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Influence of Genetic Background and Environmental Factors on the Skin and Gut Microbiota of Atlantic salmon (*Salmo salar*) Fry

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Abstract

All fish live in symbiosis with complex microbial communities, and the gastrointestinal system (GI) is a natural environment for microbes. The GI microbiota is numerous and diverse, and has important functions for the host. While the GI microbiota of fishes and its effect on development and immunology have been studied for some time, the knowledge about the fish skin microbiota and its host functions is scarce. Like the GI system, the fish skin harbors a complex and diverse microbiota, which lives in close contact and interacts with the surrounding water microbial communities.

The hypothesis of this thesis was that host genotype and diet would influence both the skin and gut microbiota of Atlantic salmon (*Salmo salar*). This was studied by comparing the microbiota of individuals at 48 days after onset of external feeding, associated with two host types representing an aquaculture and a wild strain. The two host strains had been given three different diets, including either fish oil, vegetable oil, or phospholipid oil. The microbiota was characterized by PCR and subsequent Illumina sequencing of 16S rRNA amplicons.

We found that the water microbiota was more diverse than the salmon fry microbiota, and water and fish did not share the same microbiota. Further, the gut microbiota was more diverse than skin microbiota. The gut and skin microbiota were significantly different from each other, but they shared some of the most abundant operational taxonomic units (OTUs).

Host genotype significantly affected both skin and gut microbiota, indicating host selection on microbiota associated with salmon fry. Surprisingly, diet affected skin microbiota, but not gut microbiota. There was an interaction effect between genotype and diet in skin samples, indicating that the diet might have influenced the skin microbiota also through water microbiota. The skin microbiota of developing salmon might therefore be more affected by water microbiota than gut microbiota. In this thesis we showed that genotype of salmon fry influenced skin and gut microbiota more than diet.

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1 Introduction

Food security and nutrition are important challenges to overcome since hunger and malnutrition remain as severe problems in our world. The increasing population, which is expected to reach 8.5 billion in 2030, makes the challenge even bigger. Fish is a nutrient rich food which is vital, especially for rural populations with low incomes. Fisheries and aquaculture industry play important roles in world food security, both by yielding nutritious food, but also by being a source of employment leading to economic growth (FAO, 2016). The aquaculture industry is one of the fastest growing food production sectors and accounts for 50 % of the fish production used for food in the world.

Norway is an important contributor to the world's aquaculture industry. In 2015, 1.38 tons of fish for food were produced by aquaculture in Norway, and 1.30 of these tons were the production of Atlantic salmon (*Salmo salar)* (SSB, 2016a). The value of the Norwegian farmed salmon has increased tremendously the past ten years, and salmon from Norwegian aquaculture farms were sold for 60 billion Norwegian kroner in 2016 (SSB, 2016b). This makes salmon production an important income source for our country.

There are several challenges in aquaculture, and one of them is diet. Today, the aquaculture industry use fish meal as an important lipid and protein source for fish, but the global supply of fish meal is not sufficient to the growing aquaculture sector. Finding alternative protein sources for production of carnivorous fish would therefore be one way to make the production more sustainable (Desai et al., 2012). A more plant based diet would be desirable, but studies have shown negative effects on both the gastrointestinal tracts and the microbiota of fish (Desai et al., 2012, Krogdahl et al., 2003, Krogdahl et al., 2010). This is associated with challenges such as inflammation in the gut and deficiency of omega-3 fatty acids DHA and EPA in fish meat (Sprague et al., 2016, Desai et al., 2012).

The health promoting long chained omega-3 fatty acids like DHA and EPA needs to be taken in through diet. Today a plant based diet do not yield the same amounts of the health promoting omega-3 fatty acids in fish meat (Sprague et al., 2016). Thus, exploring alternative lipid sources in sustainable feeds is therefore necessary to maintain the high concentrations of EPA and DHA. Diet may also affect the fish associated microbiota, and we know that

microbiota is important for health. The effect of microbiota on fish health has not been very focused upon in the aquaculture industry, but the interest is growing. Increased knowledge in this field may improve fish health, sustainability and production.

The Food and Agriculture Organization of the United Nations (FAO), works for a sustainable aquaculture development in the world. The industry needs to be sustainable to make a proper contribution to the food production. Improved knowledge about how the host associated microbiota influence fish health may lead to new strategies in aquaculture improving microbial conditions, and thereby leading to better fish health and a more sustainable industry. Thus, establishing different effects on fish associated microbiota, such as host genetics and environmental factors including diet and water quality must be done.

1.1 The Gastrointestinal Microbiota

Vertebrates are colonized by and live in close contact with microorganisms. The gastrointestinal (GI) system serves as a natural environment for microorganisms and is mainly dominated by bacteria (Rawls et al., 2004).

The GI microbiota in mammals has been studied for some time and has been shown to be a central part of several biological functions such as contribution to digestion and synthesis of nutrients, in addition to the development of the immune and gastrointestinal system (Sekirov et al., 2010, Kamada et al., 2013). Function of GI microbiota in vertebrates are conserved and the microbiota colonizing mammals are similar to the microbiota colonizing fish, but the composition of the microbiota is not the same (Rawls et al., 2004). Furthermore, GI microbiota is part of the defense against pathogens. Commensal bacteria outdo pathogens for nutrients, as well as producing signal molecules and other products which inhibit pathogens (Abt and Pamer, 2014). The commensal microbiota is important for the immune system of fish as well. It stimulates the mucus production, as well as production of antimicrobial factors, and contribution to regulation of immunological responses depicted in Figure 1.1 (Abt and Pamer, 2014, Hill et al., 1990, Atarashi et al., 2015).

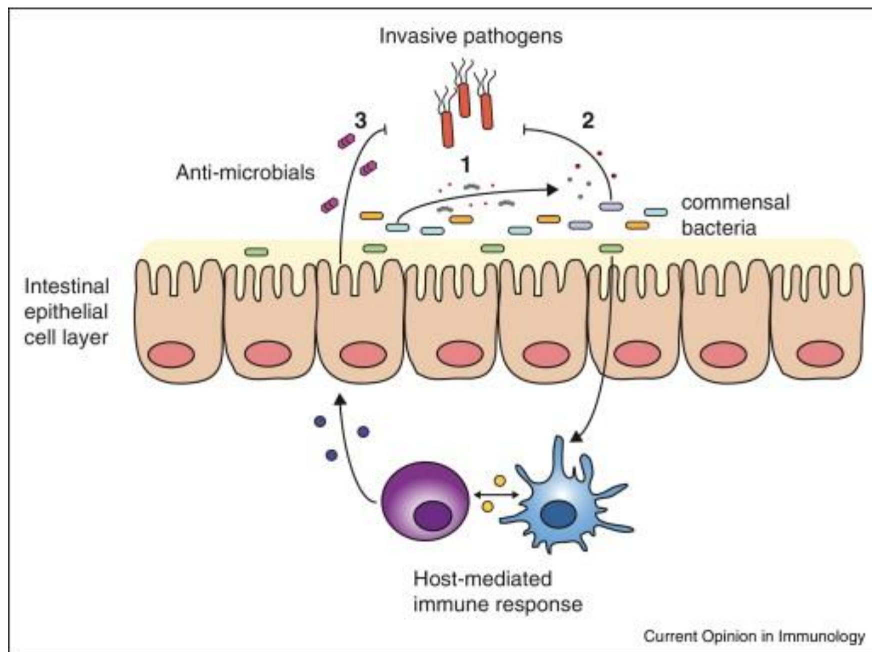

Figure 1.1: An overview of commensal bacteria-mediated mechanisms of protection against intestinal pathogens in . (1) Nutrients consumed by commensal microbiota, depriving pathogens of a niche. (2) Inhibition of pathogens by microbiota-derived metabolic by-products (3) Host immune cells stimulated by commensal bacteria leading to expression of host defense factors (Abt and Pamer, 2014).

Gnotobiology is the study of animals with a known microbiome and is based on bacteria-free (axenic) host individuals which is colonized in a controlled way with specific microorganisms. This has been a useful way to study the role of bacteria colonizing the host. The effects of microbiota are studied comparing gnotobiotic animals and so-called conventional animals with a normal microbiome. Gnotobiotic studies have revealed important information about the role of GI microbiota. For example, a gnotobiotic study of GI microbiota in mice revealed that the microbiota has an effect on nutrient uptake and storage. Microbiota from obese mice had an increased capacity to harvest energy from the diet, and transfer of the microbiota from obese mice to the gnotobiotic mice resulted in increased levels of body fat, showing that the trait is transmissible (Turnbaugh et al., 2006).

The establishment of GI microbiota in fish is affected by several factors. These include the developmental stage of fish, gut structure, surrounding environment, rearing conditions, including diet, and other stress factors like antibiotics and other pollutants (Giatsis et al., 2015, Nayak, 2010, Stephens et al., 2016). Among other things, dietary supplements as pre- and probiotics affect the gut microbiota in fish and are used as health promoting substances in aquaculture (Nayak, 2010). As mentioned, fish live in ecosystems with a high load of

microorganisms and when fish larva hatch they come in direct contact with the microbes (Rawls et al., 2004). The colonization starts right after hatching before the larva starts eating (Nayak, 2010). The larva takes in water before it starts eating, consequently addition of microorganism to the GI system starts. The bacterial load is relatively low when the fish still live of its yolk, but there is a rapid increase when food is taken in (Gomez et al., 2013, Nayak, 2010).

GI microbiota can be divided into two groups based on its ability to bind mucus. Autochtonous microbiota bind to the mucus while allochthonous microbiota do not. The autochtonous bacteria colonize the mucus layer in the intestines, while the transient allochthonous bacteria are mostly located in the content of the intestines (Nayak, 2010, Ringø et al., 2008). Gajardo et al found differences in core microbiota between mucosa and digesta in Atlantic salmon, and suggest that studies of diet and environmental influence on gut microbiota should be differentiated between the two categories (Gajardo et al., 2016).

Freshwater fish and marine fish have been shown to have different composition of GI microbiota (Roeselers et al., 2011, Nayak, 2010, Perez et al., 2010). Freshwater fish are dominated by *Aeromonas*, *Pseudomonas* and *Bacteroides* while marine fish GI tracts are dominated by *Flavobacterium*, *Carnobacterium*, *Moraxella*, *Micrococcus*, *Vibrio* and also *Pseudomonas* (Perez et al., 2010, Nayak, 2010).

Dietary effects on microbiota associated with fish larvae have been studied for different fish species and the results are not conclusive. There has been a general acceptance that feed have an effect on fish larvae (Nayak, 2010), and microbiota associated with rainbow trout larvae was found to change according to diet after first feeding (Ingerslev et al., 2014). Nevertheless, another recent study has shown that diet did not affect the microbial composition of cod larvae, indicating that diet might not be such a strong determinant of the microbiota associated with larvae after all (Bakke et al., 2013).

Developmental stage of fish is suggested to affect gut microbiota (Stephens et al., 2016, Bakke et al., 2015, Nayak, 2010). Gut microbiota in zebrafish (*Danio rerio*) has been studied during development, and the microbial communities were found to be more similar to communities in the surrounding environment at larval stage than in adult fish indicating that environmental exposure has a larger role in early developmental stage. In addition, the composition of gut microbiota in zebrafish underwent shift during periods of diet and

environmental changes but also when diet and environment stayed the same. This indicated that physiological development affected the fish microbiota (Stephens et al., 2016).

Furthermore, host species is found to be a determinant of the GI microbiota (Li et al., 2012). In addition, there are indications of genetic host selection on the microbial composition found on Mangrove killifish larva and cod larvae (Forberg et al., 2016, Bakke et al., 2015). In addition, was gut microbiota in domesticated and recently caught zebrafish found to be very similar, indicating a core gut microbiota in zebrafish (Roeselers et al., 2011). These findings suggest that host genetics influence the gut microbiota of fish.

Several studies have explored the GI microbiota in adult Atlantic salmon. Even though there is little knowledge of microbiota in developing Atlantic salmon, the interest is increasing. Life cycle stage has been shown to affect microbiota in Atlantic salmon gut (Llewellyn et al., 2016, Zarkasi et al., 2016). Different rearing environment for Atlantic salmon parr resulted in significant differences in the composition of gut microbiota (Dehler et al., 2017). Furthermore, diet has been shown to influence the composition of GI-microbiota in Atlantic salmon (Ringø et al., 2008, Zarkasi et al., 2016). The knowledge about the Atlantic salmon microbiota is increasing, and as a result there are indications that Atlantic salmon host core bacteria in gut and skin (Llewellyn et al., 2016, Lokesh and Kiron, 2016, Gajardo et al., 2016).

The composition of the GI microbiota in fish has been studied for some time, but the true complexity of the microbiota is now being revealed by using new, culture-independent methods.

1.2 Fish skin Microbiota

The role and composition of GI microbiota in fish has been studied for a while. However, the role of skin microbiota is not that well studied. The relationship between fish skin and the surrounding microbiota is close, and fish skin is one of the first barriers between the fish and its environment. It is an important barrier towards physical environmental factors as well as pathogenic organisms (Gomez et al., 2013, Larsen et al., 2013).

The fish skin is covered with mucus, and the skin mucosa resembles the gut mucosa in several ways. The mucosal surfaces of fish gut and fish skin consist of a layer with living epithelial cells which includes goblet cells (mucus producing cells) covered in mucus (Gomez et al.,

2013). The mucus layer is continuously produced and shed, making it a dynamic structure which can remove trapped matter. The main constituent of the mucus are mucins shed from the goblet cells (Linden et al., 2008, Rakers et al., 2013). The properties of the mucus are determined by the composition of O-glycans on the mucins, which are diverse. There is different O-glycosylation patterns of mucosa from intestine and skin of salmon, indicating that different microbiota may interact with the different mucosal layers based on its properties (Jin et al., 2015). In addition the mucus contain several antimicrobial factors like cytokines and proteases (Linden et al., 2008). Skin histology of rainbow trout is shown in Figure 1.2 (Rakers et al., 2013).

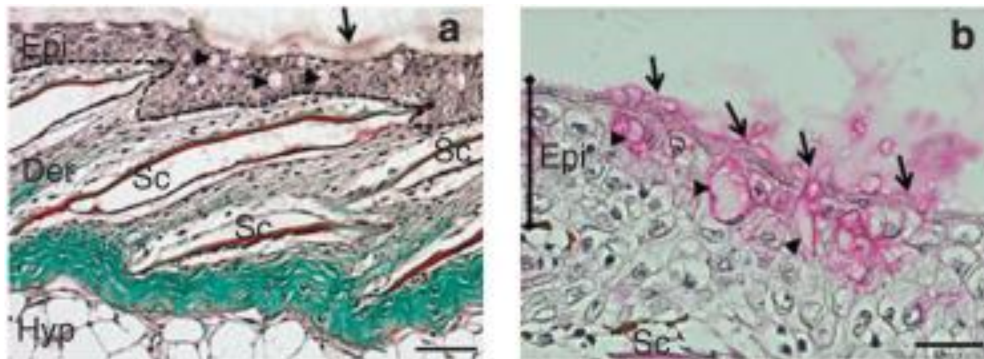

Figure 1.2: Fish skin histology. (a) Staining of rainbow trout skin. Large cells in epidermis (Epi) are mucous goblet cells (arrowheads). Secretion of mucus to the surface (arrow). Der, dermis; Hyp, hypodermis. Scale bar=50 µm. (b) Mucous goblet cells (arrowheads) are stained and secrete mucus to the outer epithelial surface (arrows). Bar=20 µm. (Rakers et al., 2013)

Fish mucus is nutrient rich, and adhesion to mucus in fish skin (or gut) is a trait which might be beneficial to the bacteria (Larsen et al., 2013). Generally the commensal bacteria are beneficial for the fish host, but pathogenic bacteria such as some species of *Vibrio* and *Flavobacterium* also carry the skin adhesion trait and may cause disease (Larsen et al., 2013).

Fish skin microbiota is highly diverse (Chiarello et al., 2015, Lowrey et al., 2015, Lokesh and Kiron, 2016), and there are variations in phyla found to be abundant in fish skin. Nevertheless, Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes have been found to be abundant in several studies (Boutin et al., 2013, Boutin et al., 2014, Lowrey et al., 2015, Lokesh and Kiron, 2016). Several factors are found to influence the skin associated microbiota such as host genetics and environmental factors.

Host genetics influence fish skin microbiota by showing host specie specificity, indicated by the fact that different fish species are colonized by distinct skin microbiota (Boutin et al., 2014, Larsen et al., 2013, Chiarello et al., 2015). Further, the skin associated microbiota is found to vary between body parts of the fish, and fish individuals (Chiarello et al., 2015). In addition, a highly diverse microbiota has been found within the epithelium layer of Rainbow trout skin (Lowrey et al., 2015).

There is little knowledge about how environmental factors influence the skin microbiota of fish, but fish skin is found to be colonized by microbiota different from the surrounding water (Austin, 2006, Chiarello et al., 2015). However, microbial diversity in brook charr skin was partially influenced by bacterial community in the surrounding water (Boutin et al., 2013). Furthermore, Atlantic salmon transitioning from freshwater to seawater reshaped the microbiota associated with the skin, indicating that environmental factors might affect the skin associated microbiota (Lokesh and Kiron, 2016).

The role of the skin microbiota is poorly understood, but it might contribute to one of the first steps in the fish's defense system against pathogens (Ángeles Esteban, 2012). Cutaneous diseases are common in fish, and further investigations of fish skin and how environmental factors influence the associated microbiota might help preventing outbreaks in farmed fish.

1.3 Approaches for investigation of microbial diversity

Traditional methods for investigation of microbial communities have been culture based, but it has been shown that only a small fraction of bacteria can be isolated by these techniques (Navarrete et al., 2008, Sekirov et al., 2010). Cultivation of bacteria demands right condition of both resources and environment. Thus lack of growth does not prove absence of bacteria in samples. Fast-growing bacteria may dominate and outcompete the slower growing bacteria in a culture environment even though it only represents a small part of the total microbial community. The development of molecular methods such as polymerase chain reaction (PCR) and sequencing has greatly increased the investigative possibility thus providing detailed characterization of the composition of microbial communities (Navarrete et al., 2008, Sekirov et al., 2010).

1.3.1 16S rRNA as Marker Gene

Analysis of the 16S rRNA gene is widely applied for taxonomic assignments and studies of phylogenetic relationships and diversity of microbial communities. The 16S rRNA gene encodes the small ribosomal subunit in bacteria, and contains both conserved and variable sequence regions. These properties, together with the fact that the gene is barely affected by horizontal gene transfer, makes it a good marker for the diversity of microbial communities (Acinas et al., 2004, James, 2010).

So-called universal primers have been designed and used to amplify the 16S rRNA gene of microbial communities by binding the conserved regions (Muyzer et al., 1993, James, 2010). The diversity of the amplification product reflects the diversity of the original microbial community of the sample, and can be further investigated by methods like denaturing gradient gel electrophoresis (DGGE) and sequencing of amplicon libraries including Illumina sequencing (Ram et al., 2011, James, 2010, Muyzer et al., 1993). There are some drawbacks to these methods. There is not necessarily a direct correlation between abundance of a given 16S rRNA sequence and abundance of the corresponding bacterial strain in the studied community. This is due to several factors. First, PCR bias such as unequal amplification efficiency for different template molecules caused by differences in primer-binding sequence, may result in more amplification of some bacterial 16S than others (Polz and Cavanaugh, 1998). Secondly, variable numbers of *rrn* operon in bacterial genomes may cause over representing of some bacteria. The *rrn* operon contain 16S rRNA gene and bacteria with more *rrn* operons will then be over represented. There may also exist sequence diversity among the multiple intragenomic copies of 16S rRNA, which will result in overestimating the diversity estimates (Acinas et al., 2004). There are alternatives to using 16S as marker gene, one is *cpn*60 which encodes a chaperonin. This gene has high resolution to distinguish species, but there are some major disadvantages such as multiple copy variation, and that the databases of this gene are less developed than for 16S rRNA. Thus 16S rRNA is generally a better choice for microbial diversity studies (Di Bella et al., 2013).

1.3.2 Denaturing Gradient Gel Electrophoresis

Denaturing gradiend gel electrophoresis (DGGE) is a fingerprinting method which is used to examine the diversity of 16S rRNA amplicons representing microbial communities.

In DGGE, DNA fragments of approximately the same length are separated by the electric current as in a denaturing gradient gel. The polyacrylamide gel of DGGE has a linear increasing denaturing gradient made by formamide and urea. During electrophoresis the helical structure of DNA molecules will stay intact until the gradient concentration where it denatures. The difference in nucleotide sequences between fragments result in different denaturing positions of the molecules, and they will therefor wander different lengths on the gel, resulting in a band pattern showing a community profile for each PCR product. Thus, the pattern reflect the microbial diversity in the original sample (Muyzer et al., 1993).

To avoid complete separation of the strands in the DNA molecules during denaturation, and avoid further migration in the gel, a GC-rich sequence (the "GC-clamp") is added to one of the primers used in PCR (Muyzer et al., 1993).

The band patterns on the gel can be analyzed statistically and the bands can be excised, re-amplified, and sequenced for taxonomic assignments. DGGE is a quick and cheap method to compare microbial community profiles among samples, for example to study dynamics of communities. The resolution and amount of taxonomic information is limited, and DGGE does not give a detailed taxonomic description. Next generation sequencing methods (NGS) have a higher resolution and give a more detailed taxonomic information than DGGE bands, and is about to outcompete DGGE for microbial community analysis (Di Bella et al., 2013).

1.3.3 Illumina sequencing of 16S rRNA amplicons

Illumina sequencing by synthesis (SBS) is a NGS based on Sanger chain termination method, using fluorescently labeled deoxyribonucleotide triphosphates (dNTPs) incorporating them into a DNA template strand. Using sequential cycles Illuminas SBS technology incorporate the dNTPs during DNA synthesis. During each cycle, incorporation of a single nucleotide is identified by excitation of the fluorophore. There are four basic steps in the Illumina NGS workflow, library preparation, cluster generation, sequencing, and data analysis (Illumina Inc, 2016).

Library preparation is done according to what one wants to investigate. Amplicon libraries are made to investigate microbial communities, and an amplicon library is made by amplifying bacterial 16S rRNA regions by broad range primers for each sample in the study. Normalization of PCR amplicons are done prior to pooling, and is done to adjust amplicon

concentration for sequencing and wash away contaminants as short oligonucleotide primers and proteins. The normalized PCR amplicons are then marked with unique indices, making it possible to identify from each other. Thus, it is possible to pool a large number of PCR products and sequence multiple amplicons in one Illumina lane. The adapters are added to the ends of DNA during amplification, and include sequencing binding site, indices and regions complementary to the flow cell oligos (Illumina Inc, 2016, Di Bella et al., 2013).

After library preparation, cluster generation is performed by isothermal amplification. The Illumina technology is based upon DNA-template molecules binding to a flow cell plate through hybridization to oligonucleotides bound to the flow cell. The template molecules are then clonally amplified through bridge amplification which makes clusters of identical DNA molecules. This occurs simultaneously for millions of clusters (Illumina Inc, 2016, Di Bella et al., 2013).

The first read of sequencing is started with the extension of the first sequencing primer. For each cycle only one of the four fluorescently tagged dNTPs are incorporated, based on the sequence of the template. After each addition, the clusters get excited by a light source which leads to emittance of a fluorescent signal characteristic for each of the nucleotides. This is the process called sequencing by synthesis (Di Bella et al., 2013). Indeces are read, and a second read is performed for pair-end sequencing which results in better alignments of the reads (Illumina Inc, 2016). After sequencing the resulting sequence reads can be sorted according to samples by the unique sequence indices (Illumina Inc, 2016).

Illumina sequencing of 16S rRNA amplicons is a powerful method for studying microbial diversity. It gives a very detailed description of microbial communities, including taxonomic information and is now a commonly used approach to characterize microbial diversity.

1.4 Hypothesis and aims

The colonization and establishment of microbial communities depends on the mucosal composition and the process of establishing the microbiota is believed to be influenced by both environmental factors and by host factors, such as genotype and developmental stage. The main hypothesis of this project is that the skin and gut microbiota of the salmon fry will be affected by environmental factors like water and feed, and by the genetic background of the host.

Previous experiments in the research group have shown that PCR amplification of the 16S rRNA gene from salmon fry is challenging, probably due to the presence of inhibitors and low fraction of bacterial DNA in the DNA template.

The first objective in this project was to optimize the PCR conditions for amplification of 16S rRNA fragments from gut and skin samples.

The second aim was to investigate how host genotype influences the skin and gut microbiota, and the third objective was to investigate whether the diet influenced the skin and gut microbiota in salmon fry.

These objectives will be investigated by using an approach based on PCR amplification of the 16S rRNA gene for samples of rearing water and individual fishes. Sequence diversity of the amplicons will be examined using Illumina sequencing.

2 Materials and Methods

2.1 Experimental Design

Samples analyzed in this study came from a first feeding experiment with Atlantic salmon (*Salmo salar*) which was performed on Frøya before this master project was started. Two genetic groups of salmon was used, wild and aquaculture Atlantic salmon. Feeding was started at 800 day degrees after hatching, and this was defined as experimental day 0. The fry was fed three different diets, fish oil (FO), vegetable oil (VO), and phospholipid oil (PL). Except for the lipid source, the oils, the ingredients in the different diets were identical. The ingredients and nutrient components of the diets are presented in Appendix A. Two replicate tanks for each genotype and diet were set up, which led to a total of twelve tanks. From each tank, 3 replicate fish were sampled, giving a total of 6 fish given the same treatment. Gut and skin samples were picked randomly and did not necessarily belong to the same fish. A schematic overview of the tanks is presented in Table 2.1.

Table 2.1: Schematic setup of tanks in Frøya experiment. Genotype of fish, diet, tank name for identification of samples and number of individuals sampled from each tank.

Genotype	Aquaculture						Wild					
Diet	Fish Oil		Vegetable Oil		Phospholipid Oil		Fish Oil		Vegetable Oil		Phospholipid Oil	
Tank name	AFO		AVO		APL		WFO		WVO		WPL	
Tank	A	B	A	B	A	B	A	B	A	B	A	B
Individuals	3	3	3	3	3	3	3	3	3	3	3	3

2.2 Sampling

Sampling was done at day 48 of the experiment. Fish skin, gut and water were sampled.

Water was sampled by filtrating 50 mL water through a 0.22 µm Dynaguard filter. The filters were stored at -20ºC. The fish were anaesthetized and the gut was dissected out and transferred to a 2 mL cryo tube and stored - 20ºC. The sampling of skin was performed after the gut was removed. The fish was rinsed with sterile water, and tweezers and scalpel was used to remove the skin from the body. The head was removed, and the fish was cut along the abdomen. The flesh was removed as much as possible by trying to rip of the skin with tweezers. Resting flesh was scraped off with a scalpel. The skin samples were stored at -18ºC.

2.3 DNA extraction

Total DNA from the samples were extracted by using either Powersoil DNA isolation Kit (MO BIO laboratories Inc, reffered to as MoBio kit) or QIAamp DNA Mini Kit (Qiagen, reffered to as Qiagen kit). DNA extraction using the MoBio kit was performed as described in the manufacturers protocol, presented in Appendix B.

DNA extraction using the Qiagen kit was performed as described in the manufacturers protocol, presented in Appendix B, with minor alterations. The samples were incubated for 3 hours for lysis of cells. In step 2a, buffer ATL was used as lysis buffer, and buffer AL was used in step 5b. Two elutions were performed with 70 µL and 60 µL buffer AE.

The DNA concentration was measured using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

2.4 Polymerase chain reaction

2.4.1 Amplification of 16S rRNA V3 region for DGGE analysis

A nested protocol for the amplification of the V3-region in 16S rRNA gene was used in order to avoid co-amplification of the salmon 18S rRNA gene (Bakke et al., 2011). Primers EUB8F and 984yR were used for external PCR, while primers 338F-GC and 518R were used for internal PCR. All primer sequences are presented in Table 2.2.

Table 2.2: Primers used for amplification of V3 and V4 region of 16S rRNA gene for DGGE analysis. Names, primer sequences and application are presented. Illumina adapter sequences are marked in red.

Primer Name	Primer sequence (5'-3')	Application
EUB8F	5'- AGA GTT TGA TCM TGG CTC AG -3'	External, V3 region
984yR	5'- GTA AGG TTC YTC CGC GT -3'	External, V4 region
338F-GC	5'- cgcccgccgcgcgcggcgggcggggcgggggcacggggggACT CCT ACG GGA GGC AGC AG -3'	Internal, V3 region
518R	5'- ATT ACC GCG GCT GCT GG -3'	Internal, V3 region
Ill-338F	5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNNN CCT ACG GGW GGC AGC AG-3'	V3 region
Ill-805R	5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN GAC TAC NVG GGT ATC TAA KCC-3'	V4 region
Ill-515F	5'- TCG TCG GCA GCG TCA GAT GTC TAT AAG AGA CAG NNNN GTG CCA GCM GCC GCG GTA A-3'	V4 region
Ill-803R	5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN CTA CVV GGG TAT CTA AKC CBK-3'	V4 region

PCR amplifications were performed using Taq polymerase (VWR) or Phusion Hot Start DNA polymerase (Thermo Scientific, Lithuania). Compositions of the mastermixes are presented in Table 2.3 and the standard cycling conditions used for PCR amplification is presented in Table 2.4. Alterations of cycling condition are presented in figure legends to gel pictures in the Results section. primeSTAR GXL polymerase was also tested, and compoisiton of the mastermix together with the cycling conditions are presented in Appendix C.

PCR decontamination kit (ArcticZymes) was performed as part of the optimization of PCR protocol to try and remove contaminant DNA, and was performed according to the manufacturers' instructions. In short, dsDNase and DTT is added to the mastermix and incubated. Incubation at 37°C activate dsDNase which decontaminate the mastermix, subsequent incubation at 60°C together with DTT inactivates the dsDNAse.

Table 2.3: Composition of mastermixes, with Phusion Hot Start polymerase and Taq polymerase, used for PCR amplification of 16S rRNA.

Phusion Hot Start			Taq		
Component	Producer	Volume (µL)	Component	Producer	Volume (µL)
5x Phusion buffer HF (7,5 mM $MgCl_2$)	Thermo Scientific	5.0	10 x key buffer (15 mM $MgCl_2$)	VWR	2.5
dNTP (10 mM)	G-Biosciences	0.5	dNTP (10 mM)	G-Biosciences	0.5
BSA (20 mg/mL)	BioLabs	0.75	BSA (20 mg/mL)	BioLabs	0.75
Phusion Hot Start - DNA polymerase (5U/µL)	Thermo Scientific	0.125	Taq polymerase (5U/µL)	VWR	0.125
$MgCl_2$ (50 mM)	Thermo scientific	0.5	$MgCl_2$ (25 mM)	Qiagen	0.5
Primer F (10 µM)	SIGMA	0.75	Primer F (10 µM)	SIGMA	0.75
Primer R (10 µM)	SIGMA	0.75	Primer R (10 µM)	SIGMA	0.75
Template DNA		1*	Template DNA		1*
H_2O		15.625	H_2O		18.125

*As template, 1 µL of undiluted DNA extracts (see section 2.3) was used.

Table 2.4: Cycling conditions for PCR reaction with the two different polymerase setups.

Phusion			Taq		
(ºC)	Time		(ºC)	Time	
98	∞		98	∞	
98	1 min		95	3 min	
98	15 s	x 25	95	30 s	x 25
53	20 s	x 25	53	30 s	x 25
72	20 s	x 25	72	1 min	x 25
72	5 min		72	10 min	
4	1 min		4	1 min	
10	∞		10	∞	

2.4.2 Amplification of 16S rRNA V4 region for Illumina amplicon sequencing

Primers with Illumina adapters were used to amplify the V4 region and V3 region of the 16S rRNA gene. Different primers were tested and primer sequences are presented in Table 2.2. The primers ultimately chosen for amplifying V4-region of 16S rRNA for Illumina sequencing were 515F and 803R. The Phusion Hot Start Polymerase was used together with the components presented in Table 2.3, except that BSA was omitted. Cycling conditions were as described in Table 2.4, but number of cycles was 36 or 38.

For the water samples, 1 µL of undiluted template and 36 cycles in PCR were used. For the wild genotype skin and gut samples, 1 µL of 1:10 diluted DNA template and 36 cycles was used in the PCR. For the aquaculture genotype gut and skin, 2 µL 1:10 diluted template and 38 cycles was used.

The PCR products were examined using agarose gel electrophoresis, by applying 1 % agarose gel with GelRed in 1 x TAE buffer (Appendix D). A volume of 5 µL of each PCR product was mixed with 1 µL DNA loading dye and placed on the gel. GeneRuler 1 kb Plus ladder (Thermo Scientific) was used as marker. 140 volt was applied to the gel, and it was run for 45-60 minutes depending on the thickness of the gel.

2.5 Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed with the phorU system from INGENY (Netherlands). An 8 % polyacrylamide gel was used with a denaturing gradient of 35 % to 55 % with urea and formamide as denaturing agents, where 100 % correspond to 7 M urea and 40 % formamide.

Two glass plates were washed with Deconex soap, rinsed and further polished with 95 % ethanol and Kimwipe paper. Together with clean spacer the two glass plates were assembled in the gel cassette and the comb was set on top. The stacking gel (0 % denaturing), 35 % denaturing gel, and 55 % denaturing gel solutions were prepared from 0 % and 80 % denaturing acrylamide solution. The 0 % and 80 % gel solutions are given in Appendix D. Solutions applied for preparation of the 35 % and 55 % denaturing gel solutions are presented in Table 2.5. The 80 % denaturing acrylamide solution was filtered through a 0.4 um filter prior to casting the gel, in order to remove urea particles.

Table 2.5: Solutions applied for preparing the 35 to 55 % denaturing gradient in DGGE gels.

Denaturing %	0 %	80 %	TEMED + 10 % APS	Total Volume
0 %	8 mL		10 + 40 µL	8 mL
35 %	13.5 mL	10.5 mL	16 + 87 µL	24 mL
55 %	7.5 mL	16.5 mL	16 + 87 µL	24 mL

Tetramethylethylenediamine (TEMED) and 10 % ammonium persulfate (APS) was then added to the gel to start the polymerization. Casting of the 55 % denaturing gel solution was started first to allow for high denaturing percentage at the bottom of the gel. Then a gradient mixer was used to mix the 55 % and 35 % denaturing gel and create the denaturing gradient. The stacking solution (0 %) was casted on top of the gel. The comb was inserted into the gel, all screws were tightened, and the gel was set for polymerization for 2 hours. Electrophoresis buffer (0.5 x TAE) was filled in the buffer tank and was heated to 60ºC.

After polymerization, the comb was removed and the gel cassette was set in the buffer tank. The wells were cleaned with a syringe with buffer from the buffer tank. PCR product was mixed with loading dye. The amount of PCR product applied depended on their band strength on agarose gel, although it was aimed at adding equal amounts of all PCR-products. The samples were applied to the wells while avoiding the six outermost wells on each side to avoid "smiling" effect. The gel was run at 60ºC and 100 V for approximately 22 hours.

The DGGE marker used was as described by Bakke et al (2013). The marker contained pooled V3 16S rDNA PCR products using pure cultures of *Staphylococcus aureus*, *Ruminococcus obeum*, *Eubacterium formicigenerans*, *Ruminococcus productus*, *Fusobacterium prauznitzii*, *Clostridium celerescans*, *Eubacterium plutii*, *Eubacterium halii*, and *Bifidobacterium longum* as templates (Bakke et al., 2013).

After electrophoresis, the gel was stained for two hours with 3 µL SYBR Gold (Invitrogen) diluted in 30 mL 1 x TAE. During staining, the gel was covered with a dark colored box. The gel was photographed under UV-light (G:BOX, Syngene).

2.6 Preparation of amplicon library for Illumina Sequencing

PCR products were generated using primers with Illumina adapters for all samples as described above (section 2.4.2). PCR products with the Illumina adapters were used for making amplicon library for Illumina sequencing. After PCR amplifications, the products were purified and normalized prior to attaching a unique sequence index for each sample.

The PCR products were normalized and purified using SequalPrep Normalization Plate (96) Kit (Invitrogen, USA) according to the manufacturer's instructions. In brief, equal amounts of DNA bind to each well and excessive nucleotides, primers and salts are washed away before DNA is eluted.

After normalization of the PCR products, a second PCR reaction was performed to add unique index sequences to each sample. Indexing was performed using Nextera XT Index Kit V2 (Illumina). The index kit contains 8 different "index 1" and 12 different "index 2". One index 1 and one index 2 are matched to a unique index pair, giving a total of 96 unique index pairs.

Components of the reaction mix are presented in Table 2.6. All components, except index sequences and template, were added to an Eppendorf tube and mixed before distribution to 96-well plate (BioRad). Indexes were added to their respective wells before templates (normalized PCR products) were added, and the plate was run in the PCR instrument (BioRad). Temperature cycles were as presented for the Phusion Hot Start polymerase in Table 2.4, but only 10 cycles was performed.

Table 2.6: Components of indexing PCR reaction of normalized products. Volume per reaction is shown.

Component	Volume (µL)
dH20	11.687
5x Phusion buffer HF (7.5 mM $MgCl_2$)	5.0
dNTP (10mM each)	0.625
Phusion Hot Start DNA polymerase	0.188
$MgCl_2$	0.5
Index 1 (DNA oligo)*	2.5
Index 2 (DNA oligo)*	2.5
Template (normalized)	2.5

* A unique pair of indexing sequences was used for each PCR product.

After the indexing PCR reactions, amplicons were normalized a second time using the same procedure as described above, and the samples were pooled together. DNA concentration was measured using NanoDrop.

The pooled sample was concentrated using Amicon Ultra Centrifugal Filter Units (Millipore, Ireland) according to the manufacturers' protocol. In addition TE buffer wash (Appendix D) at 14 000 G for 10 minutes was performed two times. The pooled sample was concentrated two times to obtain the desired concentration. Concentration and purity of the samples were measured by Qubit 3 Fluorometer (Invitrogen, Thermo Scientific).

The resulting amplicon library was sequenced on one MiSeq lane (Illumina, San Diego, CA) with V3 reagents (Illumina) employing 300 bp paired end reads at the Norwegian Sequencing Centre. PhiX library (Illumina) was blended to 50%. Data was processed using RTA 1.18.54 (Illumina).

2.7 Processing of Illumina sequencing data

The Illumina sequencing data were processed by Ingrid Bakke with the high performance USEARCH utility (version 8.1.1825) (USEARCH). The processing was carried out as implemented in the UPARSE pipeline (Edgar, 2013). The major steps in the pipeline included demultiplexing, removal of primer sequences and sequences shorter than 250 nucleotides,

quality trimming, and clustering to obtain OTU tables at 97% similarity level. The subsequent taxonomy affiliation was based on the Utax script implemented in the UPARSE pipeline with a confidence value threshold of 0.8 and the RDP reference data set (version 15).

2.8 Statistical Analysis

The Qiime pipeline (Caporaso et al., 2010) was used to determine Chao1 richness and relative abundances at different taxonomic levels. Alpha diversity indices (Chao1 and Shannons' diversity index), and relative abundances at different taxonomic levels were calculated using the Qiime script "alpha_diversity.py" (QIIME). All other analyses were performed with the program package PAST version 3.16 (Hammer et al., 2001).

Shannons' diversity index (H'), together with Chao1 and OTU richness, was used to calculate microbial diversity for each sample, called Alpha-diversity. Chao1 is a richness estimator, estimating the total number of species present in a community by non-parametric calculations (Chao, 1984). Shannons' diversity index takes into account both abundance and evenness in the samples, and is calculated from Equation 2.1. Larger values reflect communities with greater species richness and evenness (Hollister et al., 2015).

$$H' = -\sum p_i \ln p_i$$

Equation 2.1: p_i = proportion of the ith species in the community.

Comparison between communities from different samples is called beta-diversity. And a beta-diversity measure is Bray-Curtis similarity, which was calculated using PAST. Bray-Curtis similarities is based on Bray-Curtis dissimilarity which quantify dissimilarities between two samples (Bray and Curtis, 1957). It considers both species' presence/absence and the relative abundance. The dissimilarity index is between 0 and 1, where 0 represents identical samples. Bray-Curtis similarity index is 1 minus Bray-Curtis dissimilarity index, thus 1 represent identical samples.

Principle coordinate analysis (PCoA) was performed in PAST based on Bray-Curtis similarities, and used to visualize similarities/dissimilarities between the community profiles.

PCoA is a multidimentional scaling which assign each sample to a location in a multidimentional space, based on a similarity matrix, for example Bray-Curtis similarity. The distance between two samples will be smaller for more similar samples.

PERMANOVA is a non-parametric test of significant difference between community profiles from two or more groups (Anderson, 2001). In this thesis the test was based on distance calculated by Bray-Curtis similarity. Both one-way and two-way PERMANOVA were used.

Two-sample t-test was performed to test the difference between Bray-Curtis similarities.

3 Results

3.1 Optimization of PCR amplification of 16S rRNA gene regions from salmon fry gut and skin

The initial plan for this study was to characterize the microbial communities associated with the salmon skin and gut samples by using an approach involving nested PCR amplification of the V3-region for DGGE-analysis. Previous work with amplifying the V3 region in presence of eukaryote DNA has shown to be more effective with a nested amplification protocol where the first amplification is performed with primers targeting a larger region of the 16S rRNA gene (Bakke et al., 2011). The primers used for this amplification are bacteria specific, and have been shown to not co-amplify eukaryotic 18S rRNA sequences.

To optimize the PCR we tested:

- The effect of DNA extraction kit
- Nested and non-nested PCR protocols
- Different polymerases
- Different PCR enhancers
- Effect of decontamination kit
- Template amounts

3.1.1 Effect of DNA extraction kit on PCR amplification

Two different DNA extraction kits were tested for salmon skin samples to examine whether the kit affected the subsequent PCR amplification of the V3 region with the nested protocol. The effect of different template amounts on the amplification efficiency was also investigated.

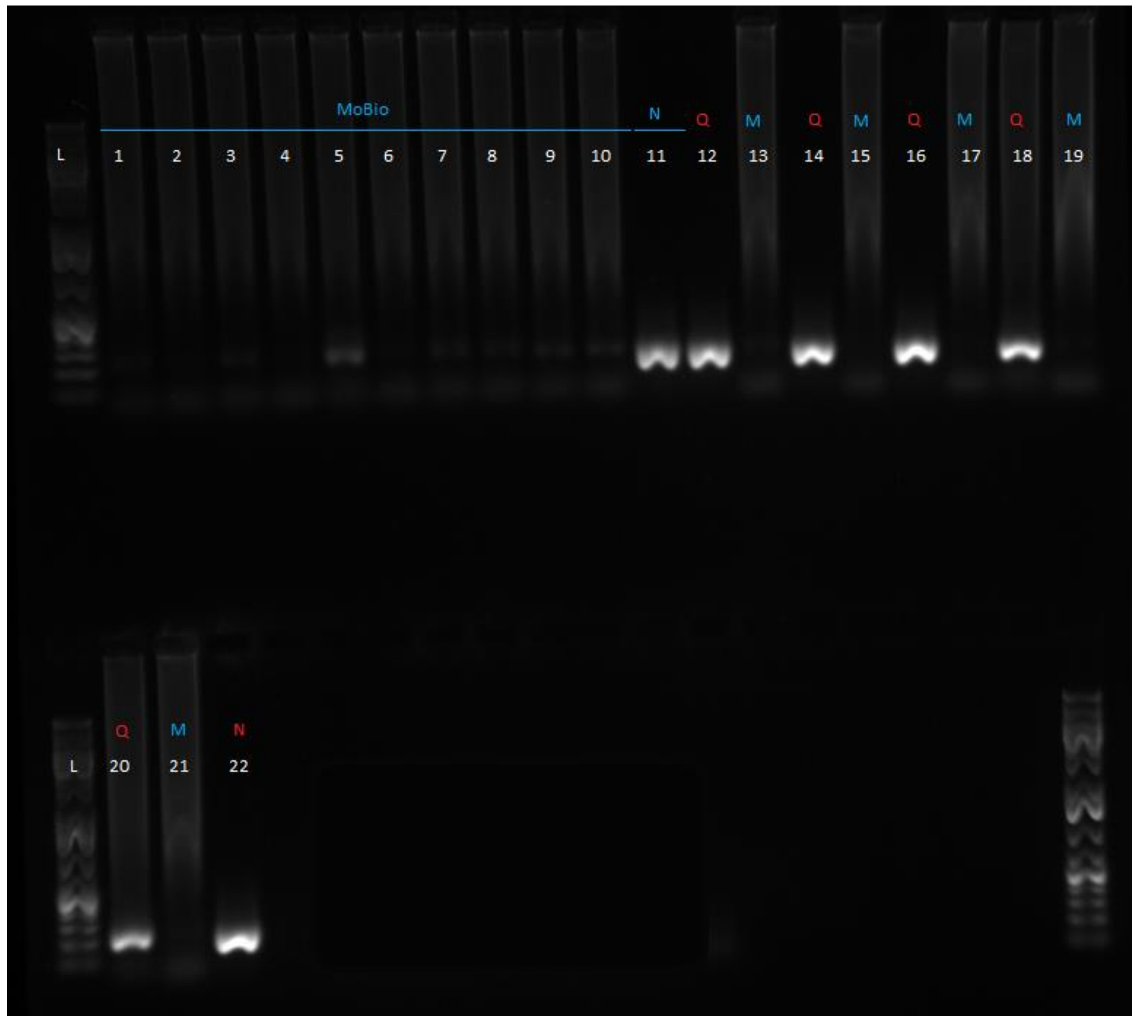

Figure 3.1: Agarose gel profiles of V3 16S rRNA products from nested PCR with MoBio and Qiagen DNA templates from different individuals. Lane 1-10: template extracted using MoBio kit, representing five individuals, where odd numbers represent skin from tail and paired numbers skin from body from their respective individual. Lanes 12, 14, 16, 18 and 20: Qiagen template. Lanes 13, 15, 17 and 19: MoBio template. Skin samples collected from five individuals, where the skin was divided along the backbone and one part extracted using MoBio kit and the other part extracted using Qiagen kit.Samples 11 and 22 show the respective negative controls.

Templates extracted using the Qiagen kit yielded stronger bands than templates extracted using the MoBio kit (Figure 3.1). There was PCR product for the negative controls, indicating presence of contaminating DNA. This is a common issue when using universal bacterial primers, since bacterial DNA often is associated with the polymerase (Iulia et al., 2013). There was more PCR product for the negative control than for the templates extracted from the MoBio kit, and approximately the same amount of PCR product in the negative control and samples extracted using the Qiagen kit (Figure 3.1).

Observing more PCR product for the negative control than for the templates extracted with the MoBio kit makes it reasonable to assume presence of inhibitors in the template generated by the MoBio kit.

Furthermore, it was not possible to know wheter products from Qiagen templates were products of specific amplification of the sample DNA or if it was contamination product (Figure 3.1). The gel indicate less problems with inhibitors using Qiagen template, and therefor this template was used for further investigations.

3.1.2 Effect of nested versus non-nested PCR protocol with PCR enhancers

A protocol with only the internal primers, 338F-GC and 518R, was tested to try to improve amplification efficiency. Due to the problems of generating PCR products for 16S rRNA, it was also investigated whether amplification efficiency could be improved by adding so-called PCR facilitators to the PCR reaction. The PCR facilitators' spermidine and glycerol were added to the PCR reactions to test their effects fir the non-nested protocol. The results from the agarose gel electrophoresis are presented in Figure 3.2.

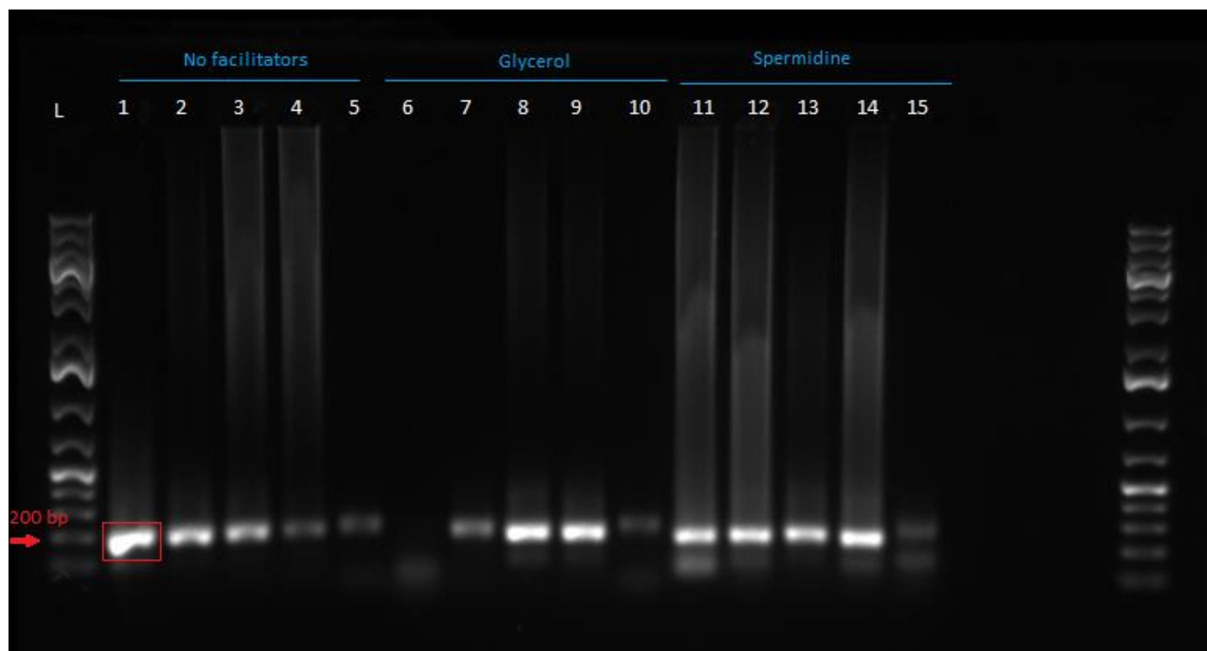

Figure 3.2: Agarose gel profiles of V3 16S rRNA amplified from salmon skin samples representing four individuals, with non-nested protocol. Lanes 1-5 no facilitators. Lanes 6-10, with glycerol, and 11-15 with spermidine. DNA templates were the same for the three sets of PCR reactions. Qiagen template: lanes 1, 2, 6, 7, 11, 12 represent the four different individuals. MoBio templates: 3, 4, 8, 9, 13 and 14, representing the four different individuals. Lanes 5, 10 and 15 show negative control for their for the relevant PCR facilitators. All reactions contained BSA.

The amplification with spermidine yielded bands for all templates (Figure 3.2). The bands representing reactions where spermidine was added appeared to be stronger than those with glycerol or without any facilitators, indicating improved amplification efficiency by the addition of spermidine. Furthermore the bands were stronger than negative control, indicating specific amplification of the template. This was also the case for some of the PCR reactions without PCR facilitator or those added glycerol (Figure 3.2). The PCR products were further analyzed by performing a DGGE (Figure 3.3), together with the PCR products obtained by nested protocol presented in Figure 3.1.

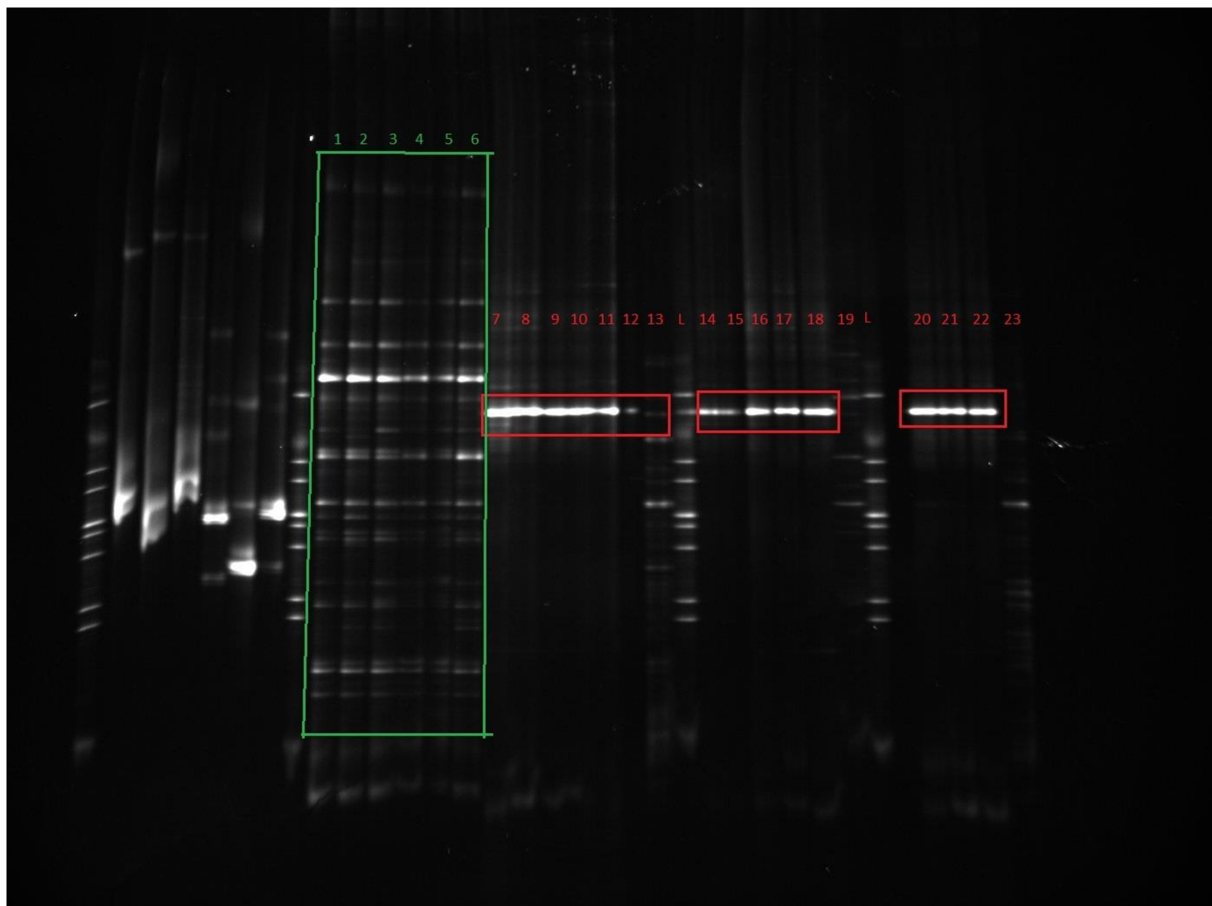

Figure 3.3: DGGE profiles for PCR amplified V3 16S rRNA fragments from salmon skin. Lanes marked green (1-6) PCR products from nested protocol (Lane 12, 14, 16, 18, 20 and 22 from Figure 3.2). Lanes marked red (7-23) PCR products from non-nested protocol. Further: 7-13 without glycerol or spermidine, 14-18 with spermidine, and 19-23 with glycerol. Lane 6, 13, 18 and 23 the respective negative controls. Qiagen templates: 1-5, 7-8, 14-15, and 19-20. MoBio templates: 9-12, 16-17, and 21-22. Lanes 10-12 represent the same PCR product.

The DGGE analysis (Figure 3.3) showed that PCR products obtained by the nested PCR protocol gave the same band pattern as the lane representing the negative control of the nested PCR. Thus, these products (lane 1-5) did not represent the specific products representing the samples, but probably contaminating DNA.

Samples amplified with the non-nested PCR protocol showed only one band in the DGGE analysis (Figure 3.3). One band in the DGGE profile indicates amplification of a sequence from only one species. The target sequence of the primers (338F and 805R) are conserved in eukaryote 18S rRNA (Bakke et al., 2011). Thus it is likely that the band represented salmon 18S rRNA since amplification of 16S rRNA should result in several bands. The nested protocol was used further to avoid amplification of the salmon DNA.

Lanes 13, 19 and 23, which represented the same PCR products as shown in the agarosegel in Figure 3.2 (lane 5, 6 and 10) showed several bands in their DGGE profiles, indicating a community. DGGE profiles in lanes 13 and 23 represented negative controls, indicating that contaminating DNA was present.

The nested PCR products analyzed by DGGE were probably contaminated, but still showed several bands in the gel, indicating amplification of microbial community 16S rRNA. Since there were indications of a microbial community, the contaminating DNA might have derived from the lab and not represent the production strain of the PCR polymerase.

3.1.3 PCR amplification with Phusion Hot Start Polymerase, PCR facilitators and DNase treatment

From previous experience in the lab, Phusion Hot Start Polymerase seems to have lower amounts of contaminating bacterial DNA (personal communication, Ingrid Bakke). Decontaminating (DNase) treatment of PCR reaction mixtures was therefore performed to see if this could resolve the contamination problems. As described above, another problem was that the template DNA probably contained PCR inhibitors. PCR enhancers (glycerol and spermidine) were therefore also used to improve the amplification efficiency. Templates were derived from three individual skin samples.

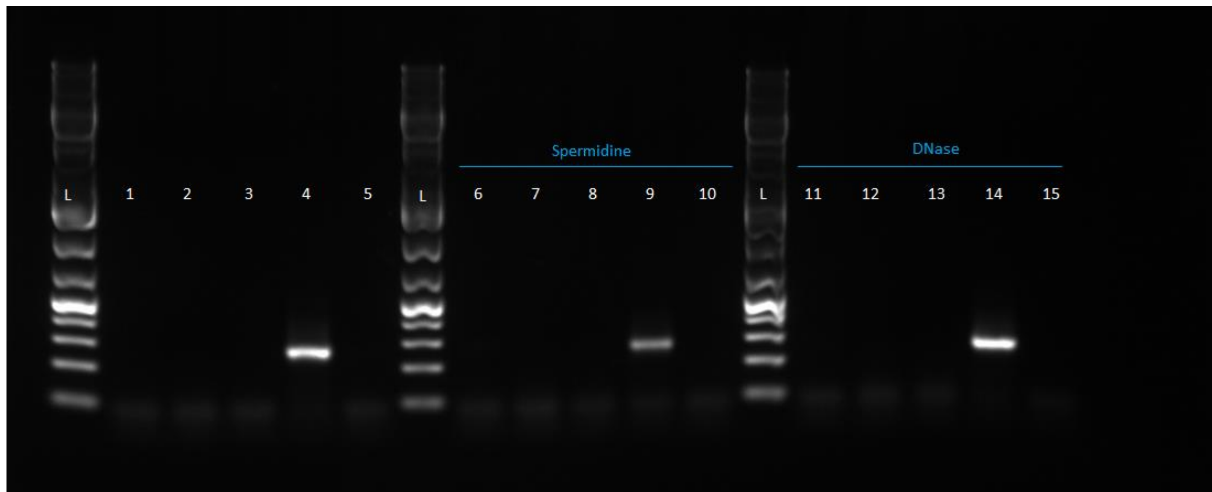

Figure 3.4: Agarose gel profiles of V3 16S rRNA with PCR products obtained by nested PCR protocol, with or without spermidine and DNase treatment. DNA templates were the same for the three different sets of PCR reactions, representing three different individuals. Phusion Hot Start polymerase was used. Lanes 1-5: no enhancers. Lanes 6-10: spermidine treatment in external and internal protocol. Lanes 11-15: DNase treatment before external amplification. Lanes 4, 9 and 14 show products for positive controls (DNA from water samples), while lanes 5, 10 and 15 show products for negative controls. 50ºC was used in external annealing and 53ºC was used in internal annealing. 24 cycles were used in both external and internal amplification. The templates were undiluted DNA from salmon skin extracted by the Qiagen kit.

No PCR products were obtained for neither of the PCR reactions (Figure 3.4), except for samples representing the positive control. There seem to be no contamination present for any of the treatments. The contamination problems described above may therefore be due to DNA associated with the Taq polymerase. The spermidine did not seem to have an enhancing effect on the amplification (Figure 3.4), despite enhancing the amplification for the non-nested PCR protocol (Figure 3.2).

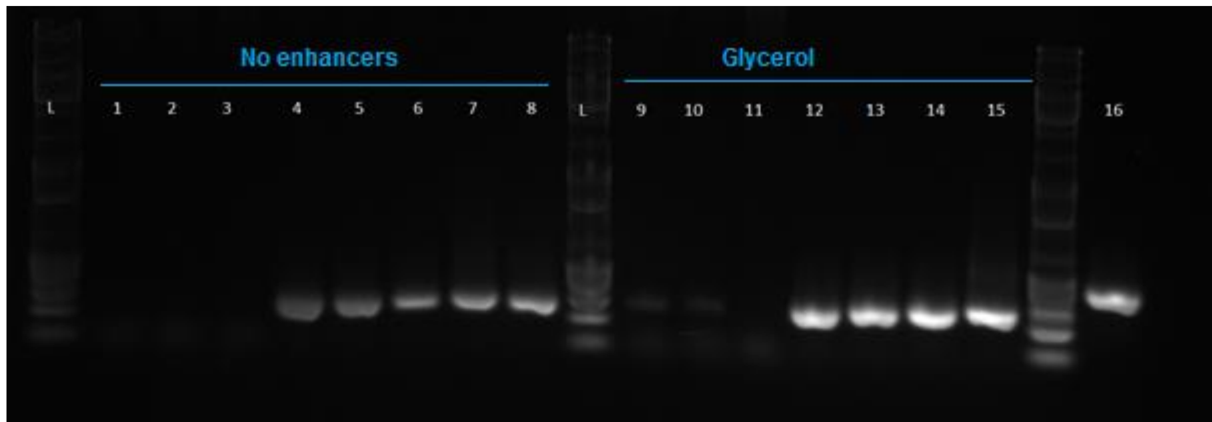

Figure 3.5: Nested PCR with Phusion Hot Start polymerase with and without glycerol treatment. Lanes 1-8 were without enhancers, and 9-16 with glycerol. Lanes 8 and 16: negative controls, sample applied to lane 16 was added after the electrophoresis was started due to a mistake. 53°C was used in both external and internal annealing. Phusion polymerase was added to each PCR tube. 25 cycles in external and internal amplification. Lanes 1-3 show undiluted template, 4-6 show 1:10 dilution of same samples. Lanes 9-11 undiluted template, 12-14 1:10 diluted template from same samples For samples with undiluted template, 2 µL template was used from the external to the internal amplification. For samples with 1:10 diluted template, 1 µL template was used from external to internal amplification. Qiagen templates from 3 individual salmon skin.

We tested if glycerol could have an enhancing effect on the amplification. Negative controls were approximately as strong as positive control and PCR products with sample templates (Figure 3.5). This again indicated a presence of contaminating DNA, which may have been caused by the higher concentrations of Phusion polymerase in these specific reactions, due to the addition of polymerase in each PCR tube, instead of the Eppendorf with the mastermix.

The PCR products obtained with 1:10 dilution of templates resulted in more PCR products than for the reactions with undiluted template. This reinforces the assumption of presence of PCR inhibitors in the template DNA.

To sum up these experiments, it is reasonable to assume that Phusion Hot Start Polymerase is less contaminated than Taq polymerase (Figure 3.4). There was no visible enhancing effect of treatments with spermidine. There was some enhancing effect of glycerol treatment, but this included enhancing of PCR product from the negative control. Dilutions (1:10) of the template seemed to result in better amplification and was used for further optimization.

3.1.4 *PCR amplification with PrimeSTAR GXL DNA polymerase*

The high-fidelity polymerase PrimeSTAR GXL was tested, in order to investigate if this polymerase could improve the amplification efficiency further. For reaction and cycling conditions, see Appendix C.

Both skin and gut templates were used for this amplification, to see if the template type would have any effect on the amplification. Templates were derived from three gut and three skin samples. Further, the effect of spermidine in both the external and internal PCR was tested.

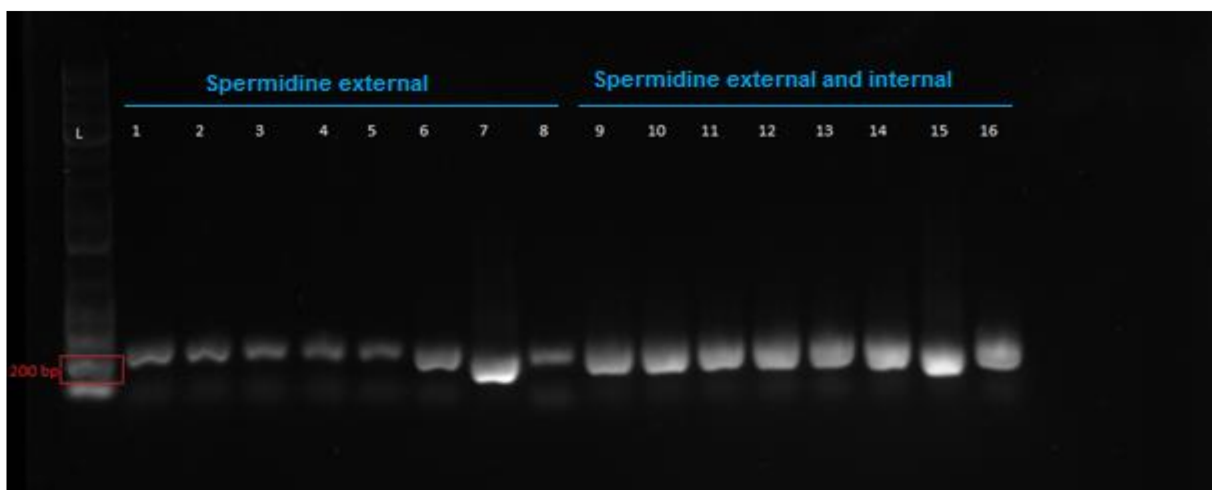

Figure 3.6: Amplification of the V3 16S rRNA region with GXL polymerase using nested protocol. Lane 1-8: PCR reactions contained spermidine only in the external amplification. Lane 9-16: PCR reactions contained spermidine in both external and internal amplification. Lanes 1-3 and 9-11: skin templates from three individuals. Lane 4-6 and 12-14: gut templates from three individuals. Lane 7 and 15 positive controls (DNA isolates from water samples). Lane 8 and 16: negative controls. All templates extracted using the Qiagen kit and diluted 1:10.

Spermidine enhanced the amplification more when used in both external and internal amplification (Figure 3.6). Amplification product in the negative control showed that contamination still was a problem (Figure 3.6). Therefore, DNase treatment was next included in the GXL polymerase protocol to eliminate contaminating DNA.

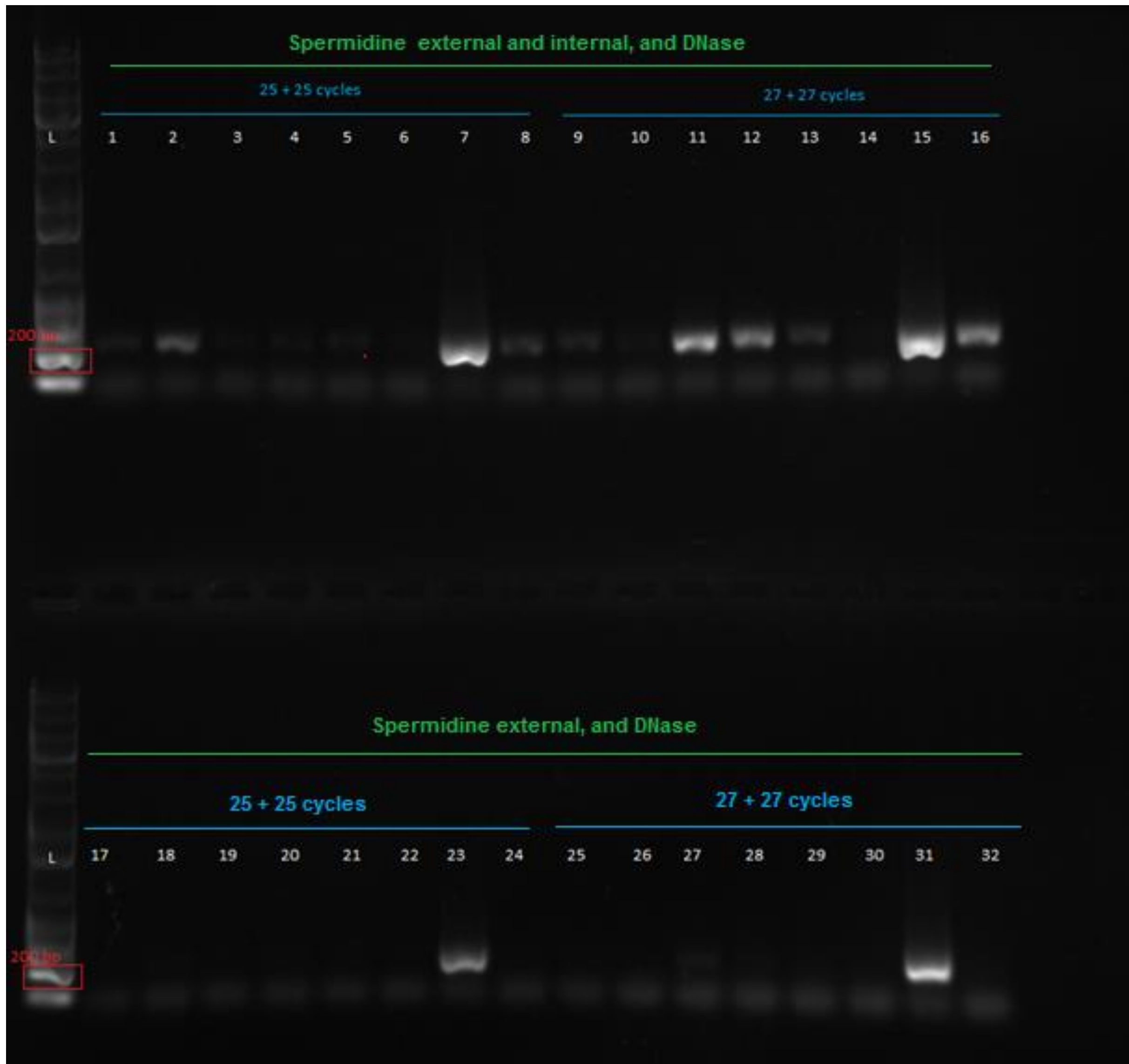

Figure 3.7: Amplification of V3 16S rRNA gene with GXL polymerase and DNase treatment using nested protocol. First row with spermidine in external and internal. Lanes 1-8: 25+25 cycles. Lanes 9-16: 27+27 cycles. Row 2 no spermidine. Lanes 17-24: 25+25 cycles. Lanes 25-32: 27+27 cycles. All samples treated with DNase. Lanes 1-3, 9-11, 17-19 and 24-26: skin templates from three different individuals (1:10 dilutions). Lane 4-6, 12-14, 20-22 and 28-30: gut templates from three individuals (1:10 dilutions). Lanes 8, 16, 24 and 32: negative controls. Lanes 7, 15, 23 and 31: positive controls (DNA from water samples).

The DNase treatment eliminated amplification of contaminating DNA (Figure 3.7). There was no observable contamination for the PCR products obtained without spermidine treatment in internal PCR. However the use of PrimeSTAR GXL DNA polymerase did not result in successful PCR amplification of the V3 region of 16S rRNA from skin or gut samples.

For the Phusion Hot start polymerase, there were almost no problems with contaminating DNA, even without the use of DNase treatment, and it was therefore used for further testing.

3.1.5 Comparing broad-coverage 16S rRNA PCR Primers

As shown above, a successful amplification of the V3 16S rRNA region was not obtained for fish samples using the nested protocol. Other primer sets, targeting the V3 and V4 regions of the 16S rRNA gene, were therefore tested. These primers had Illumina adapter sequences attached for subsequent Illumina sequencing (Table 2.2), and a PCR protocol for generation of an amplicon library was applied. This protocol did not involve nested PCR, and Phusion Hot start polymerase was used in PCR reactions without addition of PCR enhancers.

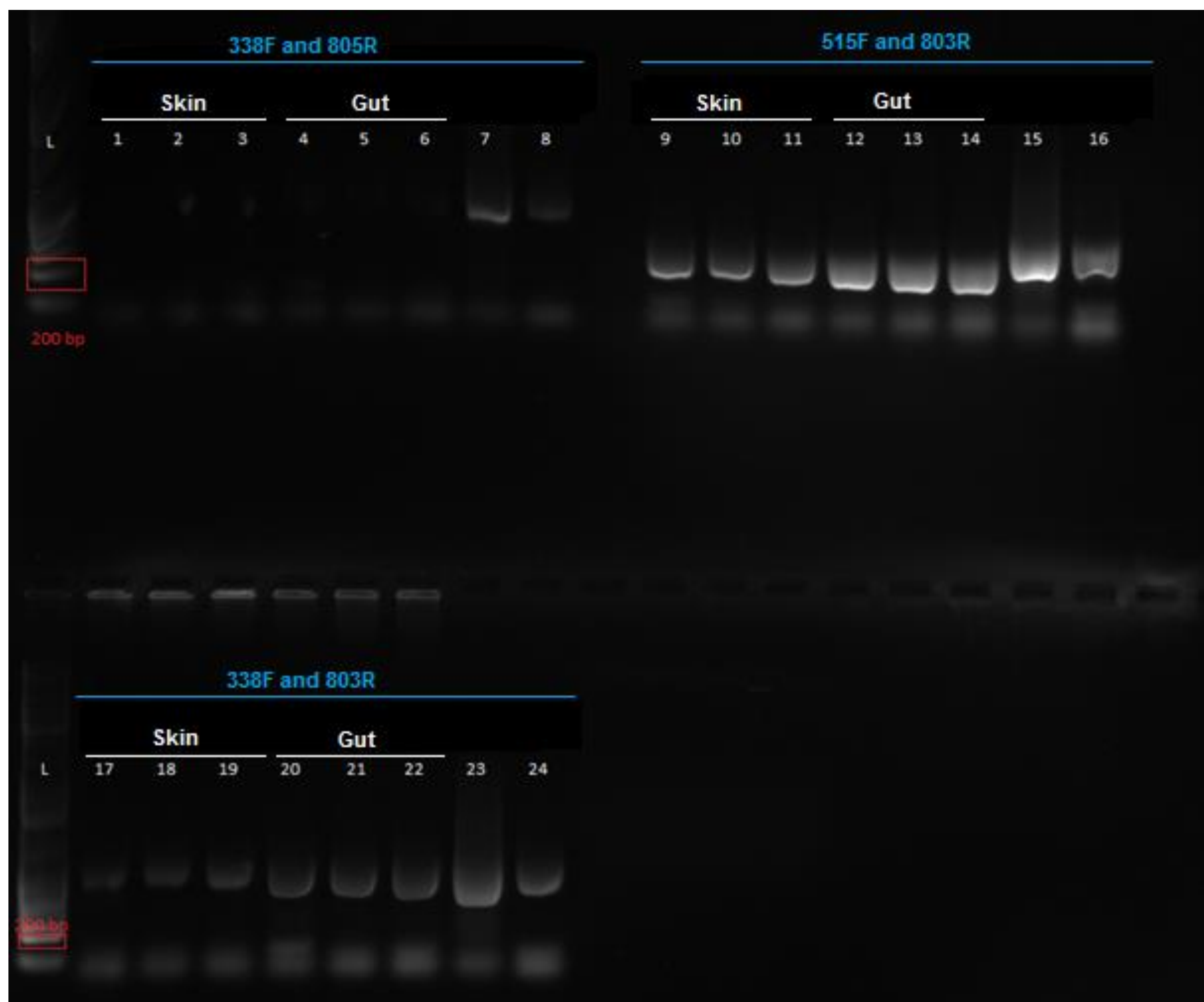

Figure 3.8: Agarose gel with 16S rRNA amplicons obtained for different primer sets for both skin and gut samples. Samples 1-8 amplified with primers 338F and 805R, samples 9-16 with 515F and 803R, and 17-24 with 338F and 803R. Phusion Hot Start polymerase was used. Lanes 1-3, 9-11 and 17-19: skin templates. Lanes 4-6, 12-14 and 20-22: gut templates. All templates extracted using Qiagen kit (1:10 dilution). Samples 7, 15 and 23: positive controls (DNA from water samples). Samples 8, 16 and 24: negative controls.

Primer set 515F and 803R resulted in PCR products for all samples, which were stronger than the product representing the negative control (Figure 3.8). This primer set target the V4 region. The two other primer sets, did not result in more PCR product for the template samples than for the negative controls (Figure 3.8). The 515F/803R primer set was further tested in PCR reactions with different template amounts.

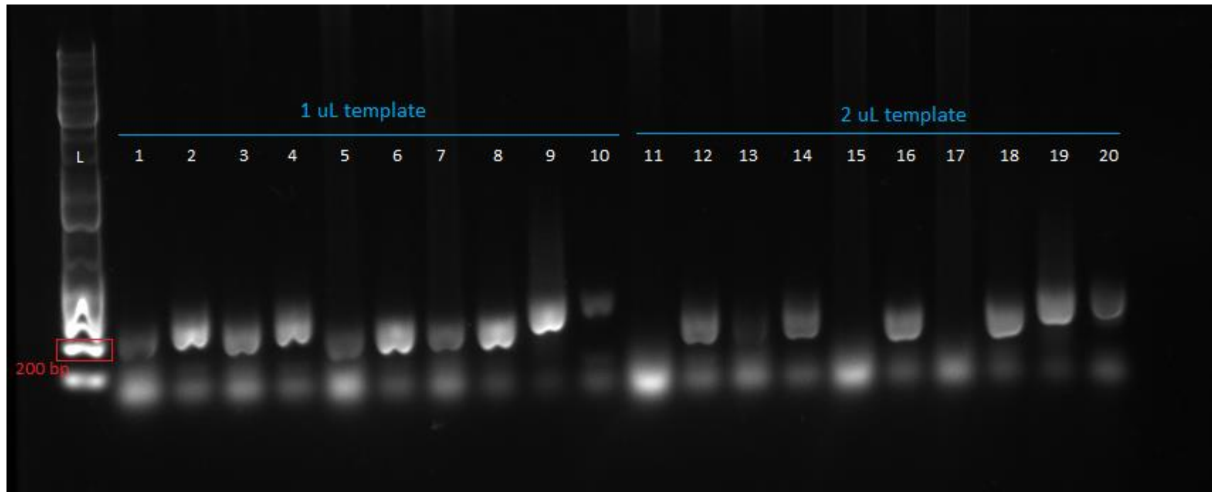

Figure 3.9: PCR amplification of V4-region of 16S. Primers 515F and 803R were used. Samples 1-10 and samples 11-20 contained 1 and 2 μL of template in a total of 20 μL reaction volume, respectively. Odd numbers: undiluted template, pair numbers: 1:10 dilutions of same template. Lanes 1-4 and 11-14: Skin template. Lanes 5-8 and 15-18: gut templates. Lanes 9 and 19: positive controls. Lanes 10 and 20: negative controls. Templates from 4 individuals, two skin, and two gut.

The most efficient amplification was obtained when using template DNA diluted 1:10 (1 μL per 20 μL reaction) (Figure 3.9).

Primer set 515F/803R (with Illumina adapters), using Phusion Hot Start polymerase and DNA extracted using the Qiagen kit, diluted 1:10, as templates, resulted in specific PCR products for both gut and skin samples. This protocol was therefor used for generating a V4 16S rRNA amplicon library for subsequent Illumina sequencing.

3.2 Illumina amplicon sequencing

As described in Experimental design (section 2.1), salmon fry representing two different strains (wild and aquaculture) were reared with three different diets, fish oil (FO), vegetable oil (VO) and phospholipid (PL), in two replicate tanks. We wanted to examine how diet and genetic background affected the skin and gut microbiota by using an Illumina amplicon sequencing approach.

3.2.1 Richness and Diversity of Microbial communities

Bacterial communities in gut and skin samples from wild and aquaculture strain *Salmo salar* were examined using Illumina MiSeq sequencing of 16S rRNA V4 amplicons. Total number of reads of the samples after quality filtering and chimera removal was 3 681154, with an average of 44 351 reads per sample.

Number of reads for water samples was 975554 with an average of 81296 ± 17153. Total number of reads for gut samples was 1139441 with an average of 33512 ± 24380. Total number of reads for skin samples was 1420830 with an average of 39467 ± 21376. The standard deviation was high for both gut and skin samples indicating differences in sequencing effort for different samples. Clustering of sequence reads into operational taxonomic units (OTU) resulted in a total of 5359 OTUs. Taxonomy assignment revealed two *Salmo salar* OTUs and 14 archaeal OTUs which were removed.

Estimated richness (Chao1) and observed number of OTUs were compared, and demonstrated that the sequencing effort across samples covered more than 70 % of the estimated bacterial richness on average. The corresponding numbers for water, skin, and gut samples were 73 %, 78.5 %, and 71.6 %, respectively. The observed numbers of OTUs and Chao1 is presented in Figure 3.10A, while Shannon's diversity index is presented in Figure 3.10B.

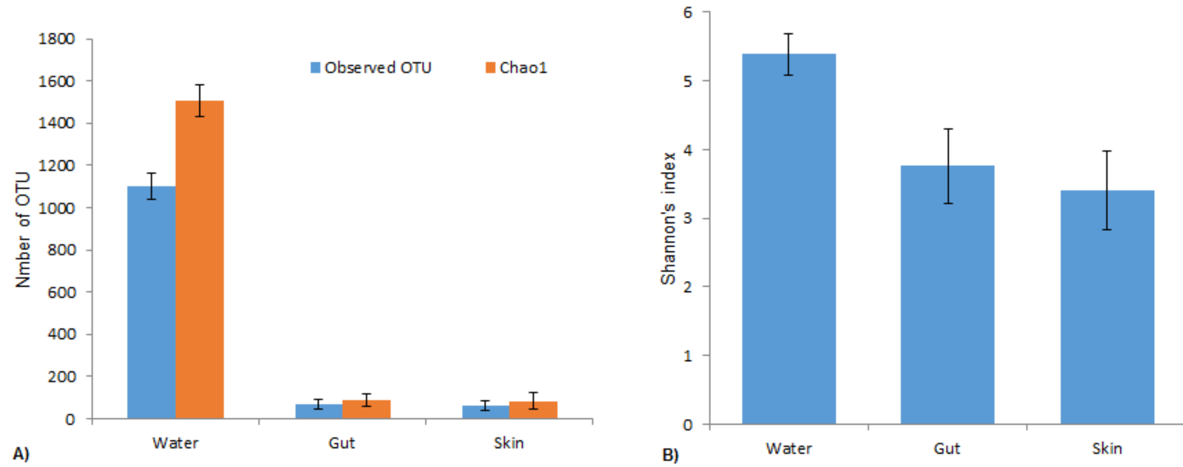

Figure 3.10.: Average diversity indices for microbiota of water, gut and skin samples. A) Number of observed OTUs and Chao1 index. B) Shannon's diversity index. Error bars indicate standard deviation of the mean.

There was a large difference in the observed number of OTUs and Chao1 between the water samples and fish samples (Figure 3.10A). The observed number of OTUs for water samples was approximately five times higher than for the gut and skin communities. Shannon's diversity index was also higher for the water microbiota than for gut and skin communities Figure 3.10B).

The observed number of OTUs and estimated richness of gut and skin microbiota from both aquaculture and wild strain samples are presented in Figure 3.11 together with Shannon's diversity index.

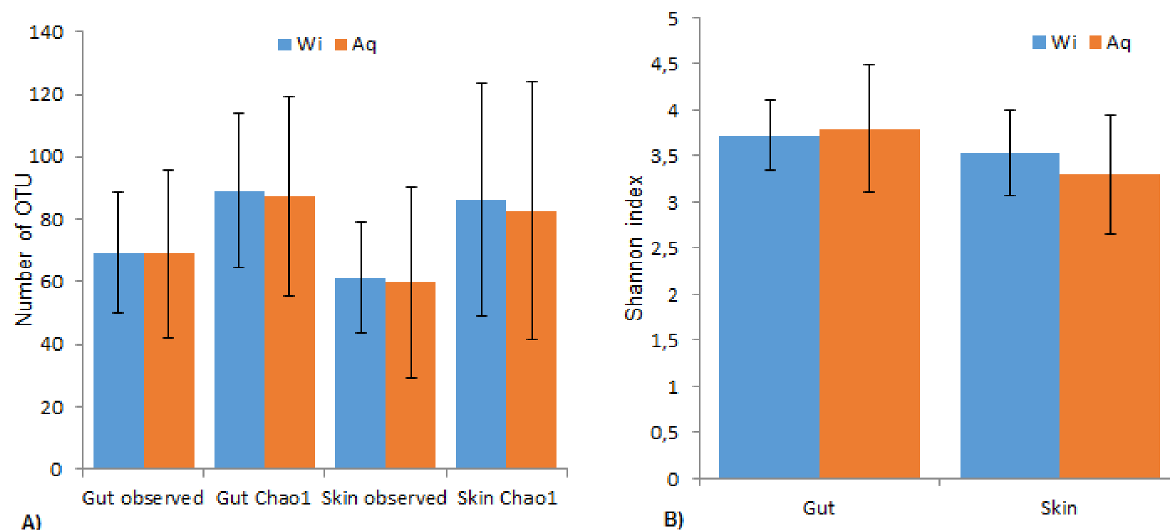

Figure 3.11: Diversity indices for gut and skin microbiota of aquaculture and wild salmon strains. A) Observed OTU richness and Chao1. B) Shannon's diversity index. Error bars indicate standard deviation of the mean. Wi: Wild strain salmon. Aq: Aquaculture strain salmon.

The diversity indices of gut and skin samples seem to be relatively similar (Figure 3.11). Gut samples seem to have a slightly higher richness, but neither observed OTU nor Chao1 were significantly different comparing skin and gut samples (t-test, $p = 0.14$ and $p = 0.66$ respectively). Shannon's diversity index on the other hand was significantly higher for gut microbiota (H' = 3.77) than skin microbiota (H' = 3.41) (t-test, $p = 0.01$).

It did not appear to be any difference in microbial diversity between aquaculture and wild salmon strains of gut samples and skin samples (Figure 3.11). This was confirmed by t-tests ($p > 0.05$).

3.2.2 Comparison of microbial communities between water, gut and skin samples

As shown above, the diversity indices showed richer microbial communities in water samples compared to fish samples. Further comparison of water microbiota and fish microbiota was done, and a figure of microbial community composition at the phylum level for water, gut and skin samples, is presented in Figure 3.12.

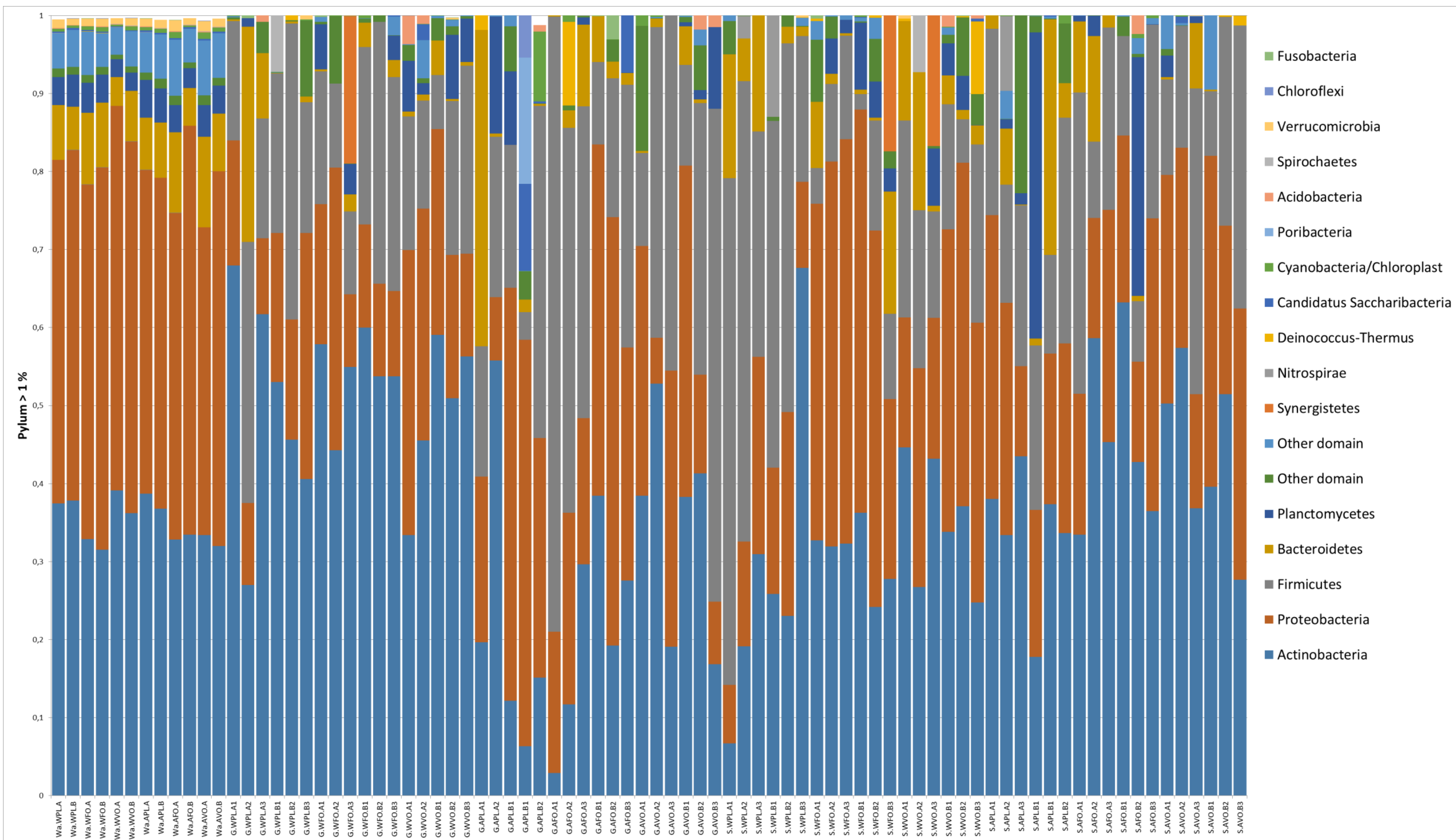

Figure 3.12: Relative abundances of bacterial phyla in individual samples. Only phyla represented with more than 1 % of the total amount of reads in minimum one of the samples were included. Wa: water sample. G: gut sample, S: skin sample. A: aquaculture strain. W: wild strain. Diets; FO: fish oil. VO: Vegetable oil. PL: phospholipid. A: tank A, B: tank B.

The bacterial phylum which was most abundant in fish samples (on average) was Actinobacteria (38 ± 16 %), followed by Proteobacteria (26 ± 13 %) and Firmicutes (24 ± 16 %) (Figure 3.12). Proteobacteria was more abundant in skin samples (27.5 %) than gut samples (24.1 %), while Firmicutes was more abundant in gut samples (26.0 %) than skin samples (22.2 %).

The bacterial communities on phylum level were notably different between water samples and fish samples. The most abundant bacterial phylum in the water samples was Proteobacteria (45 ± 4 %) followed by Actinobacteria (35 ± 3 %) and Bacteriodetes (7 ± 2 %). Firmicutes was almost absent in water samples, while Proteobacteria and Actinobacteria accounted for 80 % of the reads (Figure 3.12).

Further comparison of microbial communities between the three sample groups, water, gut, and skin, was done by principal coordinates analysis (PCoA) ordination (Figure 3.13).

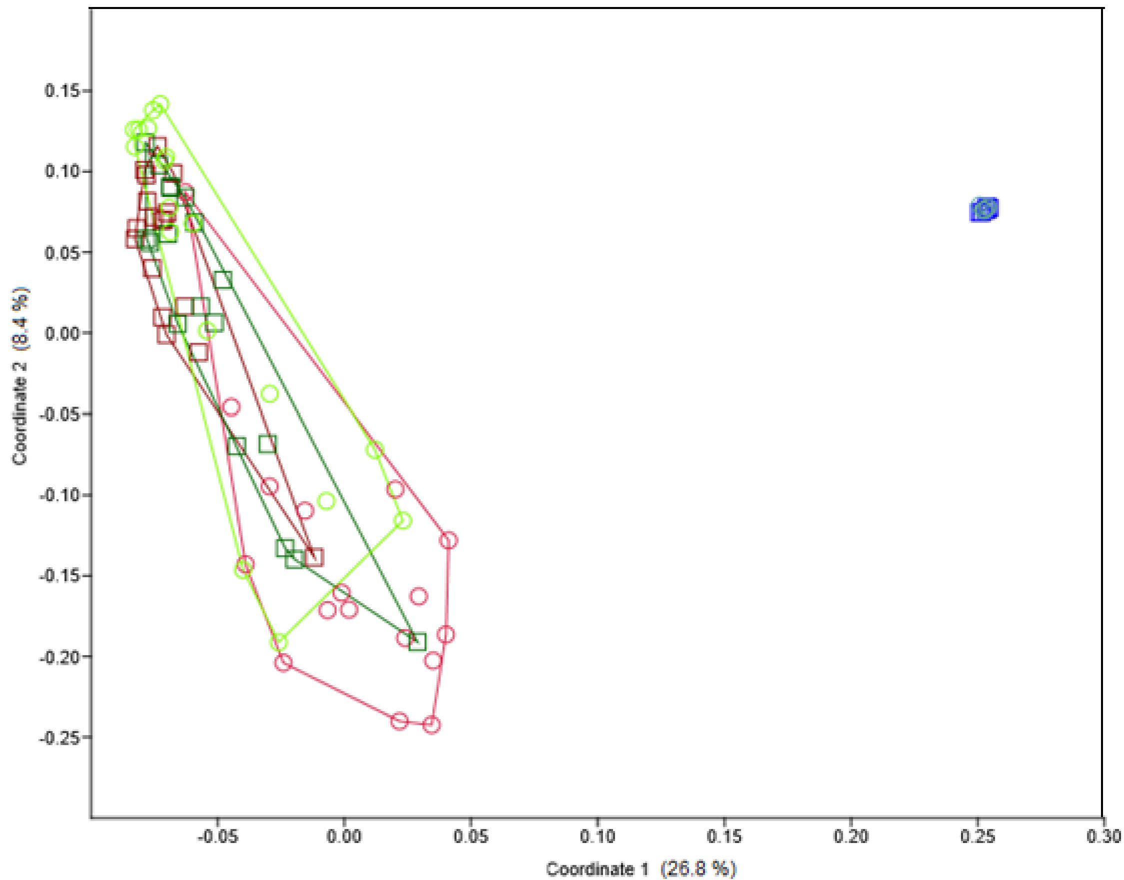

Figure 3.13: PCoA plot based on Bray-Curtis similarities for comparison of microbiota from water and fish samples. Blue circles: Water samples from tanks with aquaculture salmon. Blue squares: Water samples from tanks with wild salmon. Red circles: Gut samples from aquaculture genotype. Red squares: Gut samples from wild genotype. Green circles: Skin samples from aquaculture genotype. Green squares: Skin samples from wild genotype.

The PCoA plot corroborates the results from Figure 3.12. The first and second principal components together explained 35.2 % of the variation and there was a clear separation between samples from water microbiota and fish microbiota (Figure 3.13). This indicated that water and fish samples were very different, and it was confirmed by a one-way PERMANOVA test ($p = 0.0001$). Water samples appeared to be very similar. Gut and skin samples separated more than water samples.

3.2.3 Effect of genotype and diet on water microbiota

Water samples were compared to examine whether the different diets and genotype of salmon had any impact on the microbiota in the rearing water. The water microbiota had high OTU richness and Shannon's diversity index indicating great diversity in communities from water samples. A PCoA ordination for only water samples was done to compare the water microbiota in more detail (Figure 3.14).

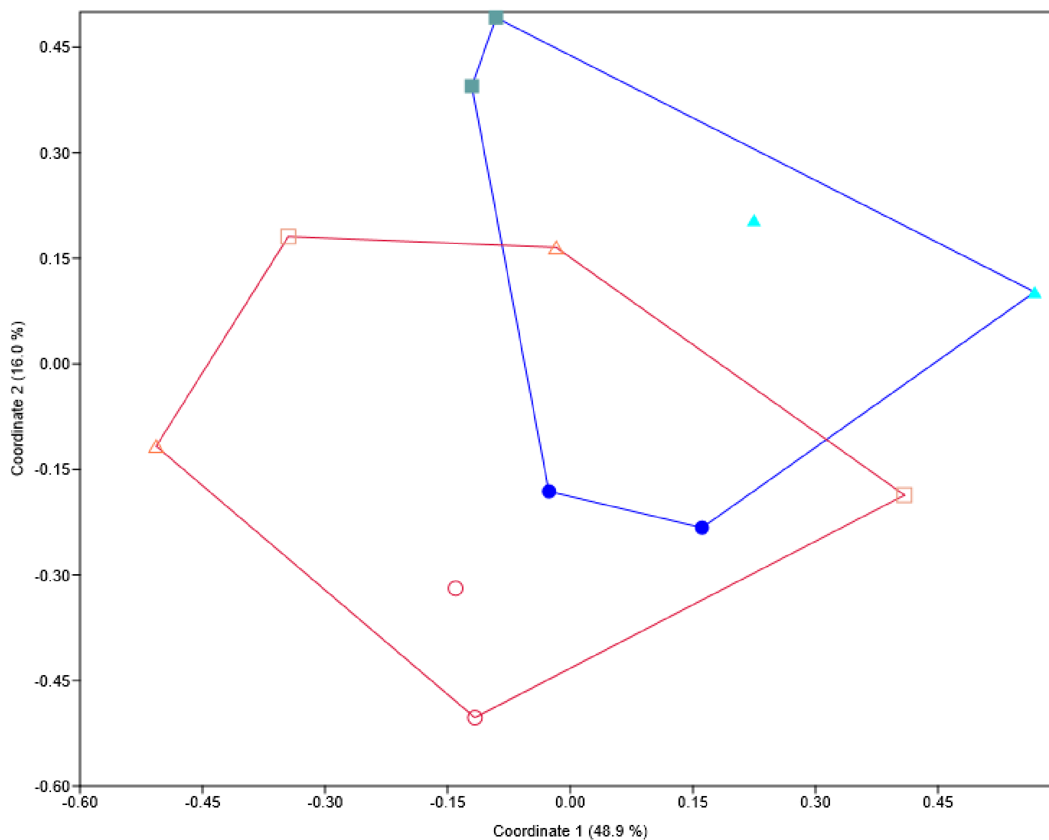

Figure 3.14: PCoA plot based on Bray-Curtis similarities of water microbiota among samples from different tanks. Blue circles: WPL tank. Blue squares: WFO tank. Blue triangles: WVO tank. Red circles: APL tank. Red squares: AFO tank. Red triangles: AVO tank. Sample labels: A: aquaculture strain; W: wild strain; FO: fish oil; VO: vegetable oil; PL: phospholipid oil.

Together, the first and second principal components explained 64.9 % of the variation (Figure 3.14). There was a tendency that the water microbiota clustered according to genotype of salmon (Figure 3.14). For microbiota from wild salmon tanks, clustering of diets appeared to be present. However, no significant differences were found for either genotype or diet

(PERMANOVA p > 0.05). Bray-Curtis similarities were calculated for comparison of community profiles between the different water samples and are presented in Figure 3.15.

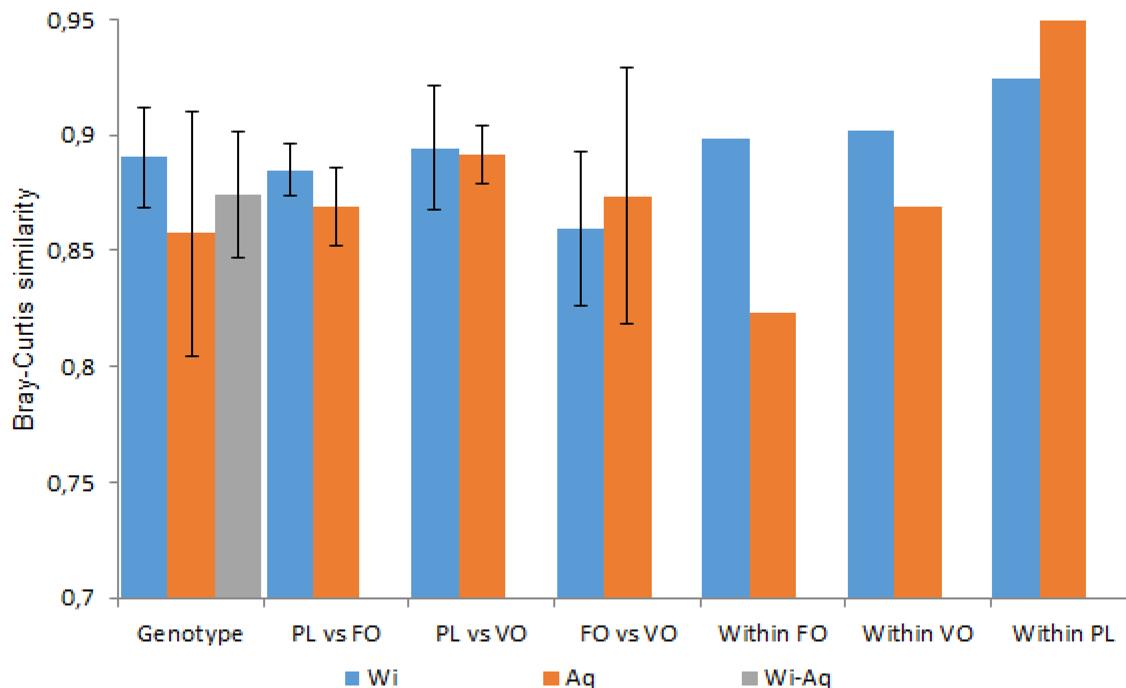

Figure 3.15: Average Bray-Curtis similarities for comparison of water microbial community profiles between water samples from tanks with; wild genotype salmon (Wi), aquaculture genotype salmon (Aq), Phospholipid diet (PL), fish oil diet (FO) and vegetable oil diet (VO). Wi-Aq represents comparisons of samples between tanks with the two genotypes. Error bars indicate standard deviation of the mean.

Bray-Curtis similarities for comparison among water samples were generally very high (Figure 3.15). There was a tendency to higher Bray-Curtis similarities between water samples from tanks with wild genotype salmon, indicating less variation between these samples. The similarity was higher between the two strains than within the aquaculture strain, indicating that microbial communities in water hosting aquaculture group was more diverse between samples (Figure 3.15).

Only two replicates for each water sample with same treatment was sequenced, so no error bars are shown for samples within each diet.

Table 3.1: The ten most abundant OTUs in water samples, together with the taxa it represents and average amount of reads.

OTU Id	Taxonomic assignment	Average amount of reads (%)
OTU_2	*Polynucleobacter*	22.3 ±3.1
OTU_9	Actinobacteria	10.5 ±1.3
OTU_21	Actinobacteria	6.9 ±0.6
OTU_15	Burkholderiales	6.6 ±1.0
OTU_18	*Sediminibacterium*	3.5 ±0.8
OTU_222	Burkholderiales	3.4 ±0.5
OTU_47	Actinobacteria	2.3 ± 0.2
OTU_44	Comamonadaceae	2.2 ± 0.3
OTU_26	Planctomycetes	2.0 ± 0.4
OTU_40	Actinobacteria	1.8 ± 0.6

The ten most abundant OTUs of the water communities are presented in Table 3.1. OTU 2 (*Polynucleobacter,* Betaproteobacteria), OTU 9 (Actinobacteria), OTU 21 (Actinobacteria) and OTU 15 (*Burkholderiales,* Betaproteobacteria) comprised on average 46 % of the total amount of reads in the water samples. These ten OTUs comprised (on average) 61.3 % of the reads from water samples.

3.2.4 Comparison of gut and skin microbiota

The PCoA plot shown in Figure 3.13 indicated that the skin and gut microbiota were relatively similar. A new PCoA ordination including only gut and skin samples was performed to examine the similarity of the skin and gut samples further.

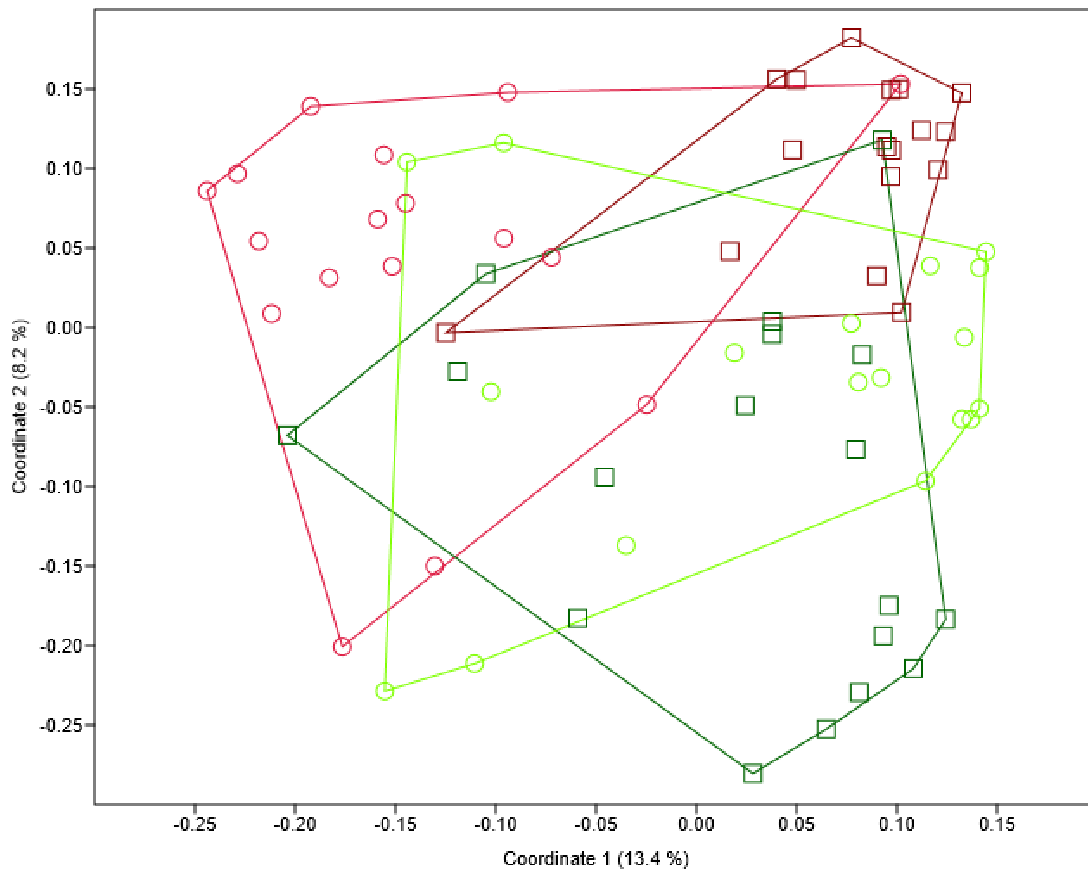

Figure 3.16: PCoA plot based on Bray-Curtis similarities for comparison of gut and skin microbiota. Green squares: Skin samples, wild genotype. Green circles: Skin samples, aquaculture genotype. Red squares: Gut samples, wild genotype. Red circles: Gut samples, aquaculture genotype.

The first and second principal components together explained 21.6 % of the variation in this dataset, and there is overlap between skin and gut samples (Figure 3.16). However a one-way PERMANOVA test based on Bray-Curtis similarities demonstrated significant difference between microbiota from gut and skin ($p = 0.0001$). Aquaculture strain gut samples were significantly different from aquaculture strain skin samples ($p = 0.0002$) and wild strain gut samples were significantly different from wild strain skin samples ($p = 0.0001$).

Average Bray-Curtis for comparisons within gut samples, within skin samples, and between gut and skin samples is shown in Figure 3.17.

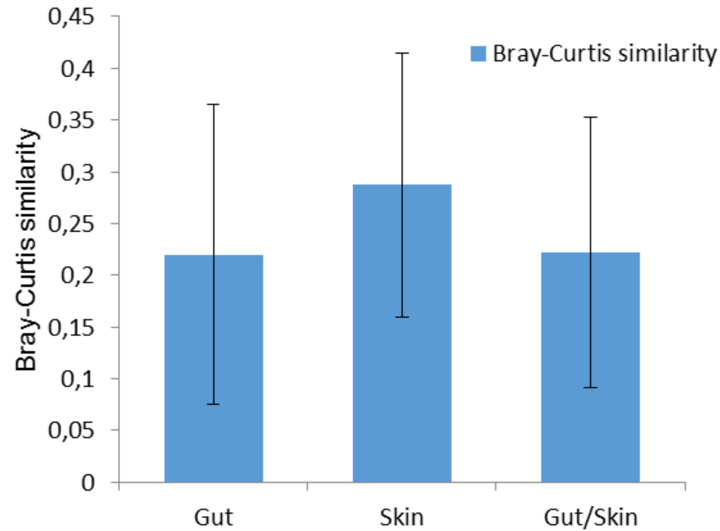

Figure 3.17: Average Bray-Curtis similarities for comparison of gut microbial community profiles, skin profiles and between gut and skin microbial community profiles.

Bray-Curtis similarities for comparisons within gut and skin samples and between gut and skin samples were all less than 0.3 (Figure 3.17). This indicated large differences in microbiota between individuals. The average Bray-Curtis similarity for comparison between gut and skin samples were the same as for comparison among gut samples (Br-C = 0.22) (Figure 3.17). Skin samples had a higher similarity (Br-C = 0.29). This indicated larger differences in gut microbiota between individuals than for skin samples. A t-test confirmed that the Bray-Curtis similarities were significantly higher for comparison among skin samples than gut samples ($p = 5.5 \times 10^{-17}$).

The most abundant sequences from the gut and skin samples were from the phyla Actinobacteria, Firmicutes and Proteobacteria (Figure 3.12). The ten most abundant OTUs from fish samples is presented in Table 3.2, and the ten most abundant OTUs for gut and skin is presented in Table 3.3.

Table 3.2: The ten most abundant OTUs in skin and gut samples combined. Presented with OTU identification and taxonomy, together with average amount of reads.

OTU ID	Taxonomy	Average amount of reads (%)
OTU_1	*Propionibacterium*	23.5 ± 14
OTU_7	*Pseudomonas*	3.4 ±6
OTU_3	Campylobacteraceae	2.9 ± 6
OTU_6	*Corynebacterium*	2.8 ± 4
OTU_4	*Streptococcus*	2.6 ± 7
OTU_5	*Exiguobacterium*	2.4 ± 5
OTU_10	*Streptococcus*	2.2 ± 4
OTU_11	*Micrococcus*	2.2 ± 3
OTU_13	*Pelomonas*	2.0 ± 3
OTU_17	Enterococcaceae	1.9 ± 5

Table 3.3: The ten most abundant OTUs in skin and gut samples. Given with OTU identification and the taxonomy, together with average amount of reads in the respective sample types.

Skin			Gut		
OTU ID	Taxonomy	Average amount of reads (%)	OTU ID	Taxonomy	Average amount of reads (%)
OTU_1	*Propionibacterium*	25.0 ±12	OTU_1	*Propionibacterium*	22.3 ±15
OTU_7	*Pseudomonas*	5.7 ±7	OTU_6	*Corynebacterium*	3.3 ±4
OTU_5	*Exiguobacterium*	4.6 ± 6	OTU_11	*Micrococcus*	2.8 ± 3
OTU_3	Campylobacteraceae	4.4 ±7	OTU_17	Enterococcaceae	2.7 ±6
OTU_4	*Streptococcus*	4.3 ± 9	OTU_14	*Staphylococcus*	2.3 ±3
OTU_10	*Streptococcus*	2.8 ± 4	OTU_13	*Pelomonas*	2.0 ± 2
OTU_32	Planctomycetes	2.5 ± 8	OTU_30	Clostridiales	1.8 ± 7
OTU_6	*Corynebacterium*	2.2 ± 4	OTU_8	Clostridiales	1.7 ± 7
OTU_13	*Pelomonas*	2.0 ± 4	OTU_23	*Weissella*	1.7 ± 4
OTU_8	Clostridiales	1.9 ±7	OTU_10	*Streptococcus*	1.5 ± 2

None of the most abundant OTUs in fish samples (Table 3.2) were among the most abundant OTUs in water communities (Table 3.1), indicating that the salmon fry do not share microbial communities with the surrounding water.

OTU 1 (*Propionibacterium,* Actinobacteria) represented 23.5 % of the fish sample reads on average (Table 3.2). It was also the most abundant specie in both gut and skin samples with average of 22.3 % and 25.0 % of the reads respectively (Table 3.3). OTU 1 was clearly more abundant than any other species in fish samples, with OTU 7 (*Pseudomonas,* Proteobacteria) as the second most abundant with an average of 3.36 % of the reads for fish samples (Table 3.2). OTU 3 (Campylobacteraceae, Proteobacteria) was the third most abundant in fish samples, it was abundant in skin samples (4.4 %) but not among the ten most abundant OTUs in gut samples. For gut samples, only OTU 23 (*Weisella*) represented the phylum Proteobacteria, all other abundant gut OTUs represented Actinobacteria or Firmicutes phyla (Table 3.3).

Some of the OTUs were among the ten most abundant in both gut and skin communities; OTU 1 (*Propionibacterium,* Actinobacteria), 6 (*Corynebacterium,* Actinobacteria), 8 (Clostridiales, Firmicutes), 10 (*Streptococcus*, Firmicutes), and 13 (*Pelomonas,* Proteobacteria) (Table 3.3). The OTUs presented in Table 3.3 comprise (on average) 55.4 % of the reads of skin samples and 42.1 % of the reads of gut samples, thus representing a amount of the reads.

SIMPER analysis was performed to identify the OTUs that contributed most to the Bray-Curtis dissimilarity between microbial communities from gut and skin samples.

Table 3.4: OTUs that contributed the most to the difference between microbiota from gut and skin samples, identified by SIMPER analysis based on Bray-Curtis dissimilarity. Top five OTUs with taxonomy, contribution percentage and mean abundance in gut and skin samples are presented.

OTU ID	Taxonomy	Contribution to dissimilarity (%)	Mean abundance Gut (%)	Mean abundance Skin (%)
OTU_1	*Propionibacterium*	10.41	21.9	25
OTU_7	*Pseudomonas*	3.55	0.87	5.71
OTU_3	Campylobacteraceae	3.22	1.3	4.4
OTU_4	*Streptococcus*	2.99	0.92	4.27
OTU_5	*Exiguobacterium*	2.95	0.027	4.6

The largest contributor to the difference between gut and skin microbiota was OTU 1 representing *Propionibacterium* (Table 3.4), the most abundant OTU in both gut and skin samples. OTU 7 (*Pseudomonas*) was the second highest contributor to the difference. This OTU was not in the ten most abundant sequences of gut samples, but was represented with an average of 5.71 % in the skin samples (Table 3.3). OTU 3 (Campylobacteraceae) contributed third most to the dissimilarity between microbiota from gut and skin samples. All OTUs in Table 3.4 had higher mean abundance in skin samples than gut samples.

3.2.5 Effect of genotype and diet on the gut microbiota

As described above, significant difference between gut and skin microbiota was found. Further analysis was performed to investigate possible effects on the gut microbiota caused by genotype or diet. A PCoA ordination was performed to compare the gut microbiota from samples representing distinct salmon strains and diets (Figure 3.18).

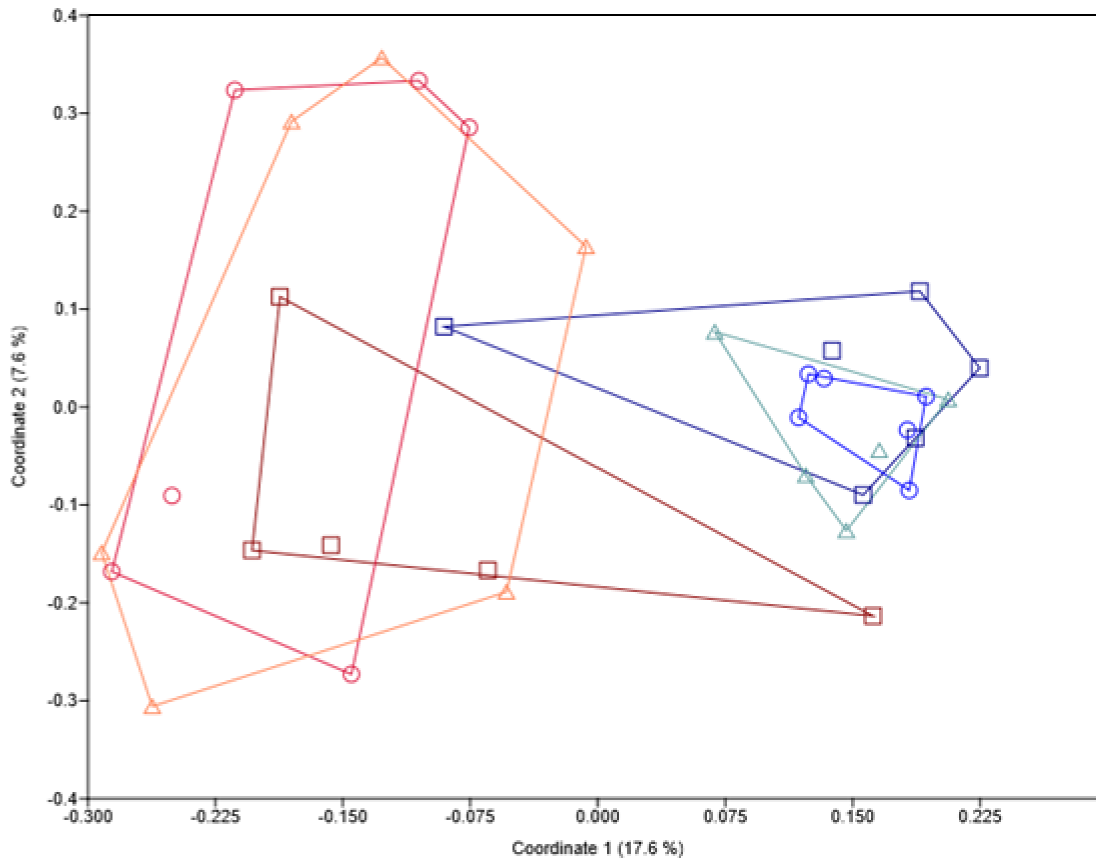

Figure 3.18: PCoA plot based on Bray-Curtis similarities for comparison of gut microbiota from different strains of salmon given different diets. Red squares: APL. Red circles: AFO. Red triangles: AVO. Blue squares: WPL. Blue circles: WFO. Blue triangles: WVO. Sample labels: A: aquaculture strain; W: wild strain; FO: fish oil; VO: vegetable oil; PL: phospholipid oil.

The first and second principal components together explained 25.2 % of the variation in this dataset (Figure 3.18). Wild and aquaculture salmon gut samples clustered separately from each other, indicating a difference between the microbial communities from the two genotypes. Microbiota from wild genotype seemed closer clustered than microbiota from aquaculture genotype. This indicates that gut microbiota from wild genotype salmon were more similar to each other than microbiota from aquaculture genotype salmon gut. There is no or little indication that the diets had any major influence on the gut microbiota (Figure 3.18).

A two-way PERMANOVA test confirmed that there was significant difference between gut microbiota of genetically different salmon ($p = 0.0001$). No significant difference according to diets was found ($p = 0.37$), and there was no interaction effect between genotype and diet

(p = 0.72). Interestingly the salmon genotype appeared to influence the gut microbiota more profoundly tan the diet.

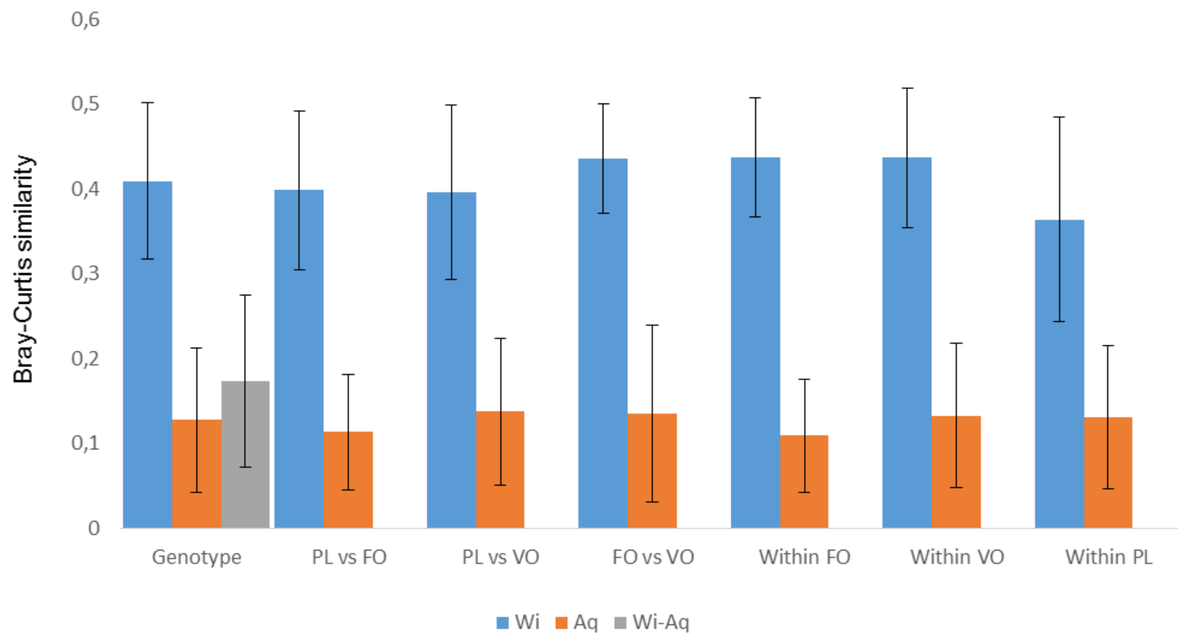

Figure 3.19: Average Bray Curtis similarities for comparison of gut microbial community profiles between samples from different genotype salmon fed different diets: Wild genotype salmon (Wi), aquaculture genotype salmon (Aq), phospholipid diet (PL), fish oil diet (FO) and vegetable oil diet (VO). Wi-Aq represent Bray-Curtis similarity between the genetic strains. Error bars indicate standard deviation of the mean.

Average Bray-Curtis similarities were calculated for comparison of microbiota from gut samples between and within groups representing different genotypes and diets (Figure 3.19). Around 4-fold higher similarities among samples from wild strain (Br-C = 0.41) than aquaculture strain salmon (Br-C = 0.13), indicated larger interindividual variation for the aquaculture group. The trend was clear for comparisons within each diet as well (Figure 3.19) A t-test confirmed that the Bray-Curtis similarities were significantly higher among skin samples from wild group than aquaculture group ($p = 3.9 \times 10^{-76}$).

To identify the OTUs which contributed most to the main differences in gut microbiota between wild and aquaculture genotype group, SIMPER analysis was performed (Table 3.5).

Table 3.5: OTUs that contributed the most to the difference between microbiota from wild strain salmon gut and aquaculture salmon gut, identified by SIMPER analysis based on Bray-Curtis dissimilarity. Top five OTUs with taxonomy, contribution percentage and mean abundance in gut and skin samples are presented.

OTU ID	Taxonomy	Contribution to dissimilarity (%)	Mean abundance Wild strain (%)	Mean abundance Aquaculture strain (%)
OTU_1	*Propionibacterium*	15.24	33.9	9.85
OTU_17	Enterococcaceae	2.94	0.79	4.82
OTU_6	*Corynebacterium*	2.81	4.14	2.68
OTU_30	Clostridiales	2.18	1.54	2.26
OTU_8	Clostridiales	2.01	1.17	2.28

OTU 1 (the most abundant OTU in gut samples) contributed most to the difference in gut microbiota between wild and aquaculture group and was much more abundant in samples from wild strain than aquaculture strain (Table 3.5). The five OTUs which contributed most to the difference were also among the most abundant OTUs in gut samples (Table 3.3). This indicated that the largest contributions to dissimilarity between the two groups were based upon difference in OTU abundance and not presence/absence of OTUs (Table 3.5). OTU 17 was the only OTU more abundant in samples from aquaculture group (Table 3.5).

3.2.6 Effect of genotype and diet on skin microbiota

As for the gut microbiota, the skin microbiota needed further analysis to examine possible effects on skin microbiota caused by genotype or diet. PCoA ordination using Bray-Curtis similarity was performed to compare the skin microbiota from samples representing distinct salmon strains and diets (Figure 3.20).

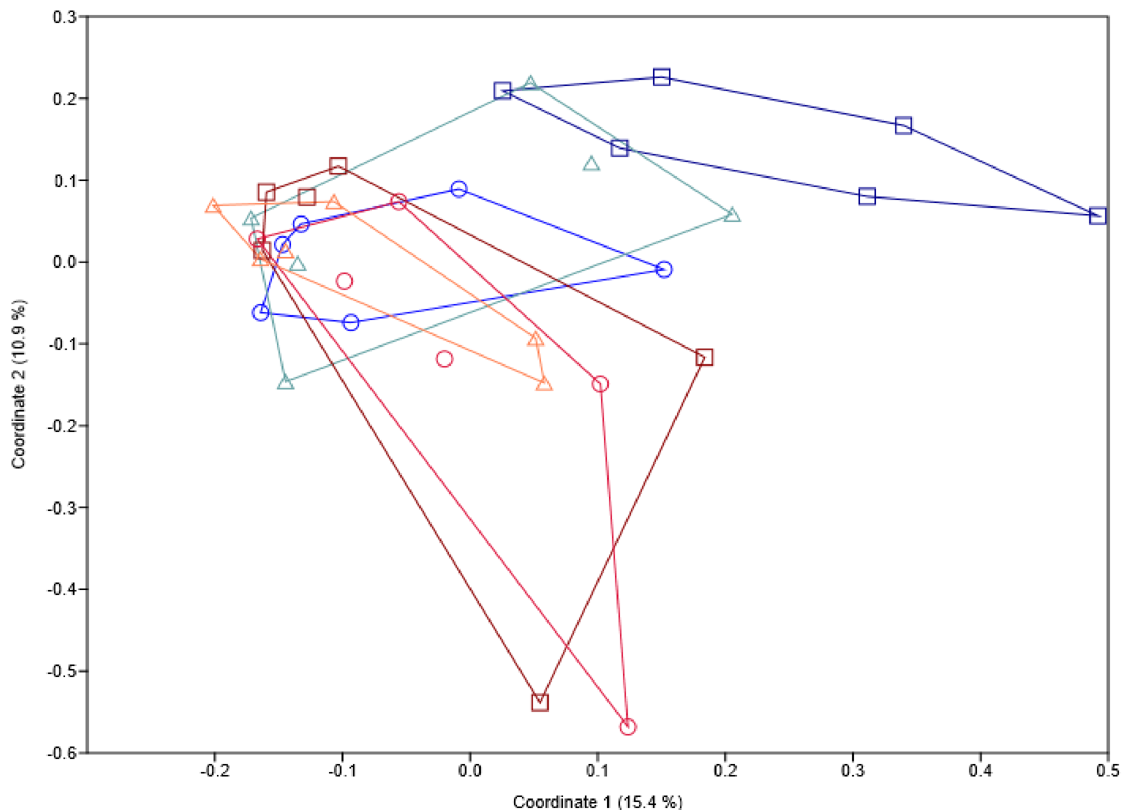

Figure 3.20: PCoA plot based on Bray-Curtis similarities for comparison of skin microbiota from different strains of salmon given different diets. Blue squares: WPL. Blue circles: WFO. Blue triangles: WVO diet. Red squares: APL. Red circles: AFO. Red triangles: AVO. Sample labels: A: aquaculture strain; W: wild strain; FO: fish oil; VO: vegetable oil; PL: phospholipid oil.

The first and second principal components together explained 26.3 % of the variation in this dataset, and it appeared to be some clustering according to genotype (Figure 3.20). Coordinates representing microbiota from wild strain salmon fed phospholipid diet (WPL, blue squares), did not cluster to the other samples. This indicated little similarity of microbiota with the other samples (Figure 3.20).

A two-way PERMANOVA was performed and the test confirmed a significant difference between the skin microbiota of different salmon strains ($p = 0.0001$). The test also showed a significant different between microbiota according to diets ($p = 0.0118$). The interaction effect between genotype and diet was also significant ($p = 0.0205$). Interestingly both salmon genotype and diet had significant influence on the skin microbiota, despite diet seeming to cluster relatively close in the PCoA ordination.

Average Bray-Curtis similarities were calculated for comparison of microbiota from skin samples between and within groups representing different genotypes and diets (Figure 3.21).

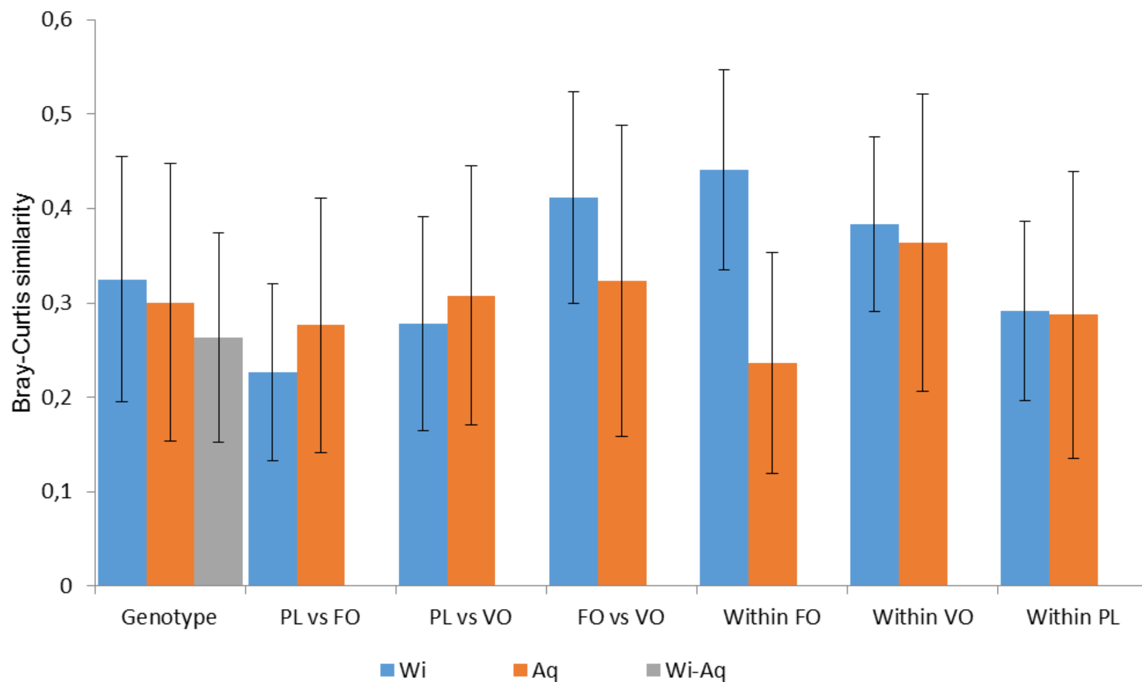

Figure 3.21: Bray-Curtis similarities for comparison of microbiota from salmon skin of different genotype salmon fed different diets. Wild genotype salmon (Wi), aquaculture genotype salmon (Aq), phospholipid diet (PL), fish oil diet (FO) and vegetable oil diet (VO). Wi-Aq represent Bray-Curtis similarity between the genetic strains. Error bars indicate standard deviation of the mean.

Bray-Curtis similarities for comparisons among samples from wild strain were relatively similar to samples from aquaculture strain (0.32 and 0.30 respectively) (Figure 3.21). There were notably higher Bray-Curtis similarities for comparison among samples within FO diet for wild group than aquaculture group. Despite this, a t-test confirmed that the similarities for comparison among samples from wild group were not significantly different from the Bray-Curtis similarities among samples from aquaculture group ($p = 0.12$). The similarities for comparison between wild and aquaculture genotype (Wi-Aq), was lower than within each of the genotypes (Figure 3.21). This indicated that skin microbiota was more similar within genotype than between.

SIMPER analysis was performed to identify the OTUs which contributed the most to the differences in skin microbiota between the wild and aquaculture genotype groups (Table 3.6).

Table 3.6: OTUs that contributed the most to the difference between microbiota from wild strain salmon skin and aquaculture salmon skin, identified by SIMPER analysis based on Bray-Curtis dissimilarity. Top five OTUs with contribution percentage and mean abundance in wild and aquaculture salmon skin microbiota are presented.

OTU ID	Taxonomy	Contribution to dissimilarity (%)	Mean abundance Wild (%)	Mean abundance Aquaculture (%)
OTU_1	*Propionibacterium*	9.42	22.4	27.7
OTU_4	*Streptococcus*	5.64	8.39	0.16
OTU_5	*Exiguobacterium*	4.98	1.64	7.56
OTU_7	*Pseudomonas*	4.84	6.88	4.54
OTU_3	Campylobacteraceae	4.75	6.05	2.74

The OTUs which contributed most to the differences in skin microbiota between wild and aquaculture group were OTU 1, 4, 5, 7 and 3 (Table 3.6). These OTUs were among the most abundant OTUs in skin samples (Table 3.3), and were also the same OTUs from SIMPER analysis of gut versus skin samples (Table 3.4). Difference in abundance of OTUs between samples from wild and aquaculture group seemed to be the main contributor to the dissimilarity in microbial communities in the two groups.

OTU 1 contributed the most to the difference, but in contrast to gut samples, OTU 1 had higher mean abundance in skin samples from aquaculture group than wild. OTU 1 and 5 were more abundant in samples from aquaculture group. OTU 4, 7 and 3 were more abundant in samples from wild group (Table 3.6).

4 Discussion

4.1 Evaluation of methods

Amplification of 16S rRNA regions by PCR was very challenging. The universal primers designed for the V3 region did not yield good results. There were problems with product in negative control, especially when using the standard Taq polymerase. Contamination of bacterial DNA probably originated from the polymerase, which has been has been shown to be a problem when using universal bacteria primers (Iulia et al., 2013). Changing the polymerase to Phusion Hot Start Polymerase improved the problems with product in the negative control.

The dominance of eukaryote DNA in the DNA preps was probably a contributor to making the amplification of bacterial DNA difficult. Furthermore it was early recognized that some kind of inhibitors were present in the DNA preps. More PCR product in negative control than for samples appeared when running many cycles. In addition the amplification gave better results when the DNA templates were diluted with MiliQ water, indicating the presence of inhibitors.

The 338F and 518R are primers which target regions conserved between eukaryotic and bacterial 18S small subunit rRNA gene, and co-amplification of eukaryote DNA is a common problem for these kinds of samples (Bakke et al., 2011). We found that a nested PCR protocol was necessary to avoid co-amplification of eukaryote 18S rRNA. When using only 338F-GC and 518R (without nested protocol) DGGE profiles showed only one band, indicating amplification of eukaryote 18S, and not a microbial community.

Changing the primers from amplifying V3 region to V4 region of 16S rRNA was the decisive factor for obtaining bacterial PCR products. This was unexpected, since the primers included long Illumina-adapter sequences and previous work done in the lab has shown that amplification with these adapter sequences often cause problems due to the formation of primer dimers. There was no need for a nested protocol using the V4 primers (515F and 803R) since they were more specific to bacteria.

V4 Illumina primers sometimes yielded products that formed double bands on the agarose gel electrophoresis. One of the bands yielded a PCR product somewhat shorter than expected. The phenomenon only occurred in amplification of fish samples, not water samples. The double bands were also present after the indexing step in the Illumina library making. This

shorter band was probably not due to primer dimers since the products were too long for this. It might have been caused by nonspecific amplification, for example from eukaryotic DNA since the bands only occurred in fish samples. Clustering of sequence reads into OTUs revealed two salmon OTUs which were removed. They were however not abundant in the community profiles for any of the samples, these were dominated by different bacterial OTUs.

The total number of reads for both gut and skin samples had high standard deviations indicating differences in the sequencing effort for different samples, which implies that the normalization had not been optimal.

16S rRNA is commonly used for targeted amplicon sequencing for bacterial diversity studies. The gene has both highly conserved regions and variable regions, making it very suitable for bacterial diversity studies. However the whole gene region is very long, making it very complex to study with DGGE and difficult to sequence with HTS techniques without extra steps. Thus one or several of the nine variable regions are usually targeted and amplified for subsequent analysis. The sequences of these variable regions vary between species. Using primers for only one region of the 16S rRNA gene may influence the richness and evenness of the communities (Di Bella et al., 2013). However, the V4 region is a good choice for regional sequencing. It is well represented in the RDP database and is capable of detecting most Bacteria and also Archea (Di Bella et al., 2013).

Another factor influencing the abundance studies using 16S rRNA as target sequence, is the variation in number of *rrn* operon in bacteria resulting in over-representation of some bacteria (Acinas et al., 2004). PCR bias in the amplification with broad range primers would also contribute to errors in abundance studies. This is due to unequal amplification efficiency for different template molecules caused by differences in primer-binding sequence (Polz and Cavanaugh, 1998). These investigative approaches are not quantitative in the meaning that abundance of one OTU or DGGE-band reflect how much there was of the corresponding bacterial strain in the samples. Even though the alpha diversity from each sample cannot be measured accurately, DGGE and amplicon sequencing are good at comparing community profiles between samples (beta diversity). The beta diversity can measure how microbial communities change over time or between different locations or habitats.

4.2 Water Microbiota

Water communities were generally found to be more diverse than gut and skin samples. Water microbiota is shown to be rich in diversity, and generally yield higher diversity indices than for fish samples (Bakke et al., 2015). Bray-Curtis similarities for comparisons among water samples were very high, implying that the microbial communities in the different tanks were very similar.

It is probable that the diet fed directly into the water in the tanks affect the microbial communities in the tanks. There might be both direct and indirect ways that the feed could affect the water microbiota. Direct; since the feed is given in the water and is food for the microbiota as well as the fish, it could alter microbiota according to the feed present. Indirect; if the gut microbiota of the fish was altered by diet, then the excretion from fish fed different diets could possibly contribute to differences in the water microbiota.

We hypothesized that the water microbiota would be influenced by the diet, but there was no significant difference in water communities between tanks that received distinct diets or housed different genotypes of salmon fry. The diets were relatively similar with the exception of lipid source (Appendix A). Thus, it may be that the differences in the diets were not big enough to select for distinct water microbiota.

4.3 Richness and Diversity of Microbial communities

The Shannon's diversity index of gut samples was significantly higher than for skin samples. There might be more niches in gut than skin because of more substrates and nutrients which may lead to more diverse communities. Although, this is not always the case, a study on Rainbow trout found that diversity was higher for skin than gut microbiota (Lowrey et al., 2015). This indicates that both skin and gut harbor diverse microbiota.

Individual differences of fish microbiota are known from other studies (Chiarello et al., 2015, Boutin et al., 2014). The relatively large standard deviations for the diversity indices, observed OTUs and Chao1, for both gut and skin samples indicated a great variance in the diversity between the salmon fry individuals. Although, there were great variations in number of reads between samples, and the higher diversities may reflect the samples with most number of reads. Nevertheless, inspection of the sequence data did not show a clear correlation between number of reads and diversity,

The Bray-Curtis similarities for comparison among skin samples were significantly higher than for comparison among gut samples. Thus, there were larger variations in microbiota between gut samples than skin samples. Gut samples harbored more diverse microbiota, which might contribute to the larger interindividual differences.

The species inventory (presence of distinct OTUs) was found to be somewhat similar for gut and skin samples, even though they were significantly different from each other, which was surprising. Gut and skin microbiota is believed to display different functions for the host and Lowrey et al investigated both skin and gut microbiota of rainbow trout, but did not find the species inventory to be similar across skin and gut samples (Lowrey et al., 2015). The salmon fry studied was sampled at day 48, which still is a very young age, and the skin and gut microbiota may be composed by similar microbiota since they are relatively newly hatched. It is reasonable to assume that the gut and skin microbiota become more distinctively different over time. There are not many studies which have examined both gut and skin microbiota of the same individuals of fish, and this needs to be done to collect more knowledge about how fish associated microbiota change in accordance to development.

4.4 Comparison between gut and skin microbiota

The water communities were highly distinct from the fish microbiota, indicating that microbiota was not shared between rearing water and fish. Most abundant phyla in water samples were Proteobacteria followed by Actinobacteria and Bacteroidetes, while Firmicutes was almost absent (Figure 3.12). The most abundant phyla found in skin and gut samples were Actinobacteria followed by Proteobacteria and Firmicutes. All these phyla have been found to be relatively abundant in fish microbiota (Nayak, 2010, Lowrey et al., 2015, Lokesh and Kiron, 2016), and the most abundant OTUs mainly represented these three phyla. Actinobacteria was represented by *Propionibacterium, Corynebacterium* and *Micrococus,* Proteobacteria by *Pelomonas*, *Pseudomonas,* and Campylobacteraceae, and Firmicutes by *Streptococcus, Exiguobacterium* and Clostridiales. OTUs representing *Propionibacterium, Corynebacterium, Streptococcus, Pelomonas* and Clostridiales were found to be relatively abundant in both skin and gut samples.

OTU 1 *Propionibacterium* (Actinobacteria) was the most abundant OTU in gut and skin samples, and was also the OTU contributing to the largest differences between gut and skin samples, and between aquaculture and wild group within gut and skin samples. It had a much

higher abundance in gut samples from wild group than aquaculture group. Interestingly it had higher abundance in skin samples from aquaculture group than from wild group. It is not easy to understand why this occurred, but it shows that the gut and skin microbiota are affected by genotype.

Propionibacterium is an anaerobic, gram positive, bacteria which is most known for their ability to synthesize propionic acid. *Propionibacterium* was found to be abundant in intraepithelial bacterial community of Rainbow trout (*Oncorhynchus mykiss*, Salmonidae) skin. It accounted for 22.5 % of the intraepithelial community, but only 3.0 % for the total skin microbiota (mucus and epidermis combined) (Lowrey et al., 2015). *Propionibacterium* was also found to be more abundant in skin mucus of un-stressed fish than stressed fish (Boutin et al., 2013). *Propionibacterium* has a low adhesion capability but have several beneficial effects on the host like stimulating immune response and increase growth of other probiotic bacteria (Boutin et al., 2013). *Propionibacterium* has also been found in Atlantic salmon fecal samples (Zarkasi et al., 2014) and gut samples (Gajardo et al., 2016). Furthermore an OTU representing *Propionibacterium* was 1 of 22 OTUs found to be part of a core microbiota, present in over 80 % of the samples, in all compartments of Atlantic salmon gut, even though it was not found to be very abundant (Gajardo et al., 2016). These results indicate that *Propionibacterium* is a part of the commensal microbiota in gut of salmon.

The high abundance of the OTU representing *Propionibacterium* in gut and skin samples from this study was not expected, as there are few studies which have identified *Propionibacterium* as a main contributor to microbial communities in fish. The high abundance may indicate that the fish had a healthy microbiota since *Propionibacterium* is known to exert beneficial effects on the host, and that the microbial communities reflect this.

It is known that microbiota between mucosal layer, epithelial layer and intestinal digesta contain different microbial communities (Gajardo et al., 2016). *Propionibacterium* was found in higher concentrations the intraepithelial layer of brook charr (*Salvelinus fontinalis*, Salmonidae) skin than in the total skin sample (Boutin et al., 2013). In this thesis DNA was isolated from the whole skin and whole intestine including the epithelial layers, and if *Propionibacterium* is more abundant in deep epithelial layers this might have contributed to the high abundance in gut and skin samples from this study. Previously done studies may not have included the epithelial layers when isolating DNA, thus not obtained all microbiota present in skin or gut.

Corynebacterium (Actinobacteria) is gram positive and aerob and it is known to be predominant in gut microbiota of fresh water fish (Nayak, 2010). An OTU representing *Corynebacterium* was found to be relatively abundant in both skin and gut samples with 2.2 % and 3.3 % average abundance, respectively. It also contributed to the difference in microbiota between gut samples from aquaculture and wild group, with higher abundance in wild group samples.

A strain of *Corynebacterium* has been detected on skin of brook charr (Boutin et al., 2014, Boutin et al., 2013). *Corynebacterium* on brook charr skin was found to be negatively influenced by stress (Boutin et al., 2013). Since the genus is known to be predominant in fish gut, this negative impact may indicate that it is a member of the commensal microbiota of healthy brook charr. *Corynebacterium* was the second most abundant OTU in gut samples, and eight most abundant in skin samples. The presence of *Corynebacterium* showed that salmon fry harbored some of the same microbiota found in other fish species, and it could be an indicator that the salmon fry were healthy and containing a so-called normal microbiota.

Two OTUs (OTU 4 and 10) representing *Streptococcus* (Firmicutes) was found to be relatively abundant in the skin samples. OTU 10 was also found to be relatively abundant in gut samples. *Streptococcus* is a gram-positive bacterium, and are mostly facultative anaerobe, but some strains are strict anaerobe (Patterson, 1996).

Streptococcus is a lactic acid bacteria, and lactic acid bacteria are found to be common in fish microbiota, especially in the digestive tract, and is seen as a part of the normal microflora (Austin, 2006, Stephens et al., 2016). Lactic acid bacteria are used as probiotics for aquaculture of fish, and different strains of *Streptococcus* are found to be probiotic (Balcázar et al., 2006, Austin, 2006, Nayak, 2010). This said, different strains of *Streptococcus* genus, such as *S. iniae*, are known to be pathogen in fish as well as humans (Yanong and Francis-Floyd, 2002, Austin, 2006). Since the salmon fry did not seem to be sick, it might suggest that the OTUs found were not pathogen. *Streptococcus* is also found in Atlantic salmon gut microbiota, so it was not surprising to find it represented in the gut samples (Zarkasi et al., 2016).

The family *Streptococcaceae* was found to be relatively abundant in skin of rainbow trout (Salmonidae) (Lowrey et al., 2015). It was interesting to see that in contrast to our findings, *Streptococcaceae* was not found to be abundant in the adult rainbow trout gut (Lowrey et al., 2015). Thus, *Streptococcus* might be a more abundant genus in young fry. The high

abundance in skin and gut samples might indicate that *Streptococcus* is a part of the commensal salmon fry microbiota. Here we only examined a "snapshot" in the development of the fry microbiota, and have no indications about the development over time.

Another OTU found to be relatively abundant in both skin and gut samples was OTU 13 representing *Pelomonas* (*Comamonadaceae*, Proteobacteria), which is gram negative and aerobic. *Pelomonas* has been identified on brook charr skin (Boutin et al., 2013), milkfish gut (Rasheeda et al., 2017) and rainbow trout gill (Lowrey et al., 2015). *Comamonadaceae* was found to be relatively abundant in skin associated microbiota from Atlantic salmon skin in freshwater (Lokesh and Kiron, 2016) and it was also found to be part of the core microbiota present in all sample sites of Atlantic salmon gut (Gajardo et al., 2016). *Pelomonas* is not well documented as a fish associated microbe, but phylogenetically similar bacteria (*Comamonadaceae*) are found to be a part of microbiota in both gut and skin in different fish species.

Two OTUs representing Clostridiales, OTU 30 and 8, was found to be relatively abundant in gut samples. OTU 8 was also found to be relatively abundant in skin samples. Clostridiales is an order of Firmicutes one of the predominant phyla of the gut microbiota in this study. Commensal Clostridia in human gut is involved in maintenance of the overall gut function (Lopetuso et al., 2013). Clostridiales is found to be a common colonizer in the GI tract of marine and freshwater fish (Nayak, 2010, Sullam et al., 2012). Thus, the finding of Clostridiales in gut microbiota was not surprising. Clostridiales has yet to be mentioned as an abundant order in fish skin so these findings are interesting. They indicate that Clostridiales is abundant in developing salmon fry gut and skin.

OTUs 7 (*Pseudomonas*), 3 (Campylobacteraceae), and 5 (*Exiguobacterium*) were among the OTUs which contributed most to the difference between gut and skin samples (Table 3.4). They were all more abundant in skin samples than gut samples, which was not surprising since gut samples had higher diversity, leading to a little less abundance per OTU. An OTU representing Planctomycetes was also relatively highly abundant in skin samples. There are no common traits among them insinuating why they could be more abundant in skin samples.

OTU 7 (*Pseudomonas,* Proteobacteria) was the second largest contributor to the dissimilarity between gut and skin samples. It was the second most abundant OTU in skin samples, but was not among the ten most abundant OTUs in gut samples (Table 3.3). *Pseudomonas* is commonly found in fish gut of both marine and freshwater fish (Nayak, 2010, Perez et al.,

2010). Despite this, OTU 7 was not very abundant in salmon fry gut samples. *Pseudomonas* was found to be a predominant bacterial genera in skin of several fish species (Striped mullet, Red snapper, Pinfish, Sand seatrout and spotted seatrout), indicating that it is a normal skin colonizer of fish (Larsen et al., 2013). Our results indicate that *Pseudomonas* is part of the commensal microbiota in salmon fry skin.

Campylobacteraceae (Proteobacteria), contain several genera. *Campylobacter* is one of them, and strains of the *Campylobacter* are known pathogens of humans even though they can be carried by healthy animals like cattle and chicken (Gerba, 2015). *Arcobacter* is another genus under Campylobacteraceae and has been found on the skin of brook charr (Boutin et al., 2013). Some genus's of *Arcobacter* are considered pathogens, but as with *Campylobacter* it is also found in healthy livestock (Forberg et al., 2016). *Arcobacter* was found in high abundance in healthy cod larvae, indicating that it might be a member of commensal microbiota of the cod larvae (Forberg et al., 2016, Bakke et al., 2015). Our results suggest that Campylobacteraceae contribute to the commensal microbiota on skin of salmon fry.

OTU 5 represented *Exiguobacterium* (Firmicutes), which is a gram-positive facultative anaerobe (Vishnivetskaya et al., 2009). OTU 5 was found to be third most abundant OTU in skin samples of the salmon fry. *Exiguobacterium* is detected in a variety of habitats, now including fish microbiota (López-Cortés et al., 2006, Boutin et al., 2013, Ringø et al., 2008, Vishnivetskaya et al., 2009). *Exiguobacterium* has been detected on skin from brook charr (Boutin et al., 2013), in the intestine of Atlantic salmon, and also on the brine shrimp *Artemia franciscana* (Ringø et al., 2008, López-Cortés et al., 2006). Our results might indicate that *Exiguobacterium* is present as a commensal bacteria on salmon fry skin.

Planctomycetes is a phylum previously found in fish gut bacterial communities (Sullam et al., 2012). It was not a very abundant phylum in salmon fry gut microbiota, but a little abundant in fish skin. Planctomycetes was present in all water samples, but the abundance varied vastly within gut and skin microbiota (Figure 3.12). There is not much literature supporting our findings, thus it is interesting that the abundance is relatively high in the skin microbiota.

An OTU representing *Micrococcus* was found to be relatively abundant in gut, but not skin samples. *Micrococcus* (Actinobacteria) is a commonly found genus among microbiota of fish gut (Nayak, 2010, Perez et al., 2010). It is gram positive and aerobe, and contains probiotic strains which is considered as control agents in aquaculture of fishes (Balcázar et al., 2006).

Thus, it was not surprising that *Micrococcus* was abundant in gut samples, and our results might suggest that *Micrococcus* colonize the gut early in the life cycle of salmon.

It was surprising to observe that the microbiota in gut and skin samples shared several abundant OTUs despite that the microbiota were significantly different. Many of the OTUs found in the skin and gut samples, represent species which are known to contribute to the normal microbiota of fish skin and gut. The most surprising result was the high abundancy of OTU 1 (*Propionibacterium*) in both skin and gut samples, which might indicate that skin and gut mucosa have similar properties at this age, and select for similar microbes. Skin and gut microbiota of same fish individuals have been little studied, and further studies are needed to confirm the similarity between the gut and skin microbiota of salmon fry.

4.5 Effects of genotype and diet on Gut Microbiota

The effect of host genetics on fish microbiota has been studied, and found to be a possible determinant for microbiota in fish gut. Bolnick et al. (2014) investigated how diet manipulations affected the microbiota in male versus female mice and stickleback. Diet was shown to influence microbiota differently in male and female. Thus, host genetics influenced the microbiota (Bolnick et al., 2014). Moreover, host species has been shown as a strong determinant of the intestinal microbiota between four cohabiting types of fish larvae (silver carp, grass carp, bighead carp and blunt snout bream) (Li et al., 2012).

In this study, the different "host genetics" was the two different genotypes of wild and aquaculture salmon. Some of the most abundant OTUs were found in both genotypes, so the microbiota appeared to be somewhat similar despite that they were significantly different between genotypes.

The Bray-Curtis similarities for comparison of gut microbiota showed very low similarities for comparisons of microbiota among aquaculture salmon, with a Bray-Curtis similarity of 0.13. The Bray-Curtis similarities for comparison of gut microbiota from wild strain salmon were much higher, with an average similarity of 0.41. This showed that there were larger interindividual variations in microbiota from the aquaculture group than for the wild group. A possible reason for this may be a stronger host selection in the wild genotype, thus stochastic processes have less influence on the microbiota.

Further, the microbiota from gut samples between the two genotypes were significantly different, indicating that host genotype has an effect on microbiota colonizing the gastro intestinal tract. The dissimilarity mainly consisted of difference in abundance of the most abundant OTUs. The OTUs contributing to the main differences was generally mora abundant in the wild strain than aquaculture strain, except for OTU 17 (Enterococcaceae) which was more abundant in the aquaculture strain (Table 3.5). This underlines that there were greater interindividual variations in the microbiota from the aquaculture group, which might lead to lower average abundance of specific OTUs.

OTU 1 (*Propionibacterium*) was more abundant in wild strain than in aquaculture strain (33.9 % versus 9.85 % respectively). Also OTU 6 (*Corynebacterium*) was more abundant in wild strain (4.14 %) than aquaculture strain (2.68 %). Both represent Actinobacteria, which was generally more abundant in wild strain gut than aquaculture gut (Figure 3.14). Actinobacteria has not been found to be among the most common phyla in gut of rainbow trout or salmon (Salmonidae) in previous studies (Desai et al., 2012, Zarkasi et al., 2016, Gajardo et al., 2016).

As discussed above, the high abundance of this phylum was related to the high abundance of *Propionibacterium* not previously found to be very abundant in fish gut. Although not very abundant, Actinobacteria was found to be more abundant in gut microbiota of parr and smolt than in adult Atlantic salmon (Llewellyn et al., 2016). Most studies which have examined gut microbiota of fish do so with aquaculture strain or laboratory fish, and this might be a reason why OTUs representing Actinobacteria is not found as one of the main contributors to gut microbiota of fish. The results from this study indicate that wild strain salmon fry contain higher abundance of the phylum Actinobacteria. In addition our results might indicate that Actinobacteria is more represented in young individuals, than adult.

OTU 17 (Enterococcaceae, Firmicutes) was notably more abundant in aquaculture genotype (4.82 %) than wild genotype (0.79 %). Also OTU 30 and 8 (Clostridiales, Firmicutes) were more abundant in aquaculture than wild strain. Firmicutes was overall more abundant in aquaculture gut microbiota than wild gut (Figure 3.14). It was interesting to observe that many of the OTUs more abundant in wild genotype represented Actinobacteria while some of the OTUs more abundant in aquaculture group represented Firmicutes, this might indicate that the different genotypes select for phylogenetically different bacteria.

One of the hypotheses of this thesis was that diet would influence the microbiota. The diet may influence the microbiota directly by altering the microbiota in the gut by selection. Different nutrients/substrates in the feed may select for different bacteria.

Feed and feeding conditions have been shown to influence the composition of GI microbiota in fish (Nayak, 2010, Bolnick et al., 2014, Desai et al., 2012, Ingerslev et al., 2014). Ringø et al showed dietary effects on gut microbiota in adult Atlantic salmon given plant based feed (Ringø et al., 2008). The study was limited to cultivable aerobic and facultative aerobic heterotrophic bacteria, and the total microbial communities were not examined, but the effect was observed nevertheless. Two different studies have shown changes in microbiota with increased abundance of Firmicutes for rainbow trout given plant based diets (Ingerslev et al., 2014, Desai et al., 2012).

Fish fed different diets do not always host significantly different microbiota. Microbiota associated with cod larvae was found to not be influenced greatly by different live feed diets and water microbiota seemed to influence the larvae microbiota more than the diet did (Bakke et al., 2015). Water microbiota is also known to influence the development of gut microbiota (Giatsis et al., 2015). There is an indirect way the diet could affect the gut microbiota, by altering the microbiota of the rearing water, which again could have had an impact on the gut microbiota. The rearing water in this experiment did not host significantly different microbiota, so this turned out to be irrelevant for this study.

The hypothesis that diet would influence the gut microbiota was not found to be valid for this experiment. One reason may be that the feed did not differ much in their contents, so it did not exert any different selection on the microbial communities in the gut.

It was a surprising finding that genotype had a greater effect on the microbiota than the diet, as described above. Previous studies on gut microbiota, done mainly on adult individuals, have shown that diet affects the microbiota. but recent studies suggest that microbiota associated with cod larvae are not significantly affected by diet (Bakke et al., 2013). The effect of genotype on fish microbiota has been relatively little studied, especially in early life stages, but this is starting to change (Li et al., 2012, Bolnick et al., 2014). Host species was shown to be a determinant of intestinal microbiota of fish larvae (Li et al., 2012) and Bakke et al suggested that strong selections in the host structured the microbiota associated with the cod larvae (Bakke et al., 2015). Our study implied that the gut microbiota in salmon is influenced by host genetics at an early developmental stage. And there seem to be some

indication that the wild genotype salmon had stronger host selection on the gut microbiota than the aquaculture genotype.

4.6 Effects of genotype and diet on skin microbiota

Previous studies has shown that fish have relatively low numbers of bacteria associated with the skin (Austin, 2006). Despite this, recent studies indicate that there is a diverse microbiota living in association with fish skin (Lowrey et al., 2015, Lokesh and Kiron, 2016, Chiarello et al., 2015). The observed number of OTUs in this study was not much lower than for gut microbiota, indicating that salmon fry harbor a relatively diverse microbial community.

The hypothesis was that the genotype would influence the microbiota. The PCoA ordination (Figure 3.20) indicated a tendency of the skin microbiota to cluster according to genotype, and the difference between skin microbiota from wild and aquaculture strain were significant.

Bray-Curtis similarities for comparison of skin microbiota within the two groups were not very different with similarities of 0.32 (± 0.13) for wild and 0.30 (± 0.15) for aquaculture strain. Thus, there were not as high interindividual variance in the aquaculture group skin as it was for the gut microbiota.

The species inventory appeared to be relatively similar, but there were big differences in the abundance of OTUs contributing to the greatest dissimilarities between the two genotypes. The five OTUs identified by the SIMPER analysis were the same five OTUs that were generally most abundant in the skin microbiota.

In contrast to gut microbiota, the OTU representing *Propionibacterium* (Actinobacteria) was more abundant in the skin of the aquaculture than the wild strain, generally Actinobacteria seemed a little more abundant in the skin from aquaculture group (Figure 3.12).

OTU 5, (*Exiguobacterium*, Firmicutes), was more abundant in aquaculture microbiota. OTU 4 (*Streptococcus,* Firmicutes) on the other hand was much more abundant in samples from wild skin. Firmicutes seemed to be a little more abundant in skin samples from wild skin than samples from aquaculture skin (Figure 3.12). Also Proteobacteria seemed a little more abundant in skin samples from wild genotype than aquaculture genotype (Figure 3.12), and OTU 7 (*Pseudomonas*, Proteobacteria) and OTU 3 (Campylobacteraceae, Proteobacteria), were more abundant in wild than aquaculture samples

Proteobacteria is known to be a very abundant phylum in skin of several fish species, including rainbow trout and Atlantic salmon (Larsen et al., 2013, Boutin et al., 2013, Lokesh and Kiron, 2016, Lowrey et al., 2015). OTUs representing Proteobacteria was shown to be present in higher abundances on brook charr skin of unstressed fish, while higher abundance of OTUs representing Actinobacteria where found on stressed fish (Boutin et al., 2013). It was interesting to observe that *Propionibacterium* (Actinobacteria) was present in higher abundance in aquaculture skin and that the two OTUs representing Proteobacteria were more abundant in wild genotype skin. Higher abundance of Proteobacteria and lower abundance of Actinobacteria may indicate that the wild genotype fish were less stressed.

In contrast to the gut microbiota, the skin microbiota was significantly affected by the diet. It is more obvious to assume that the diet would have a larger influence on the gut microbiota than the skin microbiota, both through selection and through addition of bacteria associated with the diet, but this was not found to be true in our study.

However the genotype and diet had a significant interaction effect, meaning that the effect of genotype and diet on the skin microbiota was influenced by each other. This was not found for the gut microbiota. This host genetics/diet interaction has been shown in another study, for gut microbiota, where it was shown that diet manipulations in stickleback and mice affected the gut microbiota differently in male and females (Bolnick et al., 2014).

A possible reason for this interaction may be that there were differences in the water microbiota, caused by different diets, although not significant, affecting the skin microbiota. There were very few water samples, and a lack of significance does not mean that there were no differences in the water microbiota. The fish skin is in direct contact with the rearing water were the feed is given and therefore the skin microbiota of developing salmon fry may be more easily influenced by the environmental microbes in the tank than gut microbiota. The skin microbiota may also have been directly influenced by the diet since the feed would also be present as nutrients for the skin microbiota.

Skin microbiota of salmon fry was significantly affected by both genotype of the host and by diet. There are not done many studies on skin microbiota of developing fish, and little is known about the effect of host selection on skin microbiota of fish, although there is evidence for host species specific skin microbiota (Larsen et al., 2013, Chiarello et al., 2015).

4.7 Conclusion

We succeeded in finding a PCR-protocol that worked for fish samples. Finding the "right" target sequences of 16S rRNA seemed like the decisive factor for amplifying 16S rRNA from fish samples.

The microbiota associated with the tank water samples were significantly different from the microbiota associated with the salmon fry, but was not affected significantly either by genotype of salmon fry or by diet. The water harbored a much more diverse microbiota than the fish.

Bacteria associated with the skin were significantly different from the bacteria associated with the gut, despite that they shared some of the most abundant OTUs. Further studies of samples from different points of time, representing different developmental stages, may show if the microbiota from skin and gut grow even more diverse over time.

Both skin and gut microbiota was significantly affected by the host genetics, indicating that there is selection inside the host that influence which bacteria that colonize the fish. There were greater interindividual differences within gut microbiota from aquaculture strain than wild strain, which might indicated a stronger host selection in the wild strain salmon.

The gut microbiota was surprisingly not significantly influenced by the diet, indicating that diet did not have a strong influence on the gut microbiota of developing salmon. Strong host selection might have overshadowed the effects of environmental factors such as diet.

Microbiota associated with the skin was on the other hand significantly influenced by diet. There was an interaction effect between genotype and diet, which might have indicated that the effect of diet on skin microbiota happened through the water microbiota. Thus, skin microbiota was more influenced by environmental factors than gut microbiota of developing salmon.

4.8 Further investigations

This thesis resulted in some interesting findings, but more research is needed to get a further understanding of which factors that affect the composition in fish fry.

In this project, samples from only one time point were analyzed. It could be interesting to investigate how skin and gut microbiota developed over time by analyzing samples from different time points in development. This would obtain better data to analyze the effect of genotype and diet, which might change over time. The dataset could be used to investigate if gut and skin microbiota change with developmental stage, and also to examine whether skin and gut microbiota become more divergent. There would also be more samples from water communities resulting in a better foundation to examine how diet and genetic background affects the water microbiota, and represent a better opportunity to investigate potential correlations between microbiota from water and fish.

Bacteria associated with the diet could also be characterized, in order to examine potential influence of bacteria associated with the feed. This project used samples representing all compartments of the gut, including the intestinal content, mucus, and epithelial layers. Previous studies have, however, demonstrated that the microbiota is different in these compartments, and it could therefore be interesting to investigate how the different compartments are affected by genetic background, diet and water associated microbiota.

Future investigations could additionally include a germ-free model of salmon fry which allows for investigation during controlled microbial conditions. Different host genotypes and diets could be introduced, to examine the influence on the fish health. These host-microbe effects could be examined through responses in the fish, analyzed by gene expression studies such as qPCR and transcriptomics studies.

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Appendix A Diet components

Table 5.1: Ingredients of the three different feeds, given in percentage. Differences marked with bold writing.

Ingredients	**FO**	**VO**	**PL**
	%	%	%
Fishmeal 70 LT FF Skagen	10.000	10.000	10.000
Fish protein concentrate (CPSP 90)	15.000	15.000	15.000
Squid meal	25.000	25.000	25.000
Shrimp hydrolisate	5.000	5.000	5.000
Fish gelatin	2.000	2.000	2.000
Pea protein concentrate	7.500	7.500	7.500
Wheat Gluten	12.500	12.500	12.500
Potato starch gelatinised	2.500	2.500	2.500
Fish oil	**7.200**	0.000	**3.000**
Tuna oil	**2.300**	0.000	0.000
Rapeseed oil	0.000	**2.900**	**2.500**
Linseed oil	0.000	**2.400**	0.000
Palm oil	0.000	**4.200**	0.000
Vit & Min Premix	1.500	1.500	1.500
Lutavit C35	0.030	0.030	0.030
Lutavit E50	0.120	0.120	0.120
Brewer's yeast	5.000	5.000	5.000
Betaine HCl	1.000	1.000	1.000
MAP (Monoammonium phosphate)	3.000	3.000	3.000
L-Taurine	0.350	0.350	0.350
NTNU - Phospholipids	0.000	0.000	**4.000**
Total	**100.000**	**100.000**	**100.000**

Table 5.2: Nutritional value of the three different feeds, given in percentage.

As fed basis	**FO**	**VO**	**PL**
Crude protein	65.00	65.00	65.00
Crude fat	14.10	14.10	14.10
Fiber	0.29	0.29	0.29
Starch	4.33	4.33	4.33
Ash	6.34	6.34	6.34
Gross Energy	20.43	20.43	20.43
C14	**0.83**	**0.18**	0.81
C16	2.01	2.64	1.85
C18:1n9	**1.66**	**4.42**	2.77
LNA	0.41	1.48	0.78
ALA	0.20	1.52	0.38
ARA	0.01	0.01	0.02
EPA	1.15	0.25	1.16
DHA	1.26	0.48	1.24
EPA+DHA	**2.40**	**0.73**	2.40
DHA/EPA	**1.10**	**1.90**	1.07
SFA	3.51	3.44	3.08
MUFA	5.10	5.03	4.91
PUFA	3.35	3.50	3.76
TPL	**1.31**	**1.28**	**3.23**

Appendix B DNA isolation protocols

Protocol for isolation of DNA from QIAamp DNA mini kit.

Tissues

Protocol: DNA Purification from Tissues (QIAamp DNA Mini Kit)

This protocol is for purification of total (genomic, mitochondrial, and viral) DNA from tissues using the QIAamp DNA Mini Kit.

Important points before starting

-

reduced DNA size.

-

enzymatic reactions, but will not inhibit PCR. If RNA-free genomic DNA is required, include the RNase A digest, as described in step 5a of the protocol.

Things to do before starting

-
-

instructions on page 16.

-

Do not use more than 25 mg (10 mg spleen).

Weighing tissue is the most accurate way to determine the amount.

If DNA is prepared from spleen tissue, no more than 10 mg should be used.

The yield of DNA will depend on both the amount and the type of tissue processed.

1 mg of tissue will yield approximately 0.2–1.2 µg of DNA.

2.

but lysis time will be reduced if the sample is ground in liquid nitrogen (step 2b) or mechanically homogenized (step 2c) in advance.

2a. Cut up to 25 mg of tissue (up to 10 mg spleen) into small pieces. Place in a 1.5 ml microcentrifuge tube, and add 180 µl of Buffer ATL. Proceed with step 3.

It is important to cut the tissue into small pieces to decrease lysis time.

2 ml microcentrifuge tubes may be better suited for lysis.

2b. Place up to 25 mg of tissue (10 mg spleen) in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into 1.5 ml microcentrifuge tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw, and add 180 µl of Buffer ATL. Proceed with step 3.

2c. Add up to 25 mg of tissue (10 mg spleen) to a 1.5 ml microcentrifuge tube containing no more than 80 µl PBS. Homogenize the sample using the TissueRuptor or equivalent rotor–stator homogenizer. Add 100 µl Buffer ATL, and proceed with step 3.

Some tissues require undiluted Buffer ATL for complete lysis. In this case, grinding in liquid nitrogen is recommended. Samples cannot be homogenized directly in Buffer ATL, which contains detergent.

3.

place in a shaking water bath or on a rocking platform.

Note: Proteinase K must be used. QIAGEN Protease has reduced activity in the presence of Buffer ATL.

Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h. Lysis overnight is possible and does not influence the preparation. To ensure efficient lysis, a shaking water bath or a rocking platform should be used. If not available, vortexing 2–3 times per hour during incubation is recommended.

4.

Transcriptionally active tissues, such as liver and kidney, contain high levels of RNA which will copurify with genomic DNA. RNA may inhibit some downstream enzymatic reactions, but will not inhibit PCR.

Tissues

Tissues

5a. First add 4 µl RNase A (100 mg/ml), mix by pulse-vortexing for 15 s, and incubate for 2 min at room temperature (15–25°C). Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid before adding 200 µl Buffer AL to the sample. Mix again by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of Buffer AL. In most cases it will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

5b. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

6.

15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the QIAamp Mini spin column. This precipitate does not interfere with the QIAamp procedure or with any subsequent application.

Do not use alcohols other than ethanol since this may result in reduced yields.

7.

and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.*

Close each spin column to avoid aerosol formation during centrifugation.

It is essential to apply all of the precipitate to the QIAamp Mini spin column.

Centrifugation is performed at 6000 x *g* (8000 rpm) to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through.

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 6 for safety information.

8.

and discard the collection tube containing the filtrate.*

9.

g;

14,000 rpm) for 3 min.

10.

speed for 1 min.

This step helps to eliminate the chance of possible Buffer AW2 carryover.

11.

the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.

12.

to 15%.

Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation.

Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield (see Table 5, page 25). Eluting with 4 x 100 µl instead of 2 x 200 µl does not increase elution efficiency.

For long-term storage of DNA, eluting in Buffer AE and placing at –30 to –15°C is recommended, since DNA stored in water is subject to acid hydrolysis.

Yields of DNA will depend both on the amount and the type of tissue processed. 25 mg of tissue will yield approximately 10–30 µg of DNA in 400 µl of water (25–75 ng/µl), with an A_{260}/A_{280} ratio of 1.7–1.9.

For more information about elution and how to determine DNA yield, length, and purity, refer to pages 24–25 and Appendix A, page 50.

*

Protocol for DNA isolation, MOBIO laboratories inc.

Experienced User Protocol

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1.** If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
   Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).
   Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix Solution C4 before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.
15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature.
   Note: A total of three loads for each sample processed are required.
16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x *g*.
17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.
19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.
20. Add 100 µl of **Solution C6** to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.
22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

    We recommend storing DNA frozen (-20° to -80°C). **Solution C6** contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

Thank you for choosing the PowerSoil® DNA Isolation Kit.

Appendix C GXL polymerase

Composition of mastermix using GXL polymerase	
Component	Volume (µL)
5 x primeSTAR GXL buffer	5
dNTP (2.5 mM each)	2
BSA	0.75
PrimF (10 µM)	0.75
PrimR (10 µM)	0.75
H2O	14.75
primeSTAR GXL DNA polymerase (1.25U/µL)	1*

*Wrong amount, it was supposed to be 0.5 uL per reaction

Cycling conditions for GXL polymerase		
ºC	Time	
98	2 min	
98	10 s	30 cycles
55	15 s	30 cycles
68	20 s	30 cycles
68	5 min	
4	1 min	
10	∞	

Appendix D Buffer and acrylamide solutions

50 x TAE-buffer:

Per liter:

Tris base	242g
Glacial acetic acid	57,1ml
0.5 M EDTA (pH 8,0)	100ml

Add distilled water to obtain the final volume.

Autoclave the buffer.

1 x TAE-buffer:

1960 mL Mili-Q water and 40 mL 50 X TAE buffer

Acrylamid solution (0% denaturating):

8% acrylamide in 0,5 x TAE (per 250 ml):

40% acrylamide solution (BioRadLab Inc., Ca., USA)	50 ml
50 x TAE	2.5 ml

Store the solution at 4 ^{0}C, protect from light.

Denaturating acrylamide solution (80% denaturating):

8% acrylamide, 5,6M urea, 32% formamide i 0,5 x TAE (per 250 ml):

40% acrylamide solution (BioRadLab Inc., Ca., USA)	50 ml
50 x TAE	2,5 ml
Urea	84 g
Deionized formamide	80 ml

Store the solution at 4 ^{0}C, protect from light. This solution **must** be sterile filtered before pouring the gel.

TE-buffer: 10 mM Tris-HCl, 1 mM EDTA

1 ml of 1 M Tris-HCl (pH 8.0)

0.2 ml EDTA (0.5 M)

Distilled water up to 100ml