from the sequencing machine were used for data analysis.

Sequence data analysis

Sequence data: The quality of the raw reads obtained after high-throughput sequencing were checked using FastQC (Andrews, 2010). Only the forward reads containing the V3 region of the 16S rRNA gene were used for the downstream analysis since their quality was better than the reverse reads.

Construction of operational taxonomic unit (OTU) and taxonomy tables, using the UPARSE pipeline: The forward reads were processed and analyzed by UPARSE (USEARCH version 9.2.64) software (Edgar, 2013). The reads were truncated to 240 bp, to remove the low-quality base pairs at the 3'-end and then quality-filtered. Furthermore, chimeric sequences were removed using the UCHIME algorithm (Edgar et al., 2011) and then, quality filtered sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity threshold. This threshold was chosen because higher cut-off scores may lead to overmerging of up to 15-32% (Mysara et al., 2017). It has also been suggested that 100% is the optimal identity threshold for identifying species using V4 region-targeted sequences (Edgar, 2018c). Taxonomy annotation of short 16S rRNA tags using large databases like SILVA, Greengenes, or the full RDP database may give unreliable predictions (Edgar, 2018a; Edgar, 2018b). Hence in the present study, we employed the 16S rRNA RDP training set with species names v16. The OTU sequences were assigned to different taxa using the SINTAX algorithm (Edgar, 2016) using a bootstrap cutoff value of 0.5. Afterwards, OTUs with a confidence score <1 at the domain level and the OTUs belonging to the phyla Cyanobacteria and Chlorophyta were removed. The raw 16S rRNA gene sequence data from this study has been deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB27188.

Diversity and composition analyses: Due to differences in sequencing depth, the OTU table was rarefied to the lowest number (10,604) of sequences per sample to get an even sampling depth to facilitate comparisons between the treatment groups. Furthermore, to employ content and mucus samples from the same fish, only 21 fish from each group were considered for the downstream analyses. Adding on the tank water and biofilm samples, in total 157 samples were used for the downstream analyses.

The R package 'iNEXT' v2.0.12 was used to plot the rarefaction and extrapolation curves for the species richness of the intestinal bacterial assemblage (Hsieh et al., 2016). Codes were executed to calculate and generate diversity indices, core and rare microbiota (relative abundance of core taxa and least abundant taxa) and the corresponding plots, using the R packages 'phyloseq' v1.22.3 (McMurdie and Holmes, 2013)**,** 'microbiome' v1.0.2 (Lahti et al., 2017), and their supporting packages. All the plots were visualised using the functions in 'ggplot2' v2.2.1 (Wickham, 2009). The alpha diversity plots were generated for overall species richness (OTU counts), Shannon diversity (effective number of common OTUs), and Simpson diversity (effective number of most abundant OTUs) based on the formula suggested by Jost (2006). For the beta diversity analysis of the content samples, we incorporated weighted

UniFrac distance metric because the dispersions of the different groups for this similarity index were similar. In the case of mucus samples, beta diversity was assessed using double principal coordinates analysis (DPCoA) (Fukuyama et al., 2012).

Statistical analysis

Statistical analysis was performed using R studio v3.4.3. To detect significant differences in the alpha diversity, Kruskal-Wallis test followed by Dunn's test was employed. As for the beta diversity analysis, the dispersions of the communities were checked using betadisper; thereafter Adonis (PERMANOVA) followed by pairwise comparisons was employed (999 permutations) to understand the significant dissimilarities of the communities. To detect the differentially abundant OTUs in the treatment groups, a tool for microbiome analysis- 'ANCOM' v1.1-3 (Mandal et al., 2015) was used, and 'Boruta' v5.3.0 R package (Kursa and Rudnicki, 2010) was employed to find the relevant OTUs that caused the differences in the three study groups.

Genome mining and prediction of butyrate-biosynthesis pathways

Genome mining was performed to detect the occurrence of butyrate producing genes in the genome of the significantly abundant bacteria. The genomes of the significantly abundant bacteria (selected butyrate producers) in the DI of Atlantic salmon fed AlgOS-L were retrieved from GenBank database (Table 1) and annotated using PROKKA version 1.13 (Seemann, 2014). Butyrate production abilities of the bacteria were assessed by evaluating the distribution of the pathways in each genome, i.e., by understanding the genomic arrangement of butyrate gene clusters suggested by Anand et al. (2016). Genomes were scanned for genes known to be involved in butyrate production and these sequences were then scanned in protein databases using phmmer from HMMER v. 3.1 (Finn et al., 2015) with the default E-value parameter cutoff. Phmmer uses a hidden Markov model to predict protein domains by aligning amino acids to databases such as Pfam (Finn et al., 2016). Metabolic pathways associated with SCFA production were constructed using KASS (Moriya et al., 2007). The corresponding pathway IDs were analysed as described by (Vital et al., 2014). dbCAN2 (Zhang et al., 2018) was used to annotate Carbohydrate-Active Enzymes (CAZymes) present in the genomes of the bacteria listed in Table 1. Glycoside hydrolases (GH), glycosyl transferase (GT) and polysaccharide lyases (PL) were among the carbohydrate-active enzymes that were scanned in the genomes of the mentioned bacteria.

Results

Sequencing quality

The high-throughput sequencing generated a total of 12 911 308 high-quality raw reads from all the selected samples. The reads were clustered to 2057 OTUs at 97% identity threshold. These reads were rarified, based on sample-size, to 10 604 reads/sample, and the general adequacy of the sequencing depth was perceived by drawing the rarefaction curves.

To understand the effects of AlgOS on the bacterial diversity and composition of the DI content and mucus, we describe the alterations in the AlgOS-fed fish compared to the control fish. For this, we explain the richness (i.e., counts of individual OTUs, without regard to their abundance)

and effective number of OTUs (number equivalents of entropies), and taxonomic compositional differences. Furthermore, relative abundances of the bacterial taxa are reported based on the top 20 abundant (dominant) and low abundant taxa (less abundant compared to the dominant ones). In addition, we present the significant and relevant bacterial communities of the intestinal microbiota. We also predict the butyrate production ability of certain bacteria that were significantly abundant in the AlgOS-L group.

Diversity and compositional differences of the intestinal and environmental microbiota

The species richness of the bacterial community, both in the DI content and mucus, of the AlgOS-H group was significantly lower ($P < 0.0001$ and $P < 0.001$, respectively) compared to the control group (Figures 1A, 2A). The Shannon and the Simpson diversity measures indicated that the effective number of common species and the effective number of dominant species in the AlgOS-H group were significantly lower (Shannon diversity of content $P < 0.00007$ and mucus $P < 0.017$; Simpson diversity of content $P < 0.0007$ and mucus $P < 0.018$) compared to the control group (Figures 1B, 2B, 1C, 2C). Faith's phylogenetic diversity also exhibited a similar trend; the AlgOS-H group had significantly lower ($P < 0.0001$ for content, $P < 0.002$ for mucus) diversity compared to the control group (Supplementary Figures 1A, B). DPCoA, and PCoA based on the weighted UniFrac distance matrix revealed the beta diversity of the DI bacterial communities. We detected significant differences between the control and AlgOS-fed groups (Figure 1D: F statistic = 5.8676, $R^2 = 0.188$, $P < 0.001$; and Figure 2D: F statistic = 3.783, $R^2 = 0.113$, $P < 0.005$).

The beta diversity analyses were performed for the rearing tank water, and biofilm bacterial communities corresponding to the feeding groups. DPCoA revealed that neither the bacterial communities in the water (Supplementary Figure 2, F-statistic = 0.80906, $R^2 = 0.118$, $P > 0.601$) nor those in the biofilm (Supplementary Figures 3A, F statistic = 1.3341, $R^2 = 0.1819$, $P > 0.154$) were different. DPCoA showed significant differences between biofilm and the fish-associated intestinal bacterial communities (Supplementary Figures 3B-G). Since the DNA extraction from water was performed using a different kit, we have not presented the comparison between the water bacterial communties and the intestinal bacterial communities.

Abundances of the intestinal bacteria

Proteobacteria and Spirochaetes were more abundant than the rest, in both content and mucus of AlgOS-fed groups (Figures 3A, 4A). Although Tenericutes was found to be dominant in the control group, the effect of AlgOS on these bacteria is not evident in Figures 3A, B, and 4A, B.

As for the content, Proteobacteria and Spirochaetes were dominant in the AlgOS groups compared to the control group (Figure 3B). Tenericutes, Bacteroidetes, and Firmicutes were the other dominant bacterial phyla. Bacteroidetes and Firmicutes in particular were abundant in the DI content of the control fish compared to the AlgOS-fed fish. In the mucus too AlgOS feeding increased the abundance of the dominant phyla, namely Proteobacteria and Spirochaetes (Figure 4B). Bacteroidetes, Firmicutes, and Tenericutes were also found to be dominant in the mucus, but their abundances were lower in the AlgOS-fed fish compared to the control fish.

At the genus level, *Brevinema* and *Photobacterium* (*Brevinema andersonii* and *Photobacterium phosphoreum*) were found to be the most dominant ones in the content and mucus of the AlgOS groups compared to the control group (Figures 3C, D, 4C, D). A similar trend in dominance was noted for *Aliivibrio* (*Aliivibrio logei*) too. All the other dominant genera (*Weissella*, *Sneathiella*, *Polaribacter*, *Lewinella*, *Dokdonia* and *Kordia*) were lower (in the order of 1000-2000) in the content of the AlgOS-fed fish (Figures 3C, D and Supplementary Figures 4A-E). Genera such as *Marinobacter*, *Sneathiella*, *Polaribacter*, *Lewinella* were lower (in the order of 1500-4000) in the DI mucus of the AlgOS-fed salmon (Figures 4C, D and Supplementary Figures 5A-D). *Aquabacterium*, *Bradyrhizobium*, *Methylobacterium*, *Phyllobacterium*, *Ralstonia* and *Novosphingobium* (*Aquabacterium parvum*, *Bradyrhizobium jicamae*, *Methylobacterium fujisawaense*, *Phyllobacterium myrsinaceaum*, *Ralstonia pickettii*, *Novosphingobium sediminicola*) were the abundant (in the order of 2000-10000) genera in the mucus samples of the AlgOS-L group but reduced in the AlgOS-H group compared to the control group (Figures 4C, D and Supplementary Figures 6A-F).

Core and rare bacterial taxa of the intestinal microbiota

We determined the abundance of the common core and rare taxa at prevalence and detection thresholds of 90 and 20%, respectively. The dominant genera in the content and mucus, *Brevinema* and *Photobacterium* (*P. phosphoreum* and *B. andersonii*) were found among the core members (Figures 5, 6). *Aliivibrio*, *Sneathiella* (*A. logei*, *S. glossodoripedis*) and *Mycoplasma* were also shared core taxa of the content. The common core taxa in the mucus included the aforementioned core taxa of the content (except *S. glossodoripedis)* and other genera such as *Phyllobacterium*, *Aquabacterium*, *Methylobacterium*, *Ralstonia*, *Bradyrhizobium* (*P. myrsinacearum*, *A. parvum*, *R. pickettii*, *M. fujisawaense* and *B. jicamae*). The DPCoA plot showed differential clustering of the core members of the AlgOS and control groups (content: F-statistic: 3.715, $R^2 = 0.128$, $P < 0.001$, mucus: F-statistic: 4.072, $R^2 = 0.137$ 1.0, $P < 0.01$ – Supplementary Figures 7A, B).

The significantly different bacterial communities of the intestinal microbiota

In the DI content, AlgOS-fed fish had certain groups of significantly ($P < 0.05$) abundant bacteria compared to the control fish. Firmicutes, Spirochaetes, and Proteobacteria were the abundant phyla (Supplementary Figure 8A), and Spirochaetia and Gammaproteobacteria were the significantly abundant classes (Supplementary Figure 8B). Spirochaetales, Vibrionales (order), Vibrionaceae, Brevinemataceae (family), *Brevinema*, *Photobacterium*, and *Aliivibrio* were the significantly abundant bacteria (Supplementary Figures 8C, D, E, respectively).

In the DI mucus, Betaproteobacteria was the significantly abundant class in the AlgOS-L group (Supplementary Figure 9B). Burkholderiales, Alcaligenaceae, Sphingobacteriaceae, Burkholderiaceae, Microbacteriaceae, *Achromobacter*, *Aquabacterium*, *Novosphingobium*, and *Micrococcus* were also significantly abundant in the AlgOS-L group (Supplementary Figures 9C-E). However, the phyla Actinobacteria and Proteobacteria were significantly reduced in the AlgOS-L group (Supplementary Figure 9A).

Here we report the species that were significantly different in the AlgOS-fed fish. *P. phosphoreum*, *A. logei* and *B. andersonii* were significantly abundant in the content of the

AlgOS-fed fish (Supplementary Figure 8F). *Achromobacter insolitus, Aquabacterium parvum, N. sediminicola,* and *Microbacterium ginsengiterrae* were significantly abundant in the mucus of the AlgOS-L group compared to the control group (Supplementary Figure 9F).

Relevant bacterial communities of the intestinal microbiota

Boruta analysis gave 6 and 4 relevant OTUs in content and mucus samples, respectively. These OTUs discriminate the three study groups, i.e., OTUs with higher abundance in one study group (Supplementary Figures 10A-E). In the DI content, Gammaproteobacteria, *Acetanaerobacterium*, Alteromonadaceae, Desulfuromonadales, and few taxa belonging to Bacteriodetes (*Psychroserpens jangbogonensis*, Supplementary Figure 10A, and Winogradskyella) were found to be relevant for the discrimination. An OTU of *Acetanaerobacterium* (*A. elongatum*, belonging to Firmicutes; Supplementary Figure 10B) and Gammaproteobacteria were found to be abundant in the AlgOS groups, respectively compared to control group. In DI mucus, genera such as *Phyllobacterium*, *Undibacterium* and *Microbacterium* (*Phyllobacterium myrsinacearum, Undibacterium oligocarboniphilum* and *M. ginsengiterrae*) were found to be abundant in the AlgOS-L group (Supplementary Figures 10C-E).

Occurrence of butyrate producing genes in the genome of the significantly abundant and relevant intestinal bacteria

The gene clusters associated with butyrate production−from substrates such as 4-aminobutyrate and pyruvate–were present only in *A. insolitus*, *A. parvum*, and *P. myrsinacearum*. *A. logei* is capable of producing butyrate from glutarate and *B. andersonii* has the genes for the 4-aminobutyrate pathway that is necessary for butyrate production. *A. insolitus* and *A. parvum* can produce butyrate via the pyruvate pathway. *P. myrsinacearum* can use both pyruvate and 4-aminobutyrate pathways to produce butyrate (Table 1). CAZyme families are present in the mentioned genomes; specifically, GHs and PLs families are known to participate in polysaccharide depolymerization (Kabisch et al., 2014).

Discussion

Prebiotics are intended to selectively target host microorganisms that can ferment the indigestible carbohydrate and stimulate the growth of specific bacteria to produce bioactive metabolites (Gibson et al., 2017). The beneficial bacteria and their metabolites such as SCFAs are known to provide health benefits to the hosts (Gibson et al., 2017). Furthermore, the gut microbiota can be reshaped by fine-tuning the carbohydrate food components; to improve host health status and tackle diseases, as in the case of mice fed a marine polysaccharide (Shepherd et al., 2018). In the present study, we investigated the effect of *Laminaria* sp.-derived AlgOS on the diversity and composition of bacterial communities in the intestine of Atlantic salmon. We analysed the DI content and mucus separately to understand the differences in the microbiota associated with them.

Our results revealed that AlgOS supplementation causes an overall reduction in bacterial diversity of the DI bacterial community of the fish fed 2.5% AlgOS (AlgOS-H group) compared to the control fish. However, 0.5% AlgOS supplementation (AlgOS-L group) in feed effected similar changes without lowering the bacterial diversity. In this fish group, the phyla Proteobacteria and Spirochaetes were dominant, in both DI content and mucus. Certain species of Proteobacteria (dominant), Spirochaetes (dominant), Actinobacteria (low abundance) were relatively abundant/significantly abundant bacteria in the 0.5% AlgOS-fed fish.

AlgOS shifts the diversity of the intestinal bacteria

Previous studies have shown that oligosaccharides like GOS and inulin reduced the bacterial diversity in mouse fecal samples (Cheng et al., 2017). Similarly, pectic oligosaccharides also decreased the microbial diversity and richness of caecal microbiota in mice (Bindels et al., 2015). Furthermore, simplified cecal microbiota was a characteristic of rats fed alginate compared with those fed a control diet (An et al., 2013). In contrast to our observations, an increase in the intestinal bacterial diversity under the influence of oligosaccharide supplementation has been previously reported in gilthead sea bream (Dimitroglou et al., 2010) and rats (Ou et al., 2016). On the other hand, a prebiotic blend (FOS+GOS+inulin+anthocyanins) did not alter the diversity of the gut microbiota of mice (Chen et al., 2017).

Piazzon et al. (2017) have shown that the intestinal bacteria of gilthead sea bream (*Sparus aurata*) fed more plant-derived ingredients had apparently lower Shannon index. The alpha diversity of the intestinal bacterial of largemouth bronze gudgeon (*Coreius guichenoti*) suffering from furunculosis was significantly lower compared to healthy fish (Li et al., 2016). The reduction in microbial diversity of the intestinal microbiota of AlgOS-H salmon group is intriguing and deserves further verification of the loss in the number of beneficial bacteria. It should be noted that the intestinal microbial diversity of the AlgOS-L fish group was not impacted significantly. Ecological stability in a gut environment is linked to high microbial diversity, and preservation of functions of beneficial symbionts (Chassard et al., 2008). However, high bacterial diversity and increase in cooperating microbes could jeopardize the ecological stability (Coyte et al., 2015). Hence a decrease in bacterial diversity does not always point to an unstable ecosystem. Furthermore, decrease in intestinal bacterial diversity, shifts in bacterial compositions and disruptions of community functions are associated with ill-health (Li et al., 2016). Future studies should reveal the competing and cooperating communties in the AlgOS-fed fish.

AlgOS reduces the abundance of certain Firmicutes and Bacteroidetes

Members of the phylum Bacteroidetes are prominent among the gut microbiota – they can be pathogens and have the capacity to degrade polysaccharides (Thomas et al., 2011). The metabolic functions of the Bacteroidetes that were reduced in the ALgOS-fed fish are still unknown.

Previous studies have shown that oligosaccharide can reduce the abundance of Firmicutes in the gut microbiota of mice (Petersen et al., 2010) and humans (Vigsnaes et al., 2011), as noted in the present study. In humans, dietary resistant starch was found to correlate with the abundance of Firmicutes and Bacteroidetes; the butyrate-producing Firmicutes dominate Bacteroidetes (Bacteroides) and the medium abundant Proteobacteria (Maier et al., 2017). In obese mice, FOS administration increased Bacteriodetes but decreased Firmicutes (Everard et al., 2011). Studies have reported that Firmicutes and Bacteriodetes are dominant in the fecal samples of Atlantic salmon during early summer, but later on lose their dominance to Proteobacteria (Zarkasi et al., 2014). Thus, prebiotics are likely to affect the dominant phyla of mammals and fish.

AlgOS facilitated the dominance of Proteobacteria, Spirochaetes and Actinobacteria

The significantly abundant and the relevant species indicate that AlgOS stimulated the growth of certain bacteria, especially in AlgOS-L fish. Previous studies have also demonstrated that Proteobacteria is the most abundant phylum in many marine and freshwater fishes (Roeselers et al., 2011; Li et al., 2015; Liu et al., 2016; Lokesh and Kiron, 2016), and it is also known to dominate the gut microbiota of Atlantic salmon (Gajardo et al., 2016; Lokesh et al., 2018). Therefore, it is not surprising to find Proteobacteria as one of the most abundant and dominant bacterial phyla.

Our study shows that within Proteobacteria, the salmon intestinal microbiota had a high representation of the class Gammaproteobacteria, followed by Alphaproteobacteria, Betaproteobacteria, and Deltaproteobacteria. We report some bacteria at species level to describe the plausible prebiotic effect of the test product. Prediction of taxonomy at species level is challenging since the sequences linked to cultured bacteria could vary from those predicted using algorithms connected to reference databases. It is known that the average accuracy of genus and species predictions based on V3-V5 region are 60 and 22%, respectively (Edgar, 2018a). Using a short hypervariable tag of 16S rRNA gene for taxonomy assessment comes with a limitation; insufficient depth in taxonomic resolution. We employed an RDP training set reference database using a bootstrap method to get the best possible predictions (Edgar, 2018a). Previous studies have shown that Proteobacteria are involved in metabolic pathway modules that participate in carbon and nitrogen fixation and in the stress response regulatory system (Vikram et al., 2016). Proteobacteria may also contribute to the digestive process in fish (Romero et al., 2014). *P. phosphoreum* belonging to the class *Gammaproteobacteria* is a known gut symbiont of marine fish, and this bacterium is capable of chitin digestion and uses luciferase to reoxidize reduced coenzymes and other molecules for metabolism (Nealson and Hastings, 1979). Although this species was significantly abundant in the content of the AlgOS-fed fish, we did not find a corresponding abundance in the mucus. Soybean meal can decrease the abundance of *P. phosphoreum* in the DI of Atlantic salmon (Desai et al., 2012). However, we observed an increase in abundance of this bacterium and another member of Proteobacteria (*A. logei*) as a result of AlgOS feeding.

In the mucus of AlgOS-L fish, few members of Proteobacteria including *A. parvum, B. jicamae* and *M. fujisawaense* (Supplementary Figures 6A-C) were significantly abundant. *A. parvum* is

known as a nitrate-dependent Fe(II)-oxidizing bacterium (Zhang et al., 2016). In the genus *Bradyrhizobium*, many bacteria are known to fix nitrogen (Peix et al., 2015), but currently, no information is available for *B. jicamae.* Hsiang-Yi et al. (2018) has reported the presence of *B. jicamae* in the intestinal microbiota of Anguillid eel species. Although *Methylobacterium* species are methylotrophs and they are described as agents of contamination and opportunistic infections in humans (Lai et al., 2011), details of *M. fujisawaense* are not yet reported.

The discriminatory OTUs, revealed through Boruta analysis, indicated that Gammaproteobacteria and Alteromonadaceae were abundant in the content of the AlgOS-L group. Likewise, the relevant OTUs in the mucus were abundant in the AlgOS-L group, and most of them were Proteobacteria (*P. myrsinacearum*, *U. oligocarboniphilum* (Supplementary Figures 10C, D). *P. myrsinacearum* belongs to Alphaproteobacteria; this bacterium that is associated with macroalgae is a nitrogen fixer (Gonzalez-Bashan et al., 2000). Its abundance increased in the AlgOS-L group but decreased in the AlgOS-H group, compared to the control fish. *P. myrsinacearum* has been reported in intestinal mucosa of grass carp, *Ctenopharyngodon idellus* (Huan et al., 2015). The functional relevance of *U. oligocarboniphilum* (Betaproteobacteria) to the host is not yet described.

Spirochaetes is the second most abundant and dominant phyla in the AlgOS-fed fish. *B. andersonii* was found to be the dominant species; it was also one of the core bacterial members of the DI of Atlantic salmon. Tapia-Paniagua et al. (2014) has reported *B. andersonii* in the intestinal microbiota of flat fish (*Solea senegalensis*). While Spirochaetes include species that cause disease in vertebrates, they are also known as abundant endosymbionts and lignocellulose digesters and nitrogen fixation helpers in termite guts (Kudo, 2009). However, functional information of this bacteria in the gut of fish is not yet reported. *B. andersonii* was significantly higher in the content of Atlantic salmon that consumed AlgOS-containing feeds. Our *in silico* analyses indicate that *B. andersonii* has genes that are necessary for butyrate production. In addition to the dominant Proteobacteria, some dominant/rare species of Actinobacteria, were relatively abundant/significantly abundant in the AlgOS-L fish. The abundance of *M. ginsengiterrae*, a β-glucosidase-producing bacterium (Kim et al., 2010) which belongs to Actinobacteria, increased in the AlgOS-L group. However, their functional role in the gut of the fish needs to be elucidated.

Low-AlgOS stimulated the abundance of bacteria with butyrate-producing genes in their genome

Butyrate production occurs via pyruvate by breakdown of complex polysaccharides, or via amino acids which serves as substrates for lysine, glutarate and 4-aminobutyrate pathways. However, all the pathways have a common step where crotonyl-CoA is transformed to butyryl-CoA (Vital et al., 2014). Pathway analysis suggested that the intestinal bacteria in our study have gene clusters for pyruvate and acetyl-CoA pathway; most of the reported butyrate producers are known to synthesize butyrate via pyruvate pathway (Vital et al., 2014; Anand et al., 2016). Intriguingly, the genomes we examined have CAZymes–GHs, GTs and PLs. Particularly GH3 and PL7 are known to be associated with algal polysaccharide utilization (Kabisch et al., 2014). Among the listed bacteria in our study, *P. myrsinacearum* has the genes for both pyruvate and 4-aminobutyrate pathways.

Butyrate along with other SCFAs, namely acetate and propionate are adsorbed from the intestinal lumen, and butyrate is mainly used as an energy source by the epithelial cells (Louis et al., 2014). Prebiotics are expected to hold a 'cross-feeding effect', by which they can either act as substrates to a group of butyrate-producing microorganisms or stimulate growth of other butyrate-producing bacteria that feed on the metabolites (e.g., lactate) or end product (carbohydrate fragments) of the primary butyrate producers (Belenguer et al., 2006; Ríos Covián et al., 2016). Vital et al. (2014) have reported that members of phyla, other than Firmicutes, especially Actinobacteria, Bacteroidetes, Fusobacteria, Proteobacteria, and Spirochaetes are potential butyrate producers.

Mountfort et al. (2002) have measured the SCFAs in the hindgut of three marine herbivorous fishes and related the production to the gut microbiota; the production rate of one of the predominant SCFAs i.e., acetate in three herbivorous fin fishes and terrestrial vertebrates suggests that body temperatures do not affect the fermentation systems of the metabolic groups of their gut bacteria. Kihara (2008) showed that the increased production of SCFA in the hindgut of red seabream administered with oligosaccharide lactosucrose, although carnivorous fish are known to have lower fermentation rates. In wild carnivorous freshwater fishes, *Cetobacterium* and *Halomonas* are highly abundant, while their herbivorous counterparts were enriched with *Citrobacter* and *Leptotrichia* (Liu et al., 2016). Certain bacteria present in herbivorous fish (e.g. Vibrionales, Clostridiales) (Sullam et al., 2012) are found in the DI of Atlantic salmon also (Lokesh et al., 2018). Furthermore, morphology of the digestive system will not affect the fermentation reactions in the hindgut of fishes (Mountfort et al., 2002). However, efficient fermentation of polysaccharides in the gut ecosystem requires an optimum number of certain functional bacterial groups (Chassard et al., 2008). Therefore, *in silico* and culture-based studies can provide knowledge about the contribution of fish intestinal bacteria to butyrate production. In the present study, *A. logei*, *A. insolitus*, *A. parvum,* and *P. myrsinacearum* that were found to be abundant in the content and mucus of the AlgOS-L group had genes associated with butyrate production. The role of butyrate in maintaining the host GI health has been well documented in humans and other animals (Segain et al., 2000; Fukumoto et al., 2003). Studies have shown the potential impact of dietary butyrate in fish: in carp (*Cyprinus carpio*) it improves growth (Liu et al., 2014), in gilthead sea bream (*Sparus aurata*) it might provide energy to the enteric cells and promote absorption of essential amino-acids (Robles et al., 2013), and help restore the intestinal health (Estensoro et al., 2016). Culture-dependent studies are required to ascertain the ability of the above-mentioned high abundant bacteria in AlgOS-L group in stimulating the production of butyrate in Atlantic salmon.

Environmental bacteria do not have a prominent influence on the intestinal bacteria of Atlantic salmon

In this study, we also explored the bacterial diversity of tank biofilm samples to understand if the surrounding environmental factors affected the gut microbial population of the fish. The bacterial composition of the environmental samples were found to be significantly different from the respective fish-associated microbial communities. Other studies have also reported similar differences in the host and environmental bacteria (Lyons et al., 2017a; Lyons et al.,

2017b). In this study, the surrounding environmental factors might not have affected the intestinal bacterial profile of Atlantic salmon.

Conclusion

This comprehensive characterization of the intestinal bacterial communities of Atlantic salmon has revealed that dietary supplementation of the *Laminaria* sp.-derived AlgOS caused a shift in their diversity and composition. AlgOS supplementation, at 0.5%, did not lead to any diversity shifts. AlgOS-driven sculpting led to the dominance of Proteobacteria, Spirochaetes and increased the abundance of certain Actinobacteria. Specific metabolic groups of intestinal bacteria in the distal intestine of Atlantic salmon, a carnivorous fish could possibly ferment oligosaccharides and produce SCFA. The in-silico findings on butyrate producers should be verified through further studies. This information will be useful for future studies that explore the metabolic potential of oligosaccharide-stimulated gut bacteria and their effect on the host.

Author contributions

VK, MS and JF procured the funding for the study. VK, MS, RP, JF and SG designed the study. RP provided the AlgOS. VK, MS and SG conducted the feeding experiment. SG performed the microbiota studies including the laboratory work. SG, JL, PG, VK and JF analyzed the data. YA performed the *in-silico* butyrate pathway analysis. SG wrote the manuscript with the guidance of VK. All authors read, revised and approved the manuscript.

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Conflict of interest statement

The authors declare no conflict of interest.

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Table 1. Details of the sequences used for genome mining and the associated butyrate pathways

GenBank reference ID	**NZ_AJYJ02000000**	**NZ_FOKY00000000**	**NZ_CP019325**	**NZ_LFRI00000000**	**PVBT00000000**
Species	*Aliivibrio logei*	*Brevinema andersonii*	*Achromobacter insolitus*	*Aquabacterium parvum*	*Phyllobacterium myrsinacearum*
Butyrate production pathways*					
Pyruvate pathway	×	×	✓	✓	✓
4-aminobutyrate pathway	×	✓	×	×	✓
Lysine pathway	×	×	×	×	×
Glutarate pathway	✓	×	×	×	×

*** × and ✓ indicate absence and presence of a pathway, respectively.**

Figure legends:

Figure 1: Diversity of the bacterial communities of the distal intestinal content. Boxplots show the species richness **(A)**, Shannon index **(B)**, Simpson index **(C)**. Double principal coordinate analysis plot **(D)** shows the beta diversity of the bacterial communities. Different letters indicate statistically significant differences ($P < 0.05$) between the study groups (Control group, CDC; AlgOS-L-fed group, LDC; AlgOS-H-fed group, HDC).

Figure 2: Diversity of the bacterial communities of distal intestinal mucus. Boxplots show the species richness **(A)**, Shannon index **(B)**, Simpson index **(C)**. Double principal coordinate analysis plot **(D)** shows the beta diversity of the bacterial community. Different letters indicate statistically significant differences ($P < 0.05$) between the study groups (Control group, CDM; AlgOS-L-fed group, LDM; AlgOS-H-fed group, HDM).

Figure 3: Barplots showing the abundance of the bacterial phyla **(A)**, dominant phyla **(B)**, dominant genera **(C)** and dominant species **(D)** in the distal intestinal content. The height of each bar segment represents the abundance of individual operational taxonomic units (OTUs) stacked in order from greatest to least, and separated by a thin black border line. Colour codes: Proteobacteria – green, Spirochaetes – dark blue, Bacteroidetes – light blue and pink, Firmicutes – yellow, Tenericutes – magenta. Sample names starting with − CDIC are control distal intestinal content, LDIC are AlgOS-L-fed distal intestinal content and HDIC are AlgOS-H-fed distal intestinal content.

Figure 4: Barplots showing the abundance of the bacterial phyla **(A),** dominant phyla **(B)**, dominant genera **(C)** and dominant species **(D)** in the distal intestinal mucus. The height of each bar segment represents the abundance of individual operational taxonomic units (OTUs) stacked in order from greatest to least, and separated by a thin black border line. Colour codes: Proteobacteria – green, Spirochaetes – dark blue, Bacteroidetes – light blue and pink, Firmicutes – yellow, Tenericutes – magenta. Sample names starting with − CDIM are control distal intestinal mucus, LDIM are AlgOS-L-fed distal intestinal mucus and HDIM are AlgOS-H-fed distal intestinal mucus.

Figure 5: Abundance of the core bacterial taxa in the distal intestinal content of Atlantic salmon from the three study groups. Sample names starting with − CDIC are control distal intestinal content, LDIC are AlgOS-L-fed distal intestinal content and HDIC are AlgOS-H-fed distal intestinal content.

Figure 6: Abundance of the core bacterial taxa in the distal intestinal mucus of Atlantic salmon from the three study groups. Sample names starting with − CDIM are control distal intestinal mucus, LDIM are AlgOS-L-fed distal intestinal mucus and HDIM are AlgOS-H-fed distal intestinal mucus.

Supplementary Figure legends:

Supplementary Figure 1: Boxplots showing the Faith's phylogenetic diversity of the bacterial communities of distal intestinal content **(A)** and mucus **(B)**. Different letters indicate statistically significant differences ($P < 0.05$) between the study groups.

Supplementary Figure 2: Double principal coordinate analysis plot showing the beta diversity of the tank water bacterial communities.

Supplementary Figure 3: Double principal coordinate analysis plots showing the beta diversity of the bacterial communities. Tank biofilm bacteria **(A)**, control distal intestinal content and biofilm bacteria: F-statistic = 2.0828, $R^2 = 0.0798$, $P > 0.13$ **(B)**, low distal intestinal content and biofilm bacteria: F-statistic = 2.3027, $R^2 = 0.0875$, $P > 0.86$ **(C)**, high distal intestinal content and biofilm bacteria: F-statistic = 3.7455, $R^2 = 0.1349$, $P > 0.027$ **(D),** control distal intestinal mucus and biofilm bacteria: F-statistic = 2.3912, $R^2 = 0.906$, $P > 0.074$ **(E)**, low distal intestinal mucus and biofilm bacteria: F-statistic = 9.9021, $R^2 = 0.2920$, $P > 0.002$ **(F)**, high distal intestinal mucus and biofilm bacteria: F-statistic = 6.2846, $R^2 = 0.2075$, $P > 0.012$ **(G)**.

Supplementary Figure 4: Barplots showing the abundance of bacterial species in the distal intestinal content. *Weissella cibaria* **(A)**, *Sneathiella glossodoripedis* **(B)**, *Polaribacter reichenbachii* **(C)**, *Lewinella antarctica* **(D)**, *Kordia Antarctica* **(E).**

Supplementary Figure 5: Barplots showing the abundance of bacterial species in the distal intestinal mucus. *Marinobacter salarius* **(A)**, *Sneathiella glossodoripedis* **(B)**, *Polaribacter reichenbachii* **(C)**, *Lewinella antarctica* **(D)**.

Supplementary Figure 6: Barplots showing the abundance of bacterial species in the distal intestinal mucus of AlgOS-L group. *Aquabacterium parvum* **(A)**, *Bradyrhizobium jicamae* **(B)**, *Methylobacterium fujisawaense* **(C)**, *Phyllobacterium myrsinacearum* **(D),** *Ralstonia pickettii* **(E),** *Novosphingobium sediminicola* **(F).**

Supplementary Figure 7: Double principal coordinate analysis plots showing the beta diversity of the core taxa of the distal intestinal content **(A)** and the beta diversity of the core taxa of the distal intestinal mucus **(B)**.

Supplementary Figure 8: Polar plots showing the differences in abundances of significantly different intestinal content bacteria, at different taxonomic levels. Phylum **(A)**, class **(B)**, order **(C)**, family **(D)**, genus **(E)**, and species **(F)**.

Supplementary Figure 9: Polar plots showing the differences in abundances of significantly different intestinal mucus bacteria, at different taxonomic levels. Phylum **(A)**, class **(B)**, order **(C)**, family **(D)**, genus **(E)**, and species **(F)**.

Supplementary Figure 10: Barplots showing the abundance of relevant bacterial taxa in the distal intestinal content and mucus of the AlgOS-fed groups. *Psychroserpens jangbogonensis* **(A)**, *Acetanaerobacterium elongatum* **(B)**, *Phyllobacterium myrsinacearum* **(C)**, *Undibacterium oligocarboniphilum* **(D)** and *Microbacterium ginsengiterrae* **(E).**

Figure 1.

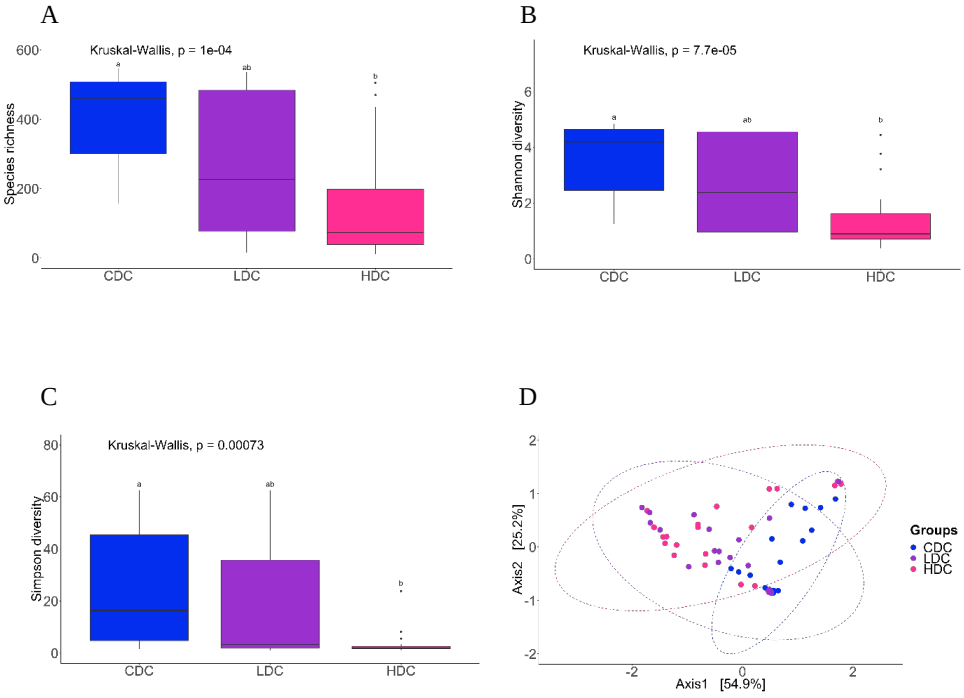
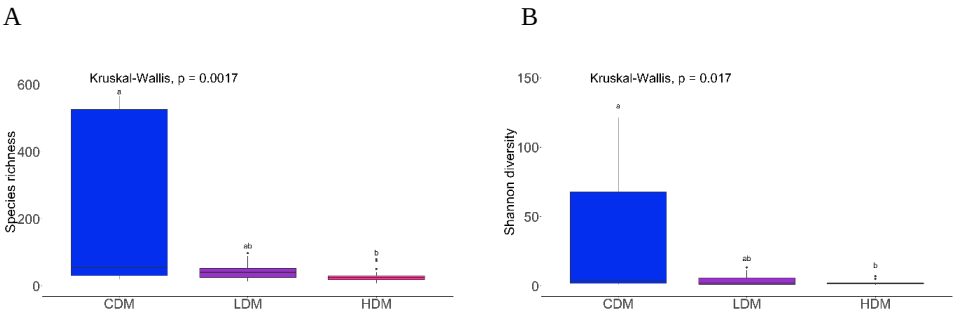


Figure 2.



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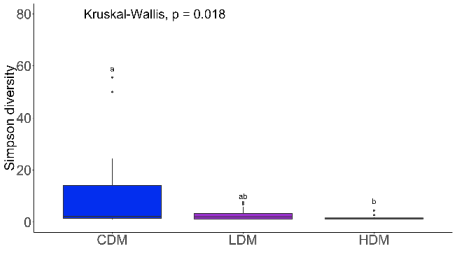

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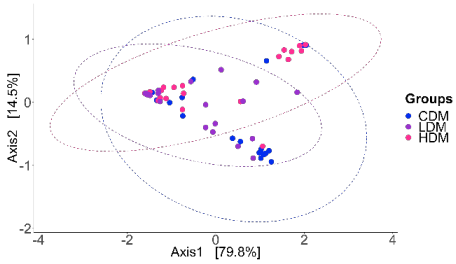
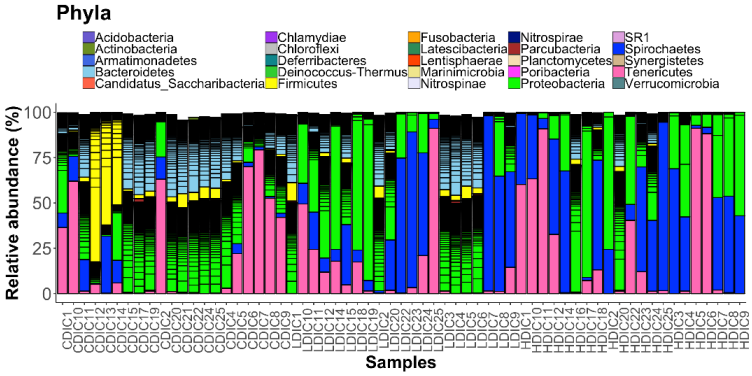


Figure 3.

A



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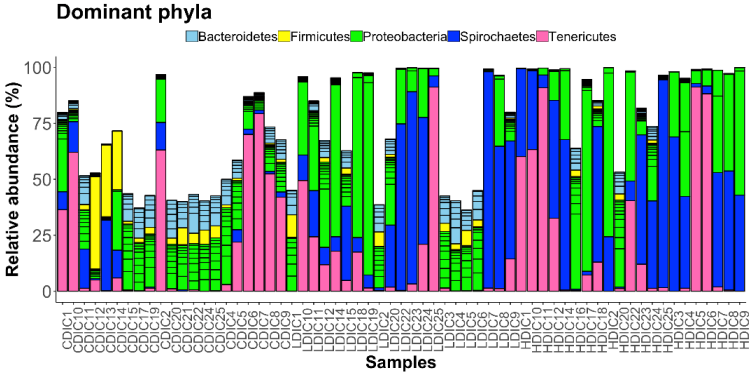

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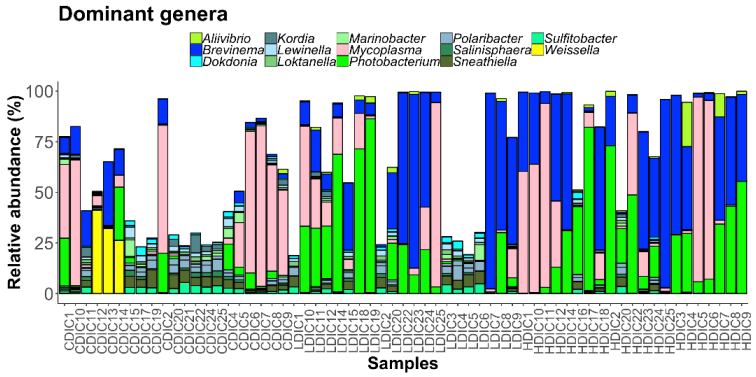

Dominant genera
Aliivibrio
Brevinema
Dokdonia
Kordia
Lewinella
Loktanella
Marinobacter
Mycoplasma
Photobacterium
Polaribacter
Salinisphaera
Sneathiella
Sulfitobacter
Weissella
Relative abundance (%)
100
75
50
25
0
Samples

D

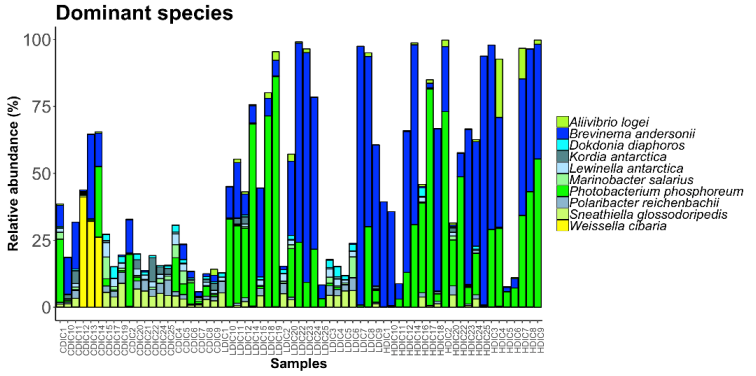
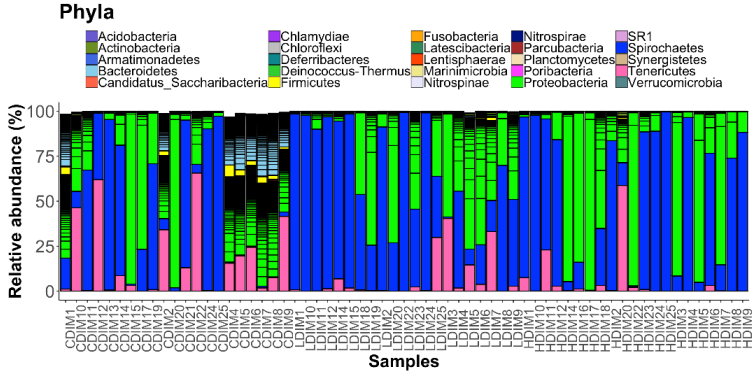
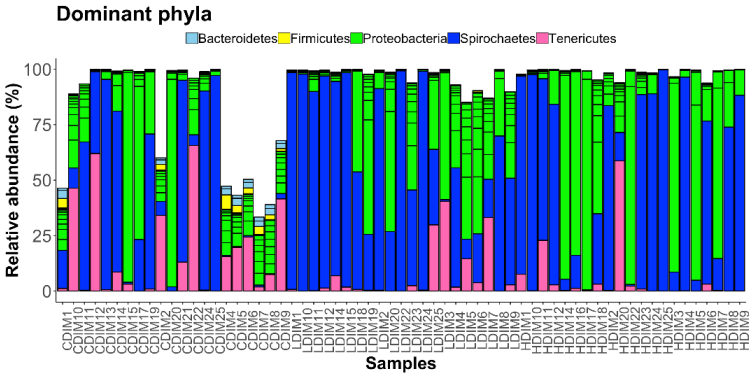

Dominant species
Relative abundance (%)
100
75
50
25
0
Aliivibrio logei
Brevinema andersonii
Dokdonia diaphoros
Kordia antarctica
Lewinella antarctica
Marinobacter salarius
Photobacterium phosphoreum
Polaribacter reichenbachii
Sneathiella glossodoripedis
Weissella cibaria
Samples

Figure 4.

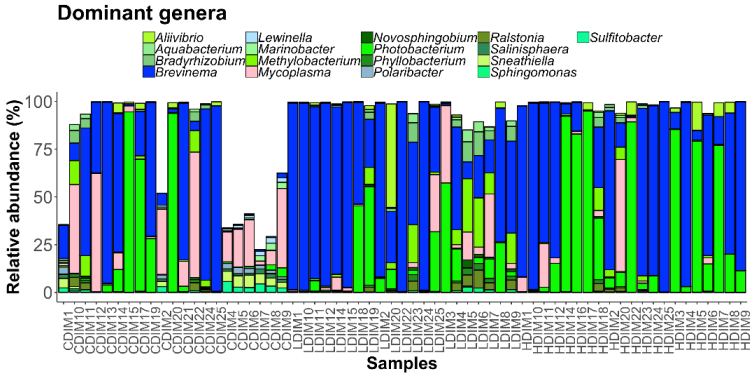
A


Phyla
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Candidatus_Saccharibacteria
Chlamydiae
Chloroflexi
Deferribacteres
Deinococcus-Thermus
Firmicutes
Fusobacteria
Latescibacteria
Lentisphaerae
Marinimicrobia
Nitrospinae
Nitrospirae
Parcubacteria
Planctomycetes
Poribacteria
Proteobacteria
SR1
Spirochaetes
Synergistetes
Tenericutes
Verrucomicrobia
Relative abundance (%)
100
75
50
25
0
Samples

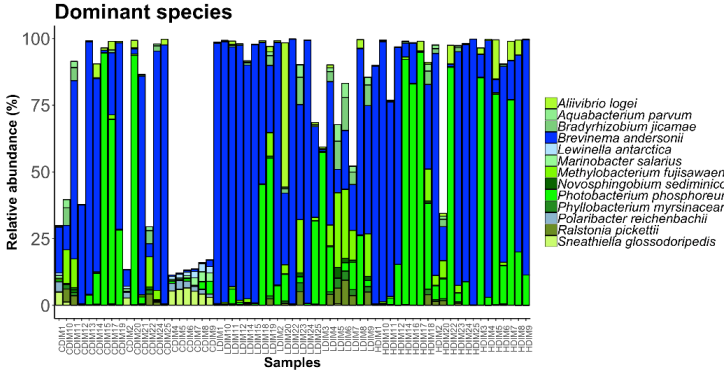
B


Dominant phyla
Bacteroidetes
Firmicutes
Proteobacteria
Spirochaetes
Tenericutes
Relative abundance (%)
100
75
50
25
0
Samples

C


Dominant genera
Aliivibrio
Aquabacterium
Bradyrhizobium
Brevinema
Lewinella
Marinobacter
Methylobacterium
Mycoplasma
Novosphingobium
Photobacterium
Phyllobacterium
Polaribacter
Ralstonia
Salinisphaera
Sneathiella
Sphingomonas
Sulfitobacter
Relative abundance (%)
100
75
50
25
0
Samples

D


Dominant species
Relative abundance (%)
100
75
50
25
0
Samples
Aliivibrio logei
Aquabacterium parvum
Bradyrhizobium jicamae
Brevinema andersonii
Lewinella antarctica
Marinobacter salarius
Methylobacterium fujisawaense
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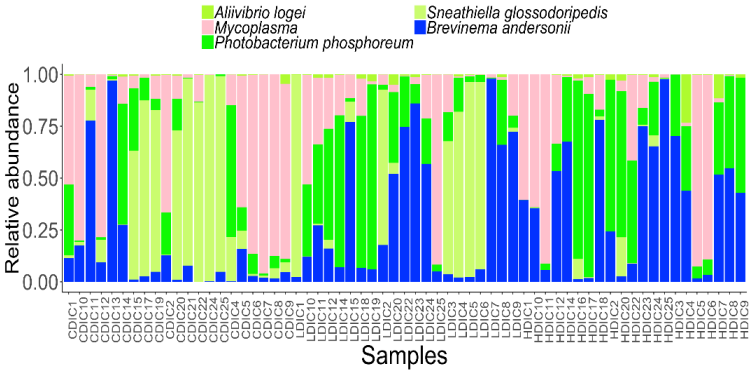
Photobacterium phosphoreum
Phyllobacterium myrsinacearum
Polaribacter reichenbachii
Ralstonia pickettii
Sneathiella glossodoripedis

Figure 5.



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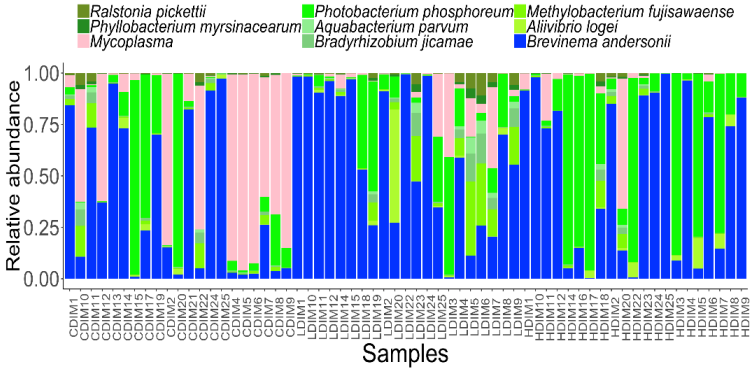
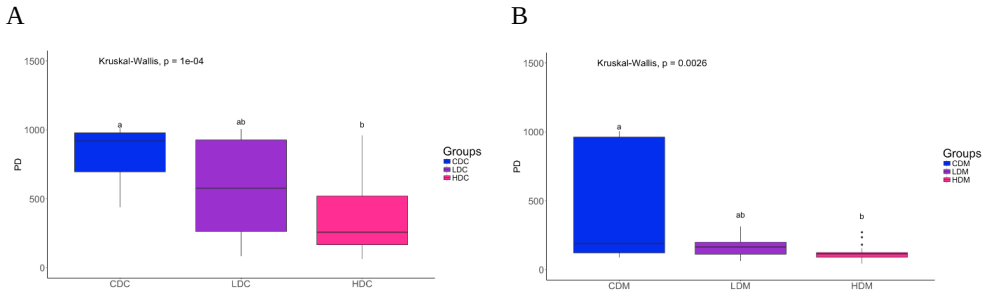


Supplementary Figure 1.



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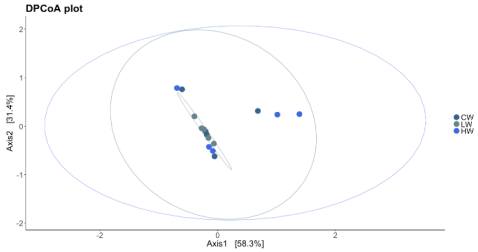

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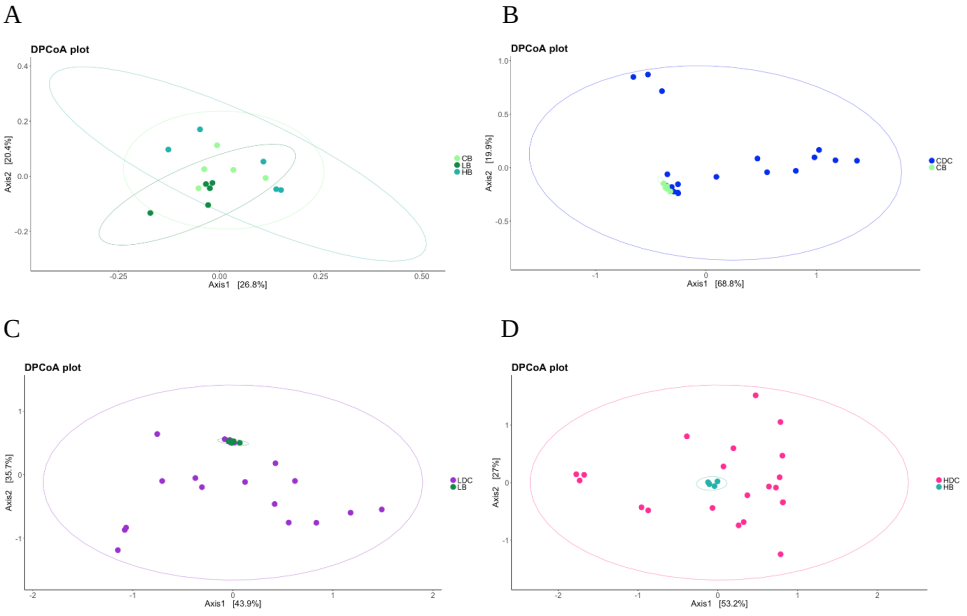

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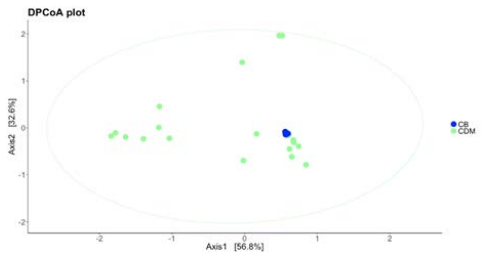

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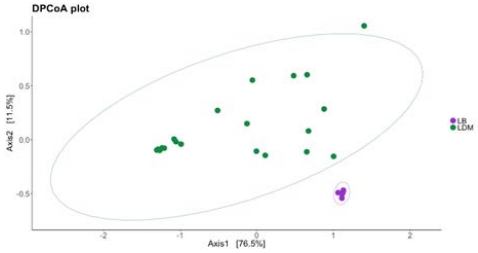

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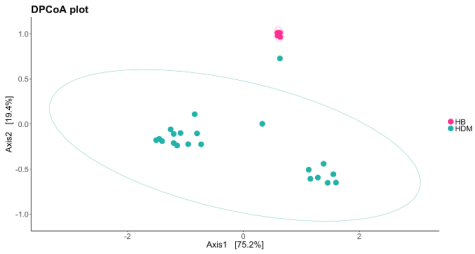
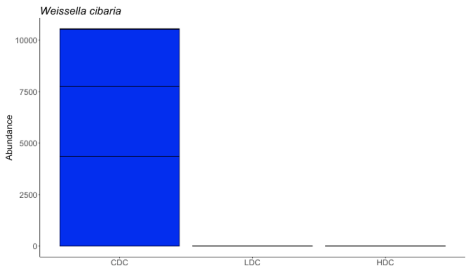


Supplementary Figure 4.

A



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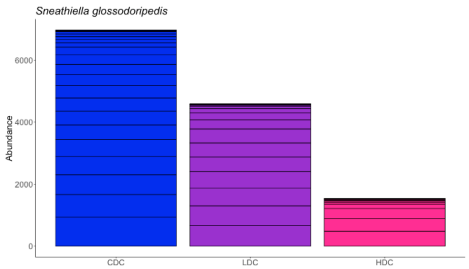

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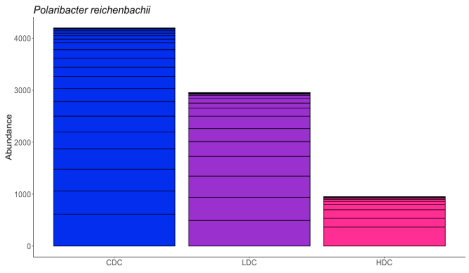

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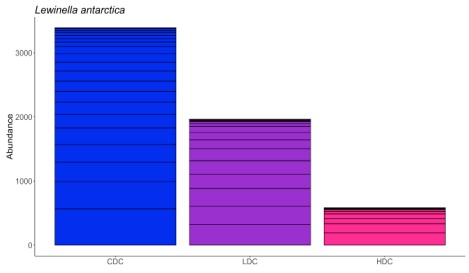

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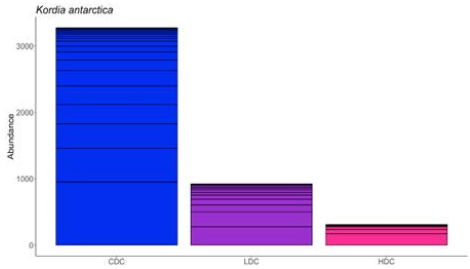
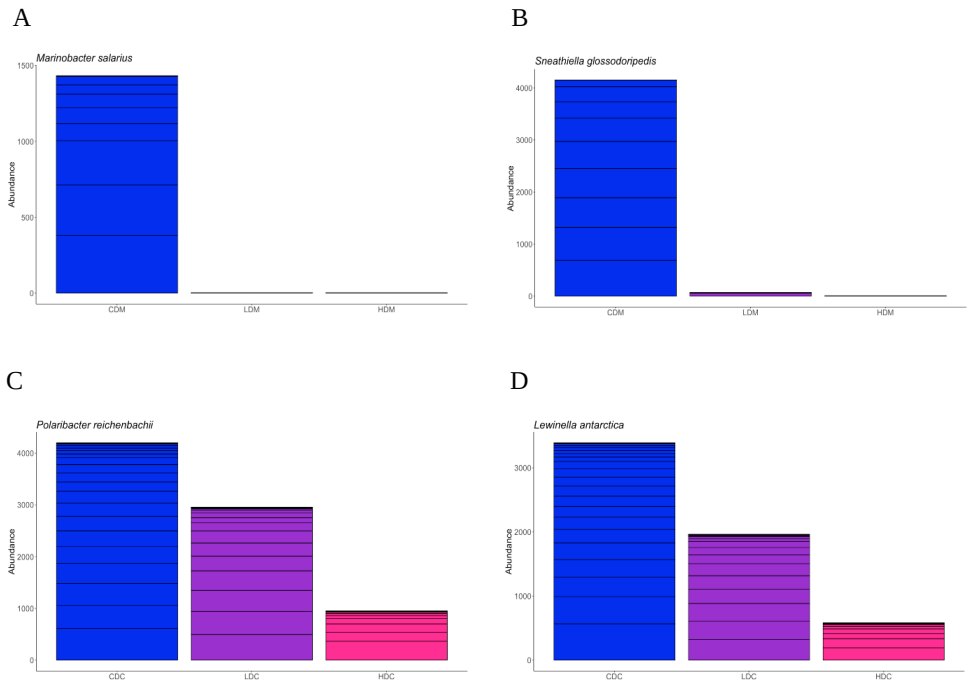


Supplementary Figure 5.



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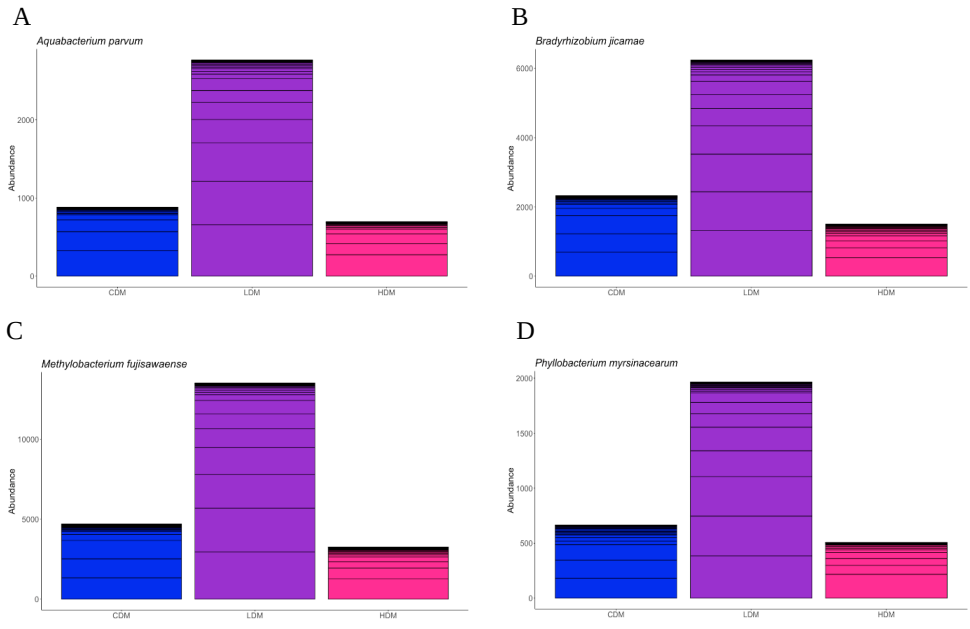

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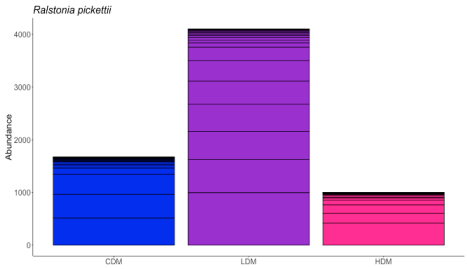

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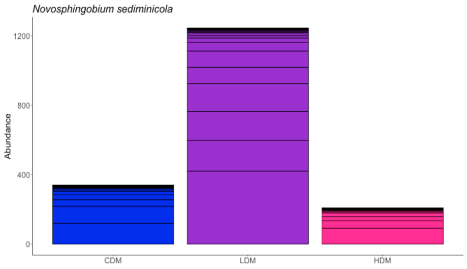
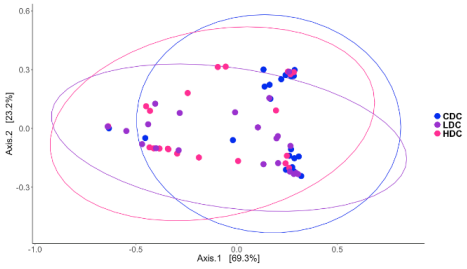


Supplementary Figure 7.

A



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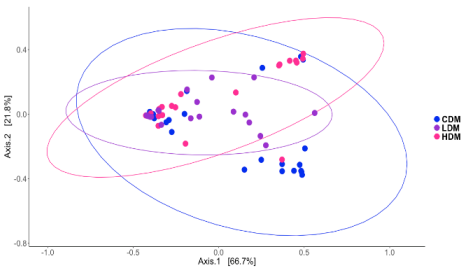
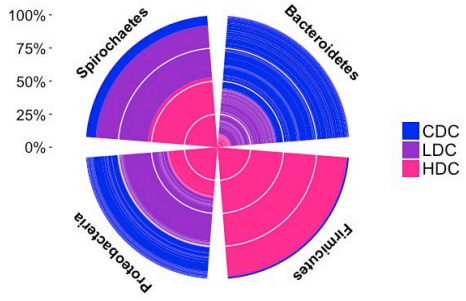


Supplementary Figure 8.

A



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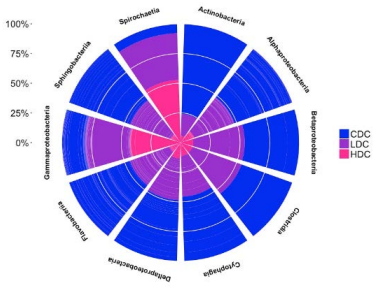

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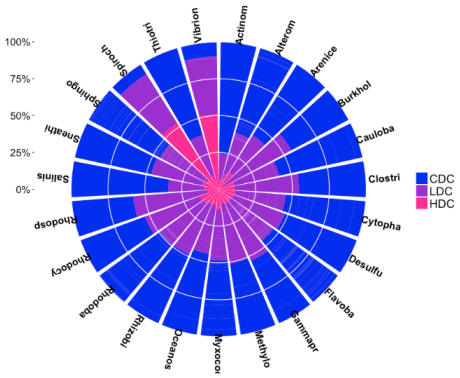

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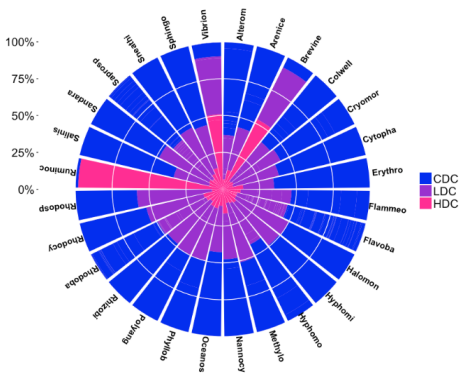

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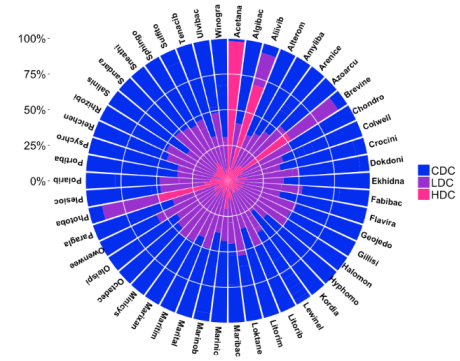

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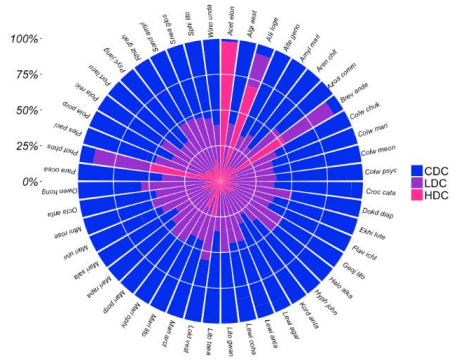


Supplementary Figure 8 abbreviations

Order

Spirocha	:	Spirochaetales
Sneathie	:	Sneathiellales
Sphingob	:	Sphingobacteriales
Rhodobac	:	Rhodobacterales
Arenicel	:	Arenicellales
Alteromo	:	Alteromonadales
Flavobac	:	Flavobacteriales
Gammapro:		Gammaproteobacteria_incertae_sedis
Caulobac	:	Caulobacterales
Sphingom	:	Sphingomonadales
Methyloc	:	Methylococcales
Cytophag	:	Cytophagales
Myxococc	:	Myxococcales
Salinisp	:	Salinisphaerales
Rhizobia	:	Rhizobiales
Vibriona	:	Vibrionales
Desulfur	:	Desulfuromonadales
Clostrid	:	Clostridiales
Thiotric	:	Thiotrichales
Rhodocyc	:	Rhodocyclales
Oceanosp	:	Oceanospirillales
Rhodospi	:	Rhodospirillales
Burkhold	:	Burkholderiales
Actinomy	:	Actinomycetales

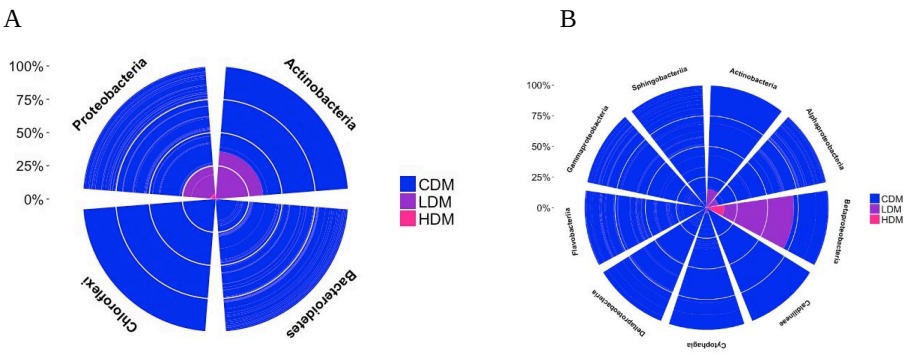
Family

Brevine	:	Brevinemataceae
Sneathi	:	Sneathiellaceae
Saprosp	:	Saprospiraceae
Rhodoba	:	Rhodobacteraceae
Arenice	:	Arenicellaceae
Alterom	:	Alteromonadaceae
Flavoba	:	Flavobacteriaceae
Hyphomo	:	Hyphomonadaceae
Sphingo	:	Sphingomonadaceae
Methylo	:	Methylococcaceae
Flammeo	:	Flammeovirgaceae
Cryomor	:	Cryomorphaceae
Polyang	:	Polyangiaceae
Nannocy	:	Nannocystaceae
Cytopha	:	Cytophagaceae
Colwell	:	Colwelliaceae
Salinis	:	Salinisphaeraceae
Phyllob	:	Phyllobacteriaceae
Erythro	:	Erythrobacteraceae
Vibrion	:	Vibrionaceae
Ruminoc	:	Ruminococcaceae
Rhodocy	:	Rhodocyclaceae
Sandara	:	Sandaracinaceae
Oceanos	:	Oceanospirillaceae
Rhodosp	:	Rhodospirillaceae
Halomon	:	Halomonadaceae
Hyphomi	:	Hyphomicrobiaceae
Rhizobi	:	Rhizobiaceae

Genus		
Brevine	:	Brevinema
Sneathi	:	Sneathiella
Arenice	:	Arenicella
Alterom	:	Alteromonas
Maribac	:	Maribacter
Sphingo	:	Sphingorhabdus
Polarib	:	Polaribacter
Flavira	:	Flaviramulus
Litorib	:	Litoribaculum
Fabibac	:	Fabibacter
Winogra	:	Winogradskyella
Kordia	:	Kordia
Crocini	:	Crocinitomix
Lewinel	:	Lewinella
Sulfito	:	Sulfitobacter
Chondro	:	Chondromyces
Ulvibac	:	Ulvibacter
Plesioc	:	Plesiocystis
Gillisi	:	Gillisia
Ekhidna	:	Ekhidna
Colwell	:	Colwellia
Hyphomo	:	Hyphomonas
Marinic	:	Marinicella
Salinis	:	Salinisphaera
Litorim	:	Litorimonas
Paragla	:	Paraglaciecola
Marinob	:	Marinobacter
Algibac	:	Algibacter
Psychro	:	Psychroserpens
Portiba	:	Portibacter
Photoba	:	Photobacterium
Dokdoni	:	Dokdonia
Acetana	:	Acetanaerobacterium
Aliivib	:	Aliivibrio
Marixan	:	Marixanthomonas
Loktane	:	Loktanella
Octadec	:	Octadecabacter
Amyliba	:	Amylibacter
Minicys	:	Minicystis
Azoarcu	:	Azoarcus
Owenwee	:	Owenweeksia
Maritim	:	Maritimimonas
Sandara	:	Sandaracinus
Oleispi	:	Oleispira
Halomon	:	Halomonas
Marital	:	Maritalea
Geojedo	:	Geojedonia
Rhizobi	:	Rhizobium
Reichen	:	Reichenbachiella
Tenacib	:	Tenacibaculum

Species		
Brev ande	:	*Brevinema andersonii*
Snea glos	:	*Sneathiella glossodoripedis*
Alte geno	:	*Alteromonas genovensis*
Mari ulvi	:	*Maribacter ulvicola*
Sphi lito	:	*Sphingorhabdus litoris*
Pola reic	:	*Polaribacter reichenbachii*
Flav icht	:	*Flaviramulus ichthyoenteri*
Lito gwan	:	*Litoribaculum gwangyangense*
Wino unda	:	*Winogradskyella undariae*
Kord anta	:	*Kordia antarctica*
Croc cata	:	*Crocinitomix catalasitica*
Lewi agar	:	*Lewinella agarilytica*
Ples paci	:	*Plesiocystis pacifica*
Ekhi lute	:	*Ekhidna lutea*
Colw meon	:	*Colwellia meonggei*
Colw psyc	:	*Colwellia psychrerythraea*
Hyph john	:	*Hyphomonas johnsonii*
Lewi anta	:	*Lewinella antarctica*
Mari lito	:	*Marinicella litoralis*
Lito taea	:	*Litorimonas taeanensis*
Para ocea	:	*Paraglaciecola oceanifecundans*
Mari sala	:	*Marinobacter salarius*
Algi aest	:	*Algibacter aestuarii*
Psyc jang	:	*Psychroserpens jangbogonensis*
Lewi coha	:	*Lewinella cohaerens*
Port lacu	:	*Portibacter lacus*
Phot phos	:	*Photobacterium phosphoreum*
Dokd diap	:	*Dokdonia diaphoros*
Acet elon	:	*Acetanaerobacterium elongatum*
Alii loge	:	*Aliivibrio logei*
Mari ophi	:	*Marixanthomonas ophiurae*
Lokt vest	:	*Loktanella vestfoldensis*
Octa anta	:	*Octadecabacter antarcticus*
Colw mari	:	*Colwellia maris*
Amyl mari	:	*Amylibacter marinus*
Mini rose	:	*Minicystis rosea*
Azoa comm	:	*Azoarcus communis*
Owen hong	:	*Owenweeksia hongkongensis*
Colw chuk	:	*Colwellia chukchiensis*
Mari rapa	:	*Maritimimonas rapanae*
Aren chit	:	*Arenicella chitinivorans*
Pola porp	:	*Polaribacter porphyrae*
Sand amyl	:	*Sandaracinus amylolyticus*
Mari arct	:	*Maribacter arcticus*
Halo alka	:	*Halomonas alkaliphila*
Mari porp	:	*Maritalea porphyrae*
Geoj lito	:	*Geojedonia litorea*
Rhiz grah	:	*Rhizobium grahamii*

Supplementary Figure 9.



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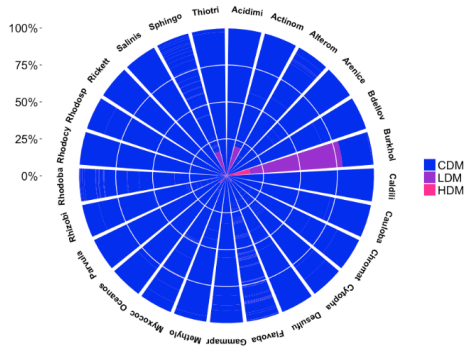

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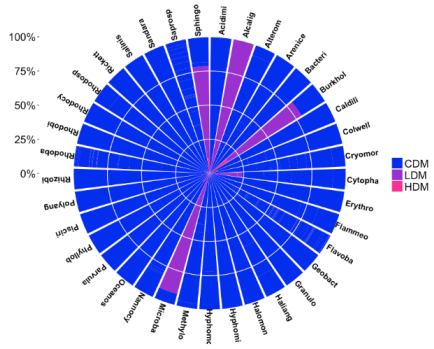

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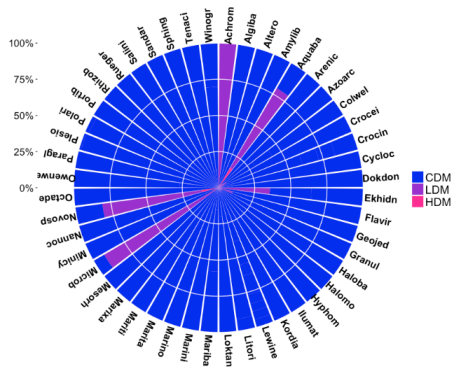

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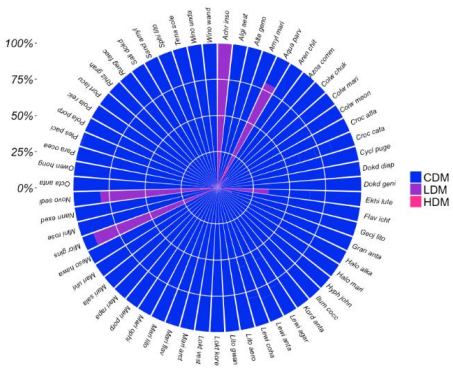


Supplementary Figure 9 abbreviations.

Order

Sphingob	:	Sphingobacterialcs
Actinomy	:	Actinomycetales
Rhodobac	:	Rhodobacterales
Arenicel	:	Arenicellales
Alteromo	:	Alteromonadales
Flavobac	:	Flavobacteriales
Gammapro:		Gammaproteobacteria_incertae_sedis
Caulobac	:	Caulobacterales
Sphingom	:	Sphingomonadales
Chromati	:	Chromatiales
Methyloc	:	Methylococcales
Cytophag	:	Cytophagales
Myxococc	:	Myxococcales
Parvular	:	Parvularculales
Rhizobia	:	Rhizobiales
Burkhold	:	Burkholderiales
Salinisp	:	Salinispbaerales
Acidimic	:	Acidimicrobiales
Ricketts	:	Rickettsiales
Desulfur	:	Desulfuromonadales
Bdellovi	:	Bdellovibrionales
Caldilin	:	Caldilineales
Thiotric	:	Thiotrichales
Rhodocyc	:	Rhodocyclales
Oceanosp	:	Oceanospirillales
Rhodospi	:	Rhodospirillales

Family

Saprosp	:	Saprospiraceae
Microba	:	Microbacteriaceae
Rhodoba	:	Rhodobacteraceae
Arenice	:	Arenicellaceae
Alterom	:	Alteromonadaceae
Flavoba	:	Flavobacteriaceae
Hyphomo	:	Hyphomonadaceae
Sphingo	:	Sphingomonadaceae
Methylo	:	Methylococcaceae
Flammeo	:	Flammeovirgaceae
Cryomor	:	Cryomorphaceae
Polyang	:	Polyangiaceae
Nannocy	:	Nannocystaceae
Parvula	:	Parvularculaceae
Cytopha	:	Cytophagaceae
Rhodobi	:	Rhodobiaceae
Colwell	:	Colwelliaceae
Alcalig	:	Alcaligenaceae
Burkhol	:	Burkholderiales_incertae_sedis
Salinis	:	Salinisphaeraceae
Acidimi	:	Acidimicrobiaceae
Phyllob	:	Phyllobacteriaceae
Rickett	:	Rickettsiaceae
Haliang	:	Haliangiaceae
Granulo	:	Granulosicoccaceae
Erythro	:	Erythrobacteraceae
Sandara	:	Sandaracinaceae
Caldili	:	Caldilineaceae
Geobact	:	Geobacteraceae
Pisciri	:	Piscirickettsiaceae
Bacteri	:	Bacteriovoracaceae
Rhodocy	:	Rhodocyclaceae
Oceanos	:	Oceanospirillaceae
Rhodosp	:	Rhodospirillaceae
Halomon	:	Halomonadaceae
Hyphomi	:	Hyphomicrobiaceae
Rhizobi	:	Rhizobiaceae

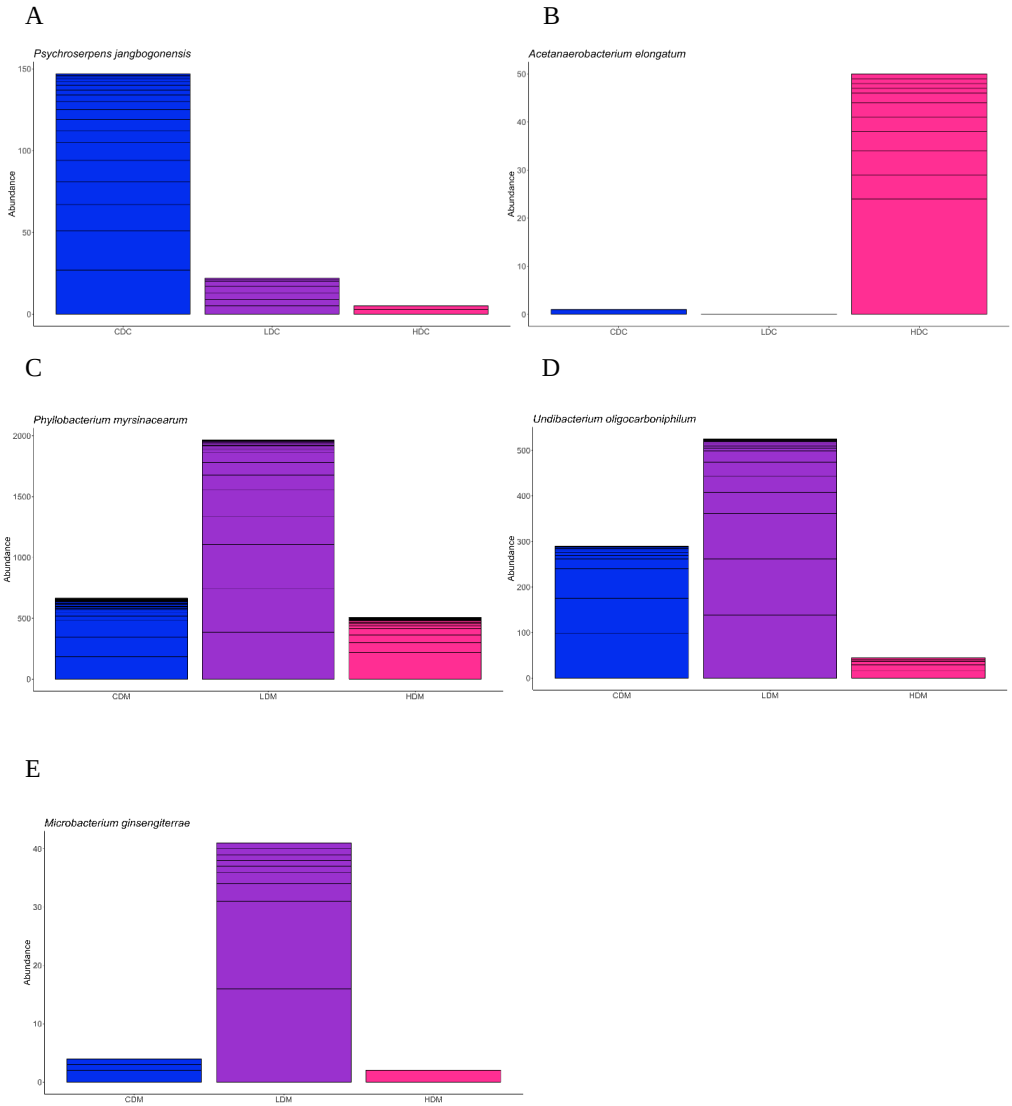
Genus

Microba	:	Microbacterium
Arenice	:	Arenicella
Alterom	:	Alteromonas
Maribac	:	Maribacter
Sphingo	:	Sphingorhabdus
Polarib	:	Polaribacter
Flavira	:	Flaviramulus
Litorib	:	Litoribaculum
Fabibac	:	Fabibacter
Winogra	:	Winogradskyella
Kordia	:	Kordia
Crocini	:	Crocinitomix
Lewinel	:	Lewinella
Sulfito	:	Sulfitobacter
Chondro	:	Chondromyces
Ulvibac	:	Ulvibacter
Plesioc	:	Plesiocystis
Gillisi	:	Gillisia
Ekhidna	:	Ekhidna
Colwell	:	Colwellia
Achromo	:	Achromobacter
Aquabac	:	Aquabacterium
Marinob	:	Marinobacter
Hyphomo	:	Hyphomonas
Marinic	:	Marinicella
Salinis	:	Salinisphacra
Ilumato	:	Ilumatobacter
Paragla	:	Paraglaciecola
Loktane	:	Loktanella
Mesorhi	:	Mesorhizobium
Haliang	:	Haliangium
Granulo	:	Granulosicoccus
Algibac	:	Algibacter
Tenacib	:	Tenacibaculum
Dokdoni	:	Dokdonia
Portiba	:	Portibacter
Owenwee	:	Owenweeksia
Sandara	:	Sandaracinus
Litoril	:	Litorilinea
Geobact	:	Geobacter
Ruegeri	:	Ruegeria
Novosph	:	Novosphingobium
Nannocy	:	Nannocystis
Marixan	:	Marixanthomonas
Cyclocl	:	Cycloclasticus
Octadec	:	Octadecabacter
Amyliba	:	Amylibacter
Halobac	:	Halobacteriovorax
Minicys	:	Minicystis
Azoarcu	:	Azoarcus
Maritim	:	Maritimimonas
Olleya	:	Olleya
Croceib	:	Croceibacter
Oleispi	:	Oleispira
Halomon	:	Halomonas
Marital	:	Maritalea
Geojedo	:	Geojedonia
Rhizobi	:	Rhizobium
Reichen	:	Reichenbachiella

Species

Micr gins	:	*Microbacterium ginsengiterrae*
Alte geno	:	*Alteromonas genovensis*
Mari ulvi	:	*Maribacter ulvicola*
Sphi lito	:	*Sphingorhabdus litoris*
Pola reic	:	*Polaribacter reichenbachii*
Flav icht	:	*Flaviramulus ichthyoenteri*
Lito gwan	:	*Litoribaculum gwangyangense*
Wino unda	:	*Winogradskyella undariae*
Kord anta	:	*Kordia antarctica*
Croc cata	:	*Crocinitomix catalasitica*
Lewi agar	:	*Lewinella agarilytica*
Ples paci	:	*Plesiocystis pacifica*
Ekhi lute	:	*Ekhidna lutea*
Colw meon	:	*Colwellia meonggei*
Achr inso	:	*Achromobacter insolitus*
Aqua parv	:	*Aquabacterium parvum*
Mari flav	:	*Marinobacter flavimaris*
Hyph john	:	*Hyphomonas johnsonii*
Lewi anta	:	*Lewinella antarctica*
Mari lito	:	*Marinicella litoralis*
Sali dokd	:	*Salinisphaera dokdonensis*
Ilum cocc	:	*Ilumatobacter coccineum*
Para ocea	:	*Paraglaciecola oceanifecundans*
Lokt kore	:	*Loktanella koreensis*
Meso hawa	:	*Mesorhizobium hawassense*
Mari sala	:	*Marinobacter salarius*
Gran anta	:	*Granulosicoccus antarcticus*
Algi aest	:	*Algibacter aestuarii*
Tena sole	:	*Tenacibaculum soleae*
Lewi coha	:	*Lewinella cohaerens*
Dokd geni	:	*Dokdonia genika*
Port lacu	:	*Portibacter lacus*
Owen hong	:	*Owenweeksia hongkongensis*
Sand amyl	:	*Sandaracinus amylolyticus*
Dokd diap	:	*Dokdonia diaphoros*
Lito aero	:	*Litorilinea aerophila*
Rueg faec	:	*Ruegeria faecimaris*
Novo sedi	:	*Novosphingobium sediminicola*
Nann exed	:	*Nannocystis exedens*
Mari ophi	:	*Marixanthomonas ophiurae*
Cycl puge	:	*Cycloclasticus pugetii*
Lokt vest	:	*Loktanella vestfoldensis*
Octa anta	:	*Octadecabacter antarcticus*
Colw mari	:	*Colwellia maris*
Amyl mari	:	*Amylibacter marinus*
Halo mari	:	*Halobacteriovorax marinus*
Mini rose	:	*Minicystis rosea*
Azoa comm	:	*Azoarcus communis*
Colw chuk	:	*Colwellia chukchiensis*
Mari rapa	:	*Maritimimonas rapanae*
Aren chit	:	*Arenicella chitinivorans*
Wino wand	:	*Winogradskyella wandonensis*
Pola porp	:	*Polaribacter porphyrae*
Croc atla	:	*Croceibacter atlanticus*
Mari arct	:	*Maribacter arcticus*
Halo alka	:	*Halomonas alkaliphila*
Mari porp	:	*Maritalea porphyrae*
Geoj lito	:	*Geojedonia litorea*
Rhiz grah	:	*Rhizobium grahamii*

Supplementary Figure 10.



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"We are what we eat." The food that we consume also feeds hundreds of trillions of complex microbes that reside in our gastrointestinal tract. This complex community of microbes is called the intestinal microbiota, and it is largely responsible for our overall health. Efforts have been directed towards dietary manipulations of human intestinal microbial community to maintain host health. A similar approach can be adopted for lower vertebrates such as fish.

This thesis describes the influence of dietary components on the intestinal bacterial community of Atlantic salmon using high-throughput sequencing. The experimental diets altered the intestinal bacterial structure of the fish. Antibiotics or probiotics in the diets increased the bacterial diversity while prebiotics decreased the overall bacterial counts. These dietary components altered the bacterial composition largely by shifting the abundance of the dominant bacterial phyla. The results reveal the potential of dietary manipulations of fish intestinal microbiota, a method which could be further explored to improve the health of farmed fish.

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