Master's thesis

NTNU
Norwegian University of Science and Technology
Department of Biotechnology and Food Science

Sofie Kjerstad Bergh

# Effect of salmon lice treatment and lice infection on bacterial colonization on Atlantic salmon skin

Master's thesis in Biotechnology
Supervisor: Ingrid Bakke
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NTNU
Kunnskap for en bedre verden

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**Trondheim May 2019** **Sofie Kjerstad Bergh**

# Abstract

The biggest challenge in the salmon aquaculture industry in Norway is the salmon louse. Nowadays, there is more conscious considering fish health and fish welfare, and more preventative measures against salmon lice. One of the preventative measurements is to add a functional component in the fish feed to prevent salmon lice infection. Preventative measures contributes to less handling and stress for the salmon by the need of fewer delousing treatments.

There are few studies on how the bacterial community composition in salmon skin mucus is affected of salmon lice infestation and treatments. In this project a functional feed was examined if it affected the composition of microbiota and the amount of bacteria in the skin mucus. Samples were collected from skin mucus before and after salmon lice infection, and from the water from the representative fish tanks. The composition of the microbial community was investigated by using DGGE analysis and Illumina sequencing of amplicons representing the variable regions V3 and V4 of the bacterial 16S rRNA gene. Amount of bacteria was estimated by determined number of copies of the 16S rDNA by using qPCR.

The fish experiment was conducted at NTNU SeaLab as a part of the research program Taskforce Salmon Lice. There was no observations of any fewer salmon lice attached to the salmon after 4 weeks post lice infection. The amount of mucus was observed to be higher for the fish fed the functional feed, but the difference was not found to be significant. In the control group, Gammaproteobacteria was most abundant in the microbial community, and the genus *Oleispira* was unique for the fish fed the control feed and the water samples in the DGGE-analysis. The bacterial class Bacilli was almost exclusively found in the skin mucus from fish fed the functional feed. Genera belonging to the Bacilli, *Streptococcus* and *Staphylococcus*, contributed most to the dissimilarities in the skin microbiota between the two feed groups. Amount of bacteria per $cm^2$ was also estimated, and there was found a significant higher amount of copies of the bacterial 16S rDNA in the skin mucus from the salmon fed the functional feed. It is clear that the functional feed affected the composition of the skin mucus microbiota community and the amount of bacteria in the mucus.

# Sammendrag

Lakselusa er den største utfordringen lakseoppdrettsnæringen står ovenfor i Norge. I dag er det mer bevissthet og flere tiltak rundt fiskehelse og fiskevelferd, og det har blitt flere forbyggende behandling mot lakselus. Et av de forbyggende tiltakene er å tilsette komponenter i fôret som skal gjøre laksen bedre rustet til å motstå påslag av lakselus. Forebyggende tiltak er med på å heve fiskevelferden ved at det blir mindre håndtering og stressende situasjoner siden at laksen må avluses sjeldnere.

Det finnes veldig få, nærmest ingen studier på om hvordan bakteriesammensetningen i lakseskinn mucuset påvirkes av lusepåslag og behandling. I denne oppgaven ble et funksjonelt fôr testet for om det påvirker sammensetningen av skinnmikrobiotaen og mengden av bakterier i skinnmucuset, sammenlignet med et kontroll-fôr. Det ble tatt prøver av skinnslimet både før og etter påslag av lakselus, samt av vann fra hver fisketank. Sammensetningen av de mikrobielle samfunnene ble undersøkt med ved hjelp av DGGE-analyser og Illuminasekvensering av PCR-produkt som representerte de variable regionene V3 og V4 i det bakterielle 16S rRNA genet. Mengden bakterier ble estimert ved å bestemme antall kopier av 16S rDNA ved hjelp av qPCR.

Fiskeeksperimentet ble utført ved NTNU SeaLab i deres fiskelaboratorium i sammenheng med forskningsprogrammet «Taskforce Salmon Lice». Det ble ikke observert noe mindre påslag av lus med bruk av det funksjonelle fôret, 4 uker etter lusesmitte. Mengden slim ble observert å være høyere for fisk som hadde fått det funksjonelle fôret, men det var ikke en signifikant forskjell. Sammensetningen av de mikrobielle samfunnene i skinnslimet var forskjellige for fisk som hadde fått funksjonelt fôr og kontroll-fôr. I skinnslimet til fisken som hadde fått kontrollfôret var det bakterieklassen gamma-proteobakterie som hadde høyest forekomst, og slekten *Oleispira* skilte seg ut som typisk for kontrollgruppa og vannprøvene ved DGGE analysen. Bakterieklassen Bacilli ble funnet nesten utelukkende i skinnet til laks som hadde fått funksjonelt fôr. Slektene *Streptococcus* og *Staphylococcus,* som hører til Bacilli bidro mest til ulikhetene i skinnmikrobiotaen mellom de to fôrgruppene. Mengden av bakterier per $cm^2$ ble også estimert, og det var et signifikant høyere antall kopier av 16S rDNA i skinnslimet til laksen som hadde fått det funksjonelle fôret. Det er tydelig at det funksjonelle fôret påvirker både sammensetning av de mikrobielle samfunnene og mengden bakterier i skinnslimet.

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# 1 Introduction

## 1.1 The Growth of The Aquaculture industry

Aquaculture is a huge part of the increasing catching and production of fish globally. While the wild catch of fish is stagnating, the aquaculture industry is continuous increasing (Svåsand T., 2015). About 73% of produced salmon in the world is farmed. Salmon is considered a healthy product since it is a good source of proteins and essential fatty acids like Omega-3 and Omega-6 fatty acids. Global health authorities recommend a daily intake of these fatty acids. The Earth is covered by 70 % of water on its surface, but only 5 % of the protein production comes from the ocean. If the prediction of the increased population in the world is fulfilled, it would require a 35 % increase in protein demand. Therefore, farming salmon would be a solution to attain food supply in the years to come due to its resource efficiency (Mowi, 2018).

In 2017 the landed value of farmed Atlantic salmon (*Salmo salar*) in Norway was 61.6 billion NOK, and the production was on 1.2 million tons salmon (SSB, 2018). In comparison, the production in 1980 was only 7,800 tons (Roll et al., 2013). The supply of salmon has increased 417 % since 1995, which gives an annual growth of 8 %. This trend is stagnating, and during recent years, it has been on 5 %. The reason for this stagnating in growth is that the industry has reached a point where the biological boundaries are pushed to its limits (Mowi, 2018).

Future growth in production and in the industry is dependent on developing new technologies and innovation. In Norway, there are regulations on ownership, and limitations on the maximum allowable biomass (MAB) of fish in the net pens (Asche et al., 2013). The Ministry of Trade, Industry and Fisheries in Norway has also implemented the traffic light system (Oct 2017) which regulates the growth in the 13 production areas along the coast. Red areas will have reduced MAB, green areas can be offered growth, and in yellow areas, the production continues, but no more allowances will be given to further growth (Ernst&Young, 2017). Also, the Norwegian government have ambitions for the blue revolution and want to increase salmon production, but it must be sustainable for the environment (Svåsand T., 2015).

In Norway, 22 000 people are working directly or indirectly in the aquaculture business (Mowi, 2018). From a global perspective, the salmon industry is quite small compared to other industries. Norway contributes to 1.7 % of the global farming of seafood, but 50 % of the salmon marked, per 2016. Still, growth and development in the salmon farming industry are at

high importance. The main contributors towards this development is a continuous high focus on disruptive innovations, and the effects of global megatrends, such as the need to feed the ever-growing world population, more health-conscious consumers and a higher focus on sustainability in food production (Ernst&Young, 2017).

## 1.2 Atlantic Salmon

The Atlantic salmon is an anadromous salmonid that has its natural habitat in the North Atlantic Ocean. It is spawning during autumn and early winter. Atlantic salmon is also iteroparous, which means it can spawn several times. Unfortunately, few do survive the first spawning. Approximately 10 % bread more than one time. The numbers of egg depend on the size of the female. On average the female lays 1500 eggs/kg body weight. The eggs are laid in the gravel in the river and are about 5-6 mm in diameter. Salmon hatch at a relatively evolved stage and then the alvein lives on a yolk sac (Jobling, 2010). When the fish is swimming freely, it has become a parr (Fig. 1.1). The parr can live in the river for over a year before wandering out to the ocean, and when this occurs and becomes a smolt (Havforskningsinstituttet, 2016). Smoltification is the phase of life when the salmon goes through a physiological and morphological change to be able to migrate from freshwater to salty sea water (Mowi, 2019). Some male salmon can get sexually mature during the parr stage without migrating out to the sea (Jobling, 2010).

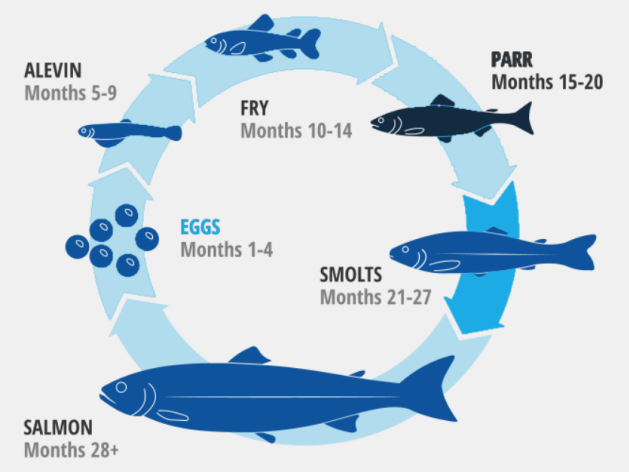


**Figure 1.1:** The lifecycle of Atlantic salmon, from egg to sexually mature. Figure from Scottich Sea Farms (2018).

The aquaculture production cycle in Norway is on 24-36 months, where 10-16 months takes place in freshwater on land and 14-22 months in seawater. Before the parr becomes a smolt, it gets vaccinated and graded. The smolts usually are around 100 g. In Norway, the smolt is released twice a year into the net pen in the seawater. While harvesting is an all year activity, most of the harvesting takes place during the last quarter of the year. When a farm is done with production, it has to wait between 2-6 months before new smolts could be transferred to the same farm. This is to regulate the salmon lice density in the production area (Mowi, 2018).

### 1.2.1 Fish welfare in aquaculture

The term welfare gives information in general about having the right conditions for growth and having a feeling of wellbeing in body and mind. Good animal welfare should protect the animals and make sure that they are adequately treated (Noble, 2018). Fish welfare is an essential subject in commercial aquaculture. Fish welfare has become so essential that it is affecting how the fish farmers make decisions and how the companies choose their strategies for future development. Many voluntary organisations, animal welfare organisations, and the government are acknowledging that fish welfare and health is as crucial in aquaculture than in other industries involving animals (Noble, 2018).

Some scientists suggest that fish can register sensory input and as a consequence, they can feel pain, fear and discomfort. Therefore in Norway, the Animal Welfare Act states that farmed fish should have a suitable living environment and should be handled in a way to secure good welfare throughout the whole life cycle (Hjeltnes B., 2018). The aquaculture industry in Norway uses welfare indicators to measure fish welfare. The indicators are based on the way the fish is experiencing its wellbeing and give an idea of how much of the fish welfare requirements are fulfilled. Environmental welfare indicators describe the environment around the fish, like the oxygen level and temperature. Animal-based welfare indicators are what the fish indicates through specific properties. For instance, the fish is emaciating, the growth rate is low and swimming abnormally, due to poor feeding conditions (Noble, 2018). Through the project “Fishwell”, a collaboration of the Institute of Marine Research, Norwegian Veterinary Institute, North University and Stirling University, a handbook was published based on knowledge of how indicators can be used to measure the level of welfare in aquaculture. This handbook is going to be a tool to work systematically to develop further welfare indicators and a protocol on how to handle different situations during farming.

Still, the losses of mortally of salmon are high. Preventative measures are put as one of the main objectives in the future for the industry. To prevent a high number of salmon lice, is an important environmental goal. Norway is divided into 13 productive areas from south to north along the coast. This division is to control the infection of salmon lice, and the lice would not leave and infest other areas (Hjeltnes B., 2018).

## 1.3 Salmon lice

The salmon lice are one of the major obstacles for growth in the Norwegian salmon aquaculture industry. The high level of salmon lice in the net pens is causing significant damage to the skin barrier and make the fish susceptible to secondary infectious diseases. Increasing levels of sea lice are caused by high host-density due to the rapid growth of the industry during the last decades. As a result, it is a higher disease transmission rate and an increased concern about the welfare for the wild salmon population nearby. The industry is experiencing challenges due to increased salmon lice control. The Norwegian Ministry of Trade, Industry and Fisheries have made a threshold on 0.5 of grown female salmon lice per fish in a farm. If a farm transcends this limit, they are forced to slaughter the fish early and lose huge valuables in weight (Brakstad et al., 2019).

The salmon louse, (*Lepeophterius salmonids* (Krøyer, 1838)), is an ectoparasite that occurs naturally in the marine environment. The parasite is host specific and in its life cycle includes 10 stages, while 8 of them is on the host (Atlantic salmon) (Kolstad et al., 2005). The life cycle of *L. salmonids* starts with two nauplii stages, a copepodite, four chalimus, two pre-adult and then a final adult stage (Fig. 1.2). Mature females hatch nauplii from egg strings that are attached. In the two nauplii and the copepod stage, the louse is free-living and survive on a yolk and other components provided maternally. The copepod is the infectious stage, and it is critical to have the ability to settle and recognise a relevant host. In the chalimus stages, it is attached to the host by a frontal filament (Llewellyn et al., 2017). Salmon lice damage the fish by feeding on their mucus, skin and blood, and the wounds they are causing increase the risk of secondary infections. Also, the salmon gets osmoregulatory dysfunction (Llewellyn et al., 2017).

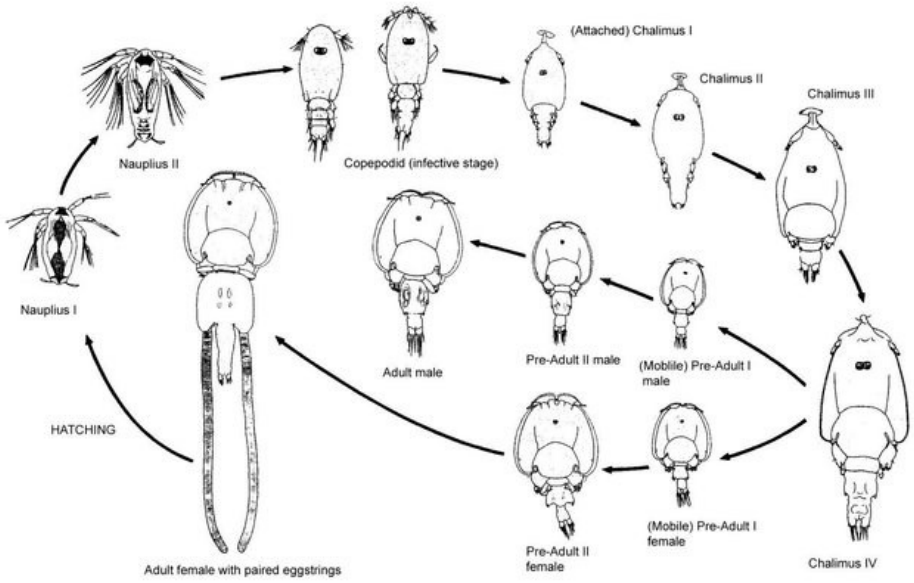


**Figure 1.2:** The life cycle of sea louse Lepeophtherius salmonids with all stages from hatching to sexually mature adult. The Nauplius I, Nauplius II and copepods are planktonic. (Ken Whelan, 2010).

The delousing treatments used today in Norway is usually a combination of several technologies (Brakstad et al., 2019). Mechanical methods are best described as non-medicinal methods, but mechanical treatments methods are the term that will be used further. The industry is in a shift from medicinal methods due to high resistance for the existing chemical to more mechanical methods. Many of the mechanical methods are only temporary solutions, and not optimised to conserve the fish welfare. At the Frisk Fisk 2019 conference in Tromsø, speakers generally agreed that mechanical methods gave lower sea lice number, but higher death rates due to secondary infections after delousing. Therefore, preventative methods are more focused on today (Brit Hjeltnes, FRISK FISK 2019).

## 1.4 Fish skin mucosa

The skin is the largest active organ in the fish immune system. It separates the individual from the environment and covers the outer surface including body and fins. The skin is one of the critical interfaces for the organism for contact and external communication with the environment. The epidermal layer protects the fish from a microorganism, physical damage and preservers hydrodynamics, and has functions to maintain physiological homeostasis like the osmotic balance. In the skin, there are multiple mucous glands (Esteban and Cerezuela, 2015).

Skin mucus forms an external layer of adherent mucus covering the living epithelial cells (Fig. 1.3). The mucus layer is produced by epidermal cells, generally by goblet cells which are unicellular glands. (Esteban and Cerezuela, 2015). In the epithelium, the mucus layer gives a chemical and a mechanical barrier against pathogenic organisms and the environment. The epidermal layer of mucus provides antimicrobial protection. This layer consists of immunoglobulins, defensins, antimicrobial peptides, lysozyme, and lectin-like agglutinins. Mucus is viscous, and with this property, it can trap and bind microbes. The rate of secretion of mucus is rapid, so the trapped microbes are "flushed" from the epidermal surface (Merrifield and Rodiles, 2015).

Still, this mucosal surface may be an adhesion site for some microbes. There are species of microbes that are resistant or are adapted to the immunological components in the fish skin mucus. Other bacterial species live in a mutualistic relationship in the mucus layer by metabolising epidermal mucus components and interfere with pathogenic colonisation by out-compete for adhesion sites and nutrients (Merrifield and Rodiles, 2015).

To determine the absolute abundance of microorganism in fish skin mucus is difficult. First, it is challenging to have a standardise sampling site of the skin. This is rarely described in research papers, and it is speculated that there are variations in the microbial communities on a different location on the body surface (Chiarello et al., 2015). Second, to avoid contamination during the sampling of epidermal tissues is demanding. The fish needs to be caught and handled to get the sampling material. Known methods to take a skin mucus sample could be by scraping the epidermal tissue, surface swabs wash or wash the whole surface of the fish with a known volume of diffluent. Which method that is the best is depending on the purpose of the investigation (Merrifield and Rodiles, 2015).

Reduced skin integrity is common for farmed Atlantic salmon. This happens especially during transfer to sea and at low water temperatures, but also the fish is exposed to a variety of stressors; transportation, vaccination, grading and infections that can harm the protective properties of the skin. The consequences are lower fish welfare, and substantial economic cost to the salmon farmers (Jensen et al., 2015).

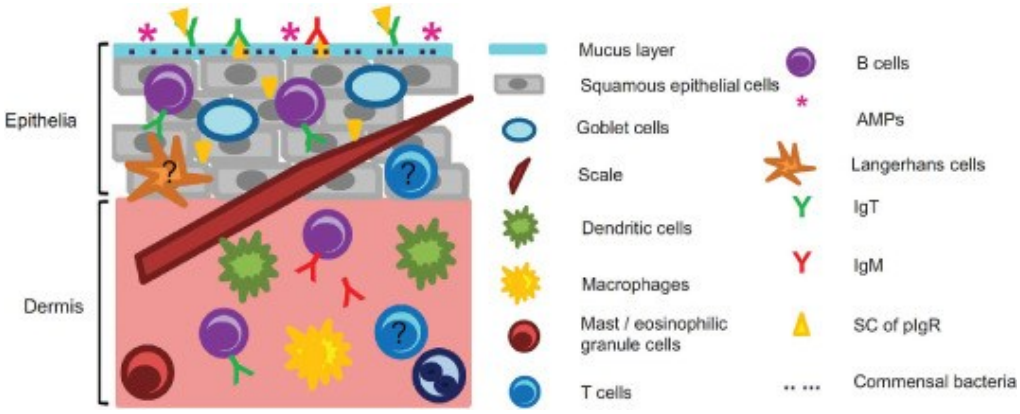


**Figure 1.3:** Teleost fish skin components with highlighting of structure and cell types (Gomez et al., 2013).

## 1.5 Sea lice treatments methods

Salmon lice are the biggest challenge in the aquaculture industry. The industry uses different methods; a medical/chemical used methods, non-medical methods, and preventative measures to cope with the major challenge. Which treatment approach that are suitable for the different fish farms depend on the size of the fish, season of the year the water exchange rate in the area, resistance documentation in the area and health situation of the fish.

### 1.5.1 Medicinal treatments

The traditional method to control the salmon lice density, and follow the restrictions on 0.5 grown female lice per fish, the aquaculture industry has used chemicals (Brakstad et al., 2019). Medicinal treatments are prescribed from a fish health biologist or a fish veterinarian, and the Norwegian Food Institute registers every treatment. Medicinal treatments are either used by adding a component to the feed or by a bath treatment. The bath treatment is conducted by taking a tarpaulin underneath the net pen and add the chemical treatment to the water, or by the use of wellboats.

Most medical treatments have been on the market for many years, and they belong to a few medicinal classes (Tab. 1.1). The reliance on a few chemicals has resulted in the frequency of resistance in salmon lice. Medical treatments are still used in all production areas and regions where it is necessary to control the population (Helgesen et al., 2019).

**Table 1.1:** Medical treatment chemicals used in delousing in Norwegian salmon aquaculture industry (Bredal, 2000). (The product name is an example for a product with that chemical, there might be more products on the market with the same chemical.)

| Classification | Chemical | Product name |
|---|---|---|
| **Pyrethroid** | Cypermethrin | Excis vet. |
| | Cis-cypermethrin | Betamax |
| | Deltamethrin | Alpha Max. |
| **Pyrethrum** | Pyrethrum extract | Py-sal vet |
| **Organic phosphorous components** | Azamethiphos | Salmosan |
| **Chitin inhibitor** | Diflubenzuron | Lepsidon vet. |
| | Teflubenzuron | Ektobann |
| **Avermectin** | Emamectin | Slice vet. |
| **Hydroperoxide** | $H_2O_2$ | PARAMOVE® |

## 1.5.2 Mechanical delousing methods

Since the first discoveries of salmon lice's resistance against the traditionally used chemicals, the salmon industry was forced to look for new solutions. This resulted in the use of mechanical treatments (Brakstad et al., 2019).

The principal to the mechanical treatments varies from applying heated seawater, flushing, flushing combined with brushes and freshwater. When using freshwater, the fish gets stressed due to interrupting the osmotic balance (Holan et al., 2017). A common feature for mechanical delousing is that the fish needs to be crowded before pumping it in the delousing system and the fish need to be handled. The crowding has a considerable impact on the welfare to the salmon. This type of treatment causes much handling of the fish, and as a consequence of this there is a risk for damage on the gills, eyes, fins and skin (Hjeltnes B., 2018). Crowding can create poor water quality with low oxygen levels, loss of scales, skin bleeding and damage due to the physical squeezing.

The documentation of the effects of the mechanical methods varies, but all methods have been suspected to impact the skin mucosal barrier of the salmon. Several of the methods have little to no documentation of the consequences. This shows the need for more objective documentation of how the methods work, the success rate and how it affects the welfare of the fish (Holan et al., 2017).

### 1.5.3 Preventative measures

The preventative measures are divided into biological and technological measures. Technological includes lice skirts, snorkel- submersible sea cage, and biological includes feed, cleaner fish, breeding and vaccines (Holan et al., 2017). Preventative measures have been in focus in the industry the past years and have led to more research and development in this field. New types of net pens e.g. OceanFarm1 from SalMar, which is a fish farm located out in the rough open sea. Other companies tries to sink the net pen, close the net pen or semi-close it to separate the lice and fish physically (Hjeltnes B., 2018).

Optimal nutrition has a significant role in maintaining a functional repairing mechanism in the fish skin and also the skin physiology (Jensen et al., 2015). A proper diet is essential to prevent diseases, maintain optimal performance and sustain proper health for the fish. In the fish farms, the fish are subject to a demanding environment. The fish is highly crowded and are subject to handling for counting of salmon lice, delousing and other reasons. These aspects have a negative impact on fish health and could have substantial economic consequences. Functional feed is the feed that has a constituent other than the essential nutrients like proteins, fatty acids, vitamins, and minerals, added. This could be probiotic, prebiotics and/or immunostimulants. The functional feed is considered to improve fish growth, health, stress tolerance and be able to be resistant to pathogens (Oliva-Teles, 2012). There are some functional feeds on the Norwegian market that are supposed to be preventative against salmon lice:

- SHIELD (20 % less attachment) – producer Skretting
- FOCUS LICE – producer Biomar
- ROBUST – producer EWOS
- PF Biofeed Aqua Forte – producer Polarfeed

SHIELD and ROBUST support the fish owns immune response while FOCUS LICE and PF Biofeed Aqua Forte should thicken the mucus layer and prevent the louse from attaching to the host. The additive Biofeed Aqua Forte has a documented effect on trout and Polarfeed has used this additive in their product PF Polar. It is this additive that is going to be further developed to

work better on Atlantic salmon and has the name Biofeed Salmo Forte. This new additive is investigated in this thesis.

### 1.5.4 Taskforce Salmon Lice

As a consequence of the demand for new methods to fight sea lice the R&D program Taskforce Salmon Lice was established and financed by Norwegian Seafood Research Fund (FHF) and lead by Yngvar Olsen from the Department of Biology at NTNU. Taskforce Salmon Lice is a project with the main goal to contribute with knowledge about how the sea louse is spreading in between farmed salmon, wild salmon and between both wild and farmed salmon. The focus is to improve the knowledge of the basic biology and ecology of the salmon louse.

One of the projects performed by Taskforce is "Improving salmon own health to fight of salmon lice". The industrial collaborators in this project were Biofeed AS in Trondheim and Pharmatech AS in Østfold. Together with research partners NTNU SeaLab and SINTEF, they aim at finding a way to improve the Atlantic salmon health to prevent attachment of salmon louse. The underlying aim in this project is that the sea lice problem in the Norwegian aquaculture needs to look at from a new angle. Instead of looking for a good method to remove the louse, it could be better to improve the fish own immune system and health through its functional feed. This will give the industry the ability to have better control and cut cost around salmon lice treatments and starvation. (FHF, 2018). This master thesis is a part of Taskforce Salmon Lice, and the fish used for sampling is from the project mentioned above.

## 1.6 Microbial community analysis with culture-independent methods

Traditional approaches for investigating the composition of microbial communities have been based on culture-dependent techniques and microscopy. Most bacteria cannot be cultivated on general media, and therefore would culture-dependent techniques not show the total diversity of the microbial community (Gilbride et al., 2006). Hence, developing culture-independent methods for characterisation of microbial communities have resulted in the application of isolating and amplifying bacterial ribosomal DNA and RNA by PCR (V. Wintzingerode et al., 1997). The small subunit ribosomal gene (16S rRNA) in bacteria is a frequently used biomarker for classifying microbes, microbial diversity analysis and phylogenetic analysis. This gene is

well suited as a biomarker due to its highly conserved and variable regions (Nikolaki and Tsiamis, 2013), along with a conserved secondary and tertiary structure (Gluick and Draper, 1992). PCR primers are designed to target these conserved regions, and enables to amplify all bacterial species present in the community in taxonomic groups, from kingdom to genus, due to the variability in the variable regions (V1-V9) (Malik et al., 2008). Databases like the Ribosomal Database Project (RDP) consist of millions of gene sequences which is mostly derived from sequencing PCR products, and is an important tool to allow researchers to analyse their rRNA sequences (Cole et al., 2013).

The sequences can be analysed with genetic fingerprinting methods, like denaturing gradient gel electrophoresis (DGGE), and to obtain community profiles and study community dynamics, or by high throughput sequencing like Illumina amplicon sequencing. In Illumina sequencing single template molecules are captured on a plate, and the templates are amplified by bridge PCR and create small clusters, and these clusters are sequenced based on fluorescence dye-labelled nucleotides. High throughput sequencing obtains higher resolution, and more taxonomic information is obtained compared to fingerprinting methods (Nikolaki and Tsiamis, 2013).

## 1.7 Hypothesis and aims

This master thesis is a part of Taskforce Salmon Lice R&D project "Improving Atlantic salmon's health to fight of salmon lice" where a functional feed was investigated. The project was performed at NTNU Sealab to examine if there was fewer louse attachment with the functional feed than with a control feed. The hypothesis in this thesis is that the two types of feed will affect the composition of skin microbiota, the quantity of bacteria in the skin mucus and skin mucus production.

In this master project the aims to see the effect of the functional feed on fish skin microbiota against a control feed on:

- Amount of mucus produced
- The composition of the skin microbiota community
- The density of bacteria in the fish skin mucus

The composition of the functional feed is confidential and will not be commented during this thesis. Methods used is DGGE fingerprint analysis, Illumina amplicon sequencing of bacterial 16S rDNA and qPCR.

# 2 Material and methods

## 2.1 Functional feed experiment design and setup

The salmon experiment for testing a functional feed towards salmon lice infection was performed in the facilities at NTNU Sealab at Brattørkaia in Trondheim. It was a part of the R&D program Taskforce Salmon Lice, and the project "Improving Atlantic salmon's health to fight of salmon lice". The projects sub-aims are divided into three work packages (WP). WP 1 investigated the functional feed effect on the development of the salmon and the quality of the salmon. Work package 2 investigated the functional feed effect on salmon louse attachment on Atlantic salmon. This thesis is a part of WP2 and was used to investigate the skin mucus microbiota. Work package three assess health parameters and will not be investigated before the aims in WP1 and WP2 is fulfilled.

In the laboriatorium at NTNU Sealab, 12 fish tanks were used in this experiment. These tanks were divided into two loops, with two separate inlet water. In each loop, there were three tanks where fish was fed the control feed, and three were fed with experimental functional feed (Fig. 2.1).

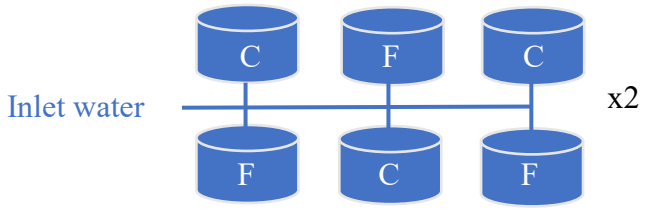


**Figure 2.1:** Setup of the experiment with functional feed. The illustration above represents one of two rings of tanks used in this experiment. The rings had separate inlet water. C : control; F : functional feed.

Each tank had ten Atlantic salmon individuals. The salmon got acclimatised for one week before starting the three weeks feed experiment. After three weeks the fish in all tanks got infested with 30-50 copepodites per fish.

During the experiment, Rolf Erik Olsen (Professor NTNU) was the principal investigator, Anna S. Båtnes (Researcher, NTNU) was primarily responsible, and Ane Nytrø (PhD candidate, NTNU) and Maria Guttu (PhD candidate NTNU) was responsible for the executing of the experiment. Nytrø, Guttu and I had the shared accountability to measure temperature and

oxygen levels in the tanks, secure enough feed in the feeding tanks and clean the tanks if necessary. During sampling Nytrø and Guttu counted and determined the gender of the salmon lice and I collected the water and skin mucus samples.

### 2.1.1 Sampling

The sampling of skin mucus was performed 26 days after starting the experimental feeding (t1: before the infestation of salmon lice) and at 29 days after infestation with salmon lice (sampling time t2). At t1, 7 individuals from two tanks with one of the representative feed treatments each, and at t2, a total of 9 individuals from each feed treatment were collected from a total of 6 tanks, three for each treatment. Water samples were taken at the same sampling times as the mucus samples. For the water samples at t1, two replicates were collected from the two tanks. At t2 only one water sample was collected from all the tanks.

Water samples were collected from the tanks by using a sterile syringe (Omnifix, 50 mL) and filtered through a 0.22 µm filter (Sterivex). These samples were stored at -20 °C. The Atlantic salmon were killed by an overdose of Benzocaine (200 mg/ml.) The length and weight were measured as shown in Appendix A. At sampling time t1, the Atlantic salmon smolt were around 212.1 gram. During the second sampling time t2, the Atlantic salmon smolt was around 348.0 gram.

The skin samples were collected by cutting a 3x6 $cm^2$ square in the skin, right under the dorsal fin with a sterile scalpel (Fig. 2.1). A sterile scalpel was used to scrape off and collect the mucus. The mucus samples were stored at – 80° C. Before extraction of the total DNA, the amount of mucus in the samples were estimated in an Eppendorf tube by comparing it against a series of similar Eppendorf tubes with a known volume of water.

**Figure 2.2:** Atlantic salmon. Illustration of where the 3x6 $cm^2$ skin was cut out beneath the dorsal fin.

### 2.1.2 DNA extraction

Total DNA was extracted from the water and mucus samples with the DNeasy Powersoil DNA Isolation Kit (Qiagen). For water samples, the Sterivex filters were taken out of the cartridge and transferred to the PowerBead Lysis tubes before following the protocol from the manufacturer (Appendix B). The water and mucus samples were extracted as described in the protocol.

## 2.2 PCR amplicons of 16S rRNA gene fragments

### 2.2.1 PCR to generate V3 amplicons for DGGE analysis

In DGGE analysis the length of DNA fragments to be analysed should not exceed around 250 base pairs. Therefore, the use of the V3 region of the 16S rRNA gene is often preferred (Personal communication, Ingrid Bakke). To study bacterial communities in or on eukaryotic organisms, we only want the amplification of the bacterial DNA. The extracted DNA is dominated by host DNA, and to avoid co-amplification of salmon 18S rDNA, a nested PCR protocol can be used. An external PCR with more bacteria specific primers and an internal PCR using the universal bacteria primers for amplifying the V3 region (Bakke et al., 2011).

The external PCR was run with 338F and 805R primers, and the internal PCR was run with the primer set 338F-GC/518R (Tab. 2.2). The external PCR run with 2 mM $MgCl_2$, 0.2 mM dNTP, 0.3 µM of both reverse and forward primer, 0.025 U $\mu L^{-1}$ Phusion Hot Start Polymerase and 1x Phusion HF Buffer™ (Thermo Scientific) with temperature cycles as shown in Table 2.1. The internal PCR run with the same condition as the external PCR, except for the primers and number of cycles. Number of cycles is presented in the results. In both external and internal PCR, the total volume was 25 µL with 1 µL DNA template added. The concentration of the template varies, and it is presented in the results. A positive and non-template control (NTC) was included in every PCR run. The positive control was used to see if the PCR reactions were successful, and the non-template control consisted of the PCR reagents, without DNA template, and it was used to examine if there was contaminating DNA in the PCR reagents. A DNA extraction control was used to control contamination associated with the DNA extraction kit.

Gel electrophoresis was used to examine the size and amount of amplicons. The PCR (4µL) was mixed with DNA loading dye (1 µL, Thermo Scientific). The loading dye contained GelRed (Biotium). This was applied on a 1 % agarose gel, and GeneRuler 1 kb Plus ladder

(Thermo Scientific) was used as the size marker on the gel. The gel was run for 1 to 1.15 hours at 100-120 V, and then the gel was photographed with GelDoc (Syngene).

**Table 2.1:** PCR cycling conditions. Steps 2-4 were repeated from 21-38 cycles. Time used for storing varies from minutes to several hours overnight.

| Step no. | PCR reaction | Time | Temperature (°C) |
|---|---|---|---|
| 1 | Denaturation | 1 min | 98 |
| 2 | Denaturation | 15 sec | 98 |
| 3 | Annealing | 20 sec | 55 |
| 4 | Elongation | 20 sec | 72 |
| 5 | Elongation | 5 min | 72 |
| 6 | Cooling | 1 min | 4 |
| 7 | Storing | | 10 |

### 2.2.2 PCR to generate V3 + V4 amplicons to Illumina sequencing

In previous work by the NTNU research group Analysis and Control of Microbial Systems (ACMS), it has been experienced difficulties with amplifying the 16S rDNA from Atlantic salmon skin mucus. To use the amplicons in diversity analysis, the bacterial DNA need to be specifically amplified. Here, this was performed by amplifying the V3 + V4 region of 16S rDNA. If amplifying only the V3 region, the PCR product would be dominated by DNA from Atlantic salmon 18S rRNA gene, due to homology between the PCR primers and the 18S rRNA gene (personal communication, Ingrid Bakke). Different PCR facilitators and cycling conditions were tested to optimise the PCR protocol for bacterial 16S rDNA from salmon skin microbiota, shown in the results.

The primers used to generate PCR amplicons for Illumina sequencing had Illumina adapter sequences in the 5' end. These adapters are necessary for the Illumina sequencing technology and indexing PCR (see Sec 2.5).

The PCR reactions were run for 38 cycles (Tab. 2.1) with the conditions described in 2.2.1, except for the primers. The primers set used were Ill-338F and Ill-805R (Tab. 2.2). These amplicons were examined with gel electrophoresis on a 1 % agarose gel.

**Table 2.2:** Primer sequences used during PCR protocols. Numbers in the primer names indicates the position of the 5' end of the primer in the *E. coli* 16S rRNA gene. The target sequences are shown in black.

| **Primer name** | **Sequence** | **Application and region of 16S rDNA gene** |
|---|---|---|
| 338F | 5' CC TAC GGG WGG CAG CAG-3' | PCR V3, V4 |
| 805R | 5'- G ACT CAN VGG GTA TCT AAK CC-3' | PCR V4 |
| Ill338F | 5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNN NCC TAC GGG WGG CAG CAG-3' | V3, V4 |
| Ill805R | 5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GNN NNG ACT CAN VGG GTA TCT AAK CC-3' | V4 |
| RT-966 | 5'-GCA ACG CGM RGA ACC TTA CCT A-3' | qPCR |
| RT-1089 | 5'-CSG GAT TAA CCS AAC ATYTCA-3' | qPCR |
| 338F-GC | 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3' | V3 region (DGGE) |
| 518R | 5'- ATT ACC GCG GCT GCT GG-3' | DGGE, V3 |
| 338F-GC-M13 | 5'-CA GGA AAC AGC TAT GAC CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG – 3' | DGGE, V3 |
| M13R | 5' - CA GGA AAC AGC TAT GAC C – 3' | Sanger sequencing |

## 2.3 Denaturing Gradient Gel-electrophoresis (DGGE)

DGGE is a genetic fingerprinting technique to analyse rDNA amplicons, which provide a profile or a band pattern representing the genetic diversity in a microbial community. The DNA fragments can have the same length but will be separated because they have different sequences. The separation is based on decreased mobility properties of partially degraded double-stranded DNA molecule in a polyacrylamide gel. The gel contains a linear concentration gradient of the DNA denaturants, urea and formamide. It is required to attach a GC-clamp of around 30 nucleotides to one of the PCR primers. This clamp consists of guanines (G) and cytosines (C) sequences, which is added to the 5'-end during the internal PCR. The GC-clamp works as a melting resistant domain that would prevent the DNA double-strand from dissociating into two single strands completely. (Muyzer and Smalla, 1998). DGGE can separate two DNA molecules with as little as single-base substitution (Sheffield et al., 1989).

DGGE was carried out for the V3 16S rDNA amplicons obtained from the nested PCR protocol. Two glass plates, with a spacer in-between and the comb on top, were assembled in the gel chamber. The gel was made of two acrylamide stock solutions, 0 % and 80% denaturing concentrations (where 100 % denaturing correspond to 7 M urea and 40 % formamide, Appendix E). The gel was casted with a gradient mixer, with the solution described in Tab. 2.3, to make a denaturing gradient. The highest gradient of 55 % on the bottom of the gel, and a medium denaturing gradient at 35 % on the top. A 0 % denaturing acrylamide gel was casted in the top cm of the gel. Ammonium persulphate (APS) and Tetramethylenediamine (TEMED) were applied to start the polymerisation of the gel. The gel was left for polymerisation for 2 hours.

**Table 2.3:** Contents and components used for casting an 8 % polyacrylamide gel for DGGE with 35 – 55 % denaturing gradient.

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

The buffer tank with 17 L buffer (0.5 x TAE) was preheated to 60 °C, while the buffer was circulating in the tank. Upon loading the samples, the buffer circulation was turned off. Loading dye (6 µL) was mixed with the PCR product (5-15 µL) before applied into the wells, and the circulation and high voltage were turned on. The acrylamide gel was run for around 22 hours at 100 V and 23-27 mA.

After electrophoresis, the gel was transferred to a plastic sheet and stained with a solution consisting of SYBR® Gold (3 µL, Invitrogen), 50 x TAE (600 µL) and 30 mL PCR-grade water. This was put in the dark and left for one hour. Every 15 min the gel was carefully tilted back and forth to even out the staining dye on top of the gel. The gel was photographed in a UV-cabinet (Syngene) with the software GelDoc.

### 2.3.1 Reamplifying of DGGE bands for Sanger sequencing

The bands of interest from the DGGE gel was excited and transferred to Eppendorf tubes with 30 µL PCR-water and vortexed. Then, the bands were reamplified with PCR using the same conditions described in Section 2.2.1, and the temperature conditions (Tab. 2.1) for 38 cycles with the primer set 338F-GC-M13 and 518R (Tab. 2.2). The PCR products were examined with gel electrophoresis on a 1% agarose gel as described earlier in Section 2.2. To purify the PCR products the QIAquick purification kit (Qiagen) was used accordingly to the manufacturers' protocol (Appendix C). The sequencing primer M13 (5 µL, 5mM) (Tab. 2.2) was mixed with 5 µL of purified PCR product and sent to The Genome and Diagnostic Centre (GATC) for Sanger sequencing.

### 2.3.2 Statistical analysis of DGGE bands

Gel2K (Norland, Department of Biology, University of Bergen, 2004) software was used to convert the DGGE band profiles into histograms (Appendix D). Peaks in the histogram represent the intensity of the band in the DGGE gel. This intensity was converted to peak area values with Gel2K, and then these values were exported to Microsoft Excel. The data set was normalised by dividing the intensity value from each band by the total intensity of all band belonging to the same lane.

## 2.4 Quantitative Polymerase Chain Reaction

Quantitative PCR is based on the principle of continuous observations of changes in fluorescence during a PCR protocol (Malinen et al., 2003). This analysis method is enabling the quantification of DNA, and it is therefore also called quantitative PCR (qPCR) (Lubbs et al., 2009). SYBR Green 1 is a dye that gives a fluorescence signal when it is bound to double-stranded DNA and is applied to fluorescent monitoring of an amplification reaction (Malinen et al., 2003). The fluorescent signal is increased proportionally during each PCR cycle and as the DNA products increase exponentially (Dorak, 2007). The output from qPCR is the conversion of fluorescent signals from the reactions to a numerical value (Ct) in the form of an amplification curve (Dorak, 2007).

In this experiment, DNA extracted from a *Vibrio* strain was used to make the standard curve. To produce DNA template from this bacterial strain to qPCR standard curve, a known sequence of 123 bp of the 16S rRNA gene amplified with the primers RT966F and RT-1082R (Tab. 2.2). The PCR reaction was performed with Phusion Hot Start Polymerase II and with the same conditions as described in Section 2.2.1. The amount of DNA in the *Vibrio* amplicon product was determined using Qbit (Invitrogen), cleansed with the QIAquick PCR purification kit (Qiagen) following the manufacturers' protocol (Appendix C), and diluted to a final concentration of 1 ng/µL. The standard curve was made with a 1:5 dilution series with 5 dilutions.

DNA extracts from fish skin mucus samples were diluted 1:100. The qPCR reagents mix consisting of the broad range primers RT966F and RT1089R (2.5 µL, 5 mM), 2x SYBR® Green mix (12.5 µL) and DNA-free water (2.5 µL) per reaction, was made and distributed (20 µL) in each well in a 96 well plate. An NTC and a germfree salmon fry sample were used as a control for contaminating DNA and amplification of salmon 18S rDNA. All samples were run in three

replicate qPCR reactions. The qPCR was performed in a QuantStudio instrument (AppliedBiosystems) with temperature steps described in Tab. 2.4.

**Table 2.4:** Temperature cycling in qPCR. Steps 2-4 were repeated for 40 cycles.

| Step no. | PCR-reaction | Temperature (°C) | Time (sec) |
|---|---|---|---|
| **1** | **Pre-incubated** | 95 | 10 |
| **2** | **Denaturing** | 95 | 15 |
| **3** | **Annealing** | 60 | 20 |
| **4** | **Elongation** | 72 | 20 |

Melting curve analysis was performed after the amplification at 72 °C (20 sec), 95 °C (15 sec), 60 °C (60 sec) and 95 °C (1 sec).

### 2.4.1 Processing of data from qPCR

Data collected with QuantStudio was processed in QuantStudio™ Design and Analysis Software v1.5.0 (AppliedBiosystems). Ct-values were calculated in QuantStuido™ and exported to Microsoft Excel. Amplicon products with significant deviation in Ct-value from others in the triplicate were excluded. By using equation 2.1, the copy number (CN) of the 16S rDNA, corresponding to the DNA concentration of the *Vibrio* DNA used in the standard curve, was calculated. The DNA length was 123 bp, and the DNA concentration of the diluted sample (0.008 ng $\mu L^{-1}$).

$$CN_{stock}\,(molecules/\,\mu L) = \frac{DNA_{cons}(g/\,\mu L) x 6.022x1023(molecules/mol)}{DNA_{length} x\, 660\,(g/mol)} \qquad (2.1)$$

A standard curve was made, to visualise the coherence between the Ct-value and copy number of 16S rDNA. The samples with the highest DNA concentration (0.04 ng $\mu L^{-1}$) was excluded from the standard curve. CN per $cm^2$ skin mucus for each mucus sample was calculated based on the standard curve. Finally, a Welch t-test for unequal mean was conducted to examine if there was a significant difference in the estimated microbial density between mucus samples from the two feed groups.

## 2.5 Amplicon library for Illumina sequencing

The preparing of the amplicon library was performed by Mia Tiller Mjøs. First, the amplicons were normalised with a SequalPrep Normalization Plate (96) kit (Invitrogen). Each amplicon was added a unique index sequence, with the Nextera Index kit (Illumina), during a PCR. For the forward primer, there were 8 unique sequence indexes and for the reverse primer it was 12 unique sequences. This makes a total of 96 different index combination, which is one for each sample in the 96 well plate. After indexing the 96-well plate was normalised again with a SequalPrep as mentioned. The indexed PCR products were pooled together with 90 other indexed PCR products and up-concentrated by an AmiconUltra 0.5 Centrifugal Filter (Mereck Millipore, Ireland) as described by the manufacturer. The PCR products were sequenced on one MiSeq lane with V3 reagents for paired-end sequencing at the Norwegian Sequencing Centre (NSC).

### 2.5.1 Processing of Illumina sequencing data

The Illumina sequencing data were processed using the USEARCH pipeline (version 10; https://www.drive5.com/usearch/) by Ingrid Bakke. The command Fastq_mergepairs was used for trimming off primer sequences, merging of paired reads, and filtering out reads shorter than 400 base pairs. Further, the process included demultiplexing and quality trimming (the Fastq_filter command with an expected error threshold of 1). Chimera removal and clustering at the 97% similarity level was performed using the UPARSE-OTU algorithm (Edgar, 2013). Taxonomy assignment was performed applying the Sintax script (Edgar, 2016) with a confidence threshold of 0.8 and the RDP reference data set (version 16). Then, the OTU table was manually inspected, and OTUs that represented Atlantic salmon and other eukaryote genes were excluded from the table. Further, dominating OTUs in non-template controls were excluded. The resulting OTU table was normalised to 17 500 number of reads per sample by first determining the fraction of the OTUs for each sample, and then multiply with the relevant number of reads. Finally, rounding off the read numbers to integers. The Usearch commands Alpha_div and Sintax_summary were used to calculate α-diversity indices and generate taxa summary tables (at various taxonomic levels as specified with the results), respectively.

The software PAST v3.20 (Hammer, 2001) was used to analyse the OTU-table, and to investigate the microbial composition, with statistical analysis, of the control feed and experimental functional feed. The α-diversity for individual skin mucus was described with

Shannon's diversity index (H') and observed operational taxonomic units (OTUs), and they had to be calculated. Shannon's diversity index is expressed by (Beisel and Moreteau, 1997);

$$H' = -\sum_{i=1}^{S}\left(\frac{n_i}{n}\right)\left(ln\frac{n_i}{n}\right) \quad (2.2)$$

where S represents the OTU richness, n is the total number of reads and $n_i$ the number of reads belonging to the relevant OTUi. Both the species richness and evenness are included in Shannon's diversity index. The observed number of OTUs in a microbial community represents the number of observed OTUs. There will be uncertainties with the detected species in this taxonomic survey because observed OTU richness will rely on the sequencing depth and does not show the true OTU number in a microbial community.

The β-diversity compares microbial community profiles between different samples. A principal coordination analysis (PCoA) plot was used to visualise the β-diversity based on the Bray-Curtis similarities. In the PCoA a Bray-Curtis matrix for all pairwise comparison of the community profiles between samples is used to plot all the samples and present it in a multidimensional coordinate system (Davis, 1986). Samples are considered similar or dissimilar depending on their distances to each other; the closer the samples were positioned in the PCoA plot the similar the microbial community in the samples.

A Similarity Percentage (SIMPER) analysis and a one-way permutational multivariate analysis of variance (PERMANOVA) test were both based on Bray-Curtis similarities. SIMPER was used to identify the OTUs that contributed to the differences in the microbial community. A PERMANOVA was performed to investigate if the differences in the microbial community between the two feed groups were significantly different (Anderson, 2001).

# 3 Results

## 3.1 Lice infection and mucus measurements

The experiment with the Atlantic salmon was carried out at NTNU SeaLab with the Taskforce Salmon Lice research group as a part of the Taskforce Salmon Lice project. They aimed to count the number of salmon louse infected the salmon, and to see if the functional feed had the preferred property in terms of preventing the salmon lice from attaching to the salmon. Skin mucus samples from three replicates of fish, from three replicated tanks for each treatment (C and F), were sampled at t2. At sampling time t1, 7 individual fish from one tank of each treatment had been sampled for skin mucus. Water from each tank was sampled at the same sampling time. There were no losses, and all fish survived the experiment. No significant difference in salmon louse numbers was found between the two groups. Thus, no indications that the functional feed affected the salmon lice infection.

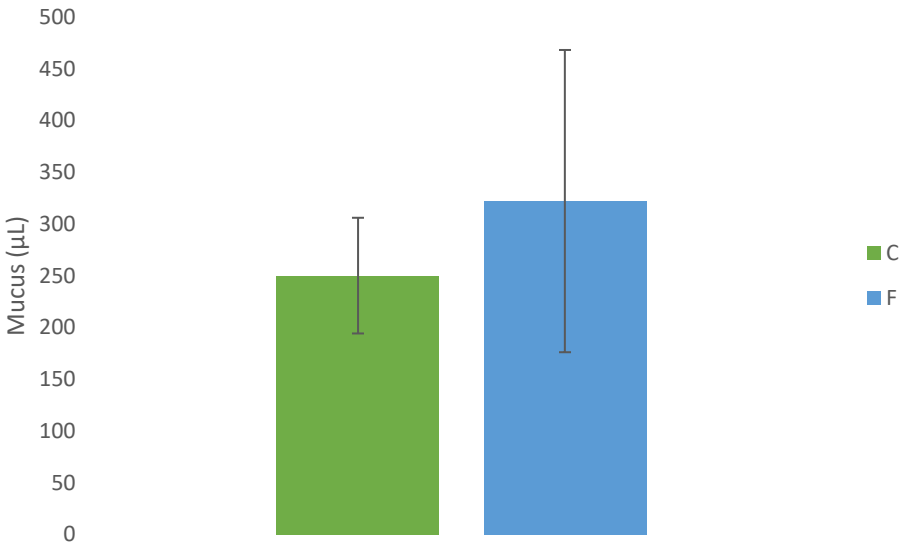


**Figure 3.1:** Amount of mucus (µL) collected from 3x6 $cm^2$ of salmon skin from 9 individuals from the control group (C) and functional feed (F) from after the lice infection. Error bars represent standard deviation .

To examine if the functional feed affected the amount of mucus, skin mucus samples were analysed by measuring the volume of mucus scraped off from 3x6 $cm^2$ fish skin. There seems to be more mucus in the group with functional feed (Fig 3.1), but the standard deviation was

large. A t-test showed that it was no significant difference in the amount of mucus on the 3x6 $cm^2$ skin sample between the two feed groups.

## 3.2 Optimisation of PCR amplification of 16S rDNA in Atlantic salmon skin mucus

The NTNU ACMS research group have experienced problems when amplifying the 16S rDNA from the Atlantic salmon skin mucus earlier. In this project, the same was experienced when amplifying the V3 + V4 region of the 16S rDNA from the skin mucus samples. Dilution of the DNA extract was performed to see if it had any effect on the inhibitions of the PCR reaction. A non-template control indicated that there was contaminating DNA in the PCR reagents, but PCR reactions with DNA extracted from skin mucus was still not amplified (Fig. 3.2). The samples M13-M24 (Fig 3.2) did not show in any PCR product, which indicates that something was inhibiting the PCR reaction.

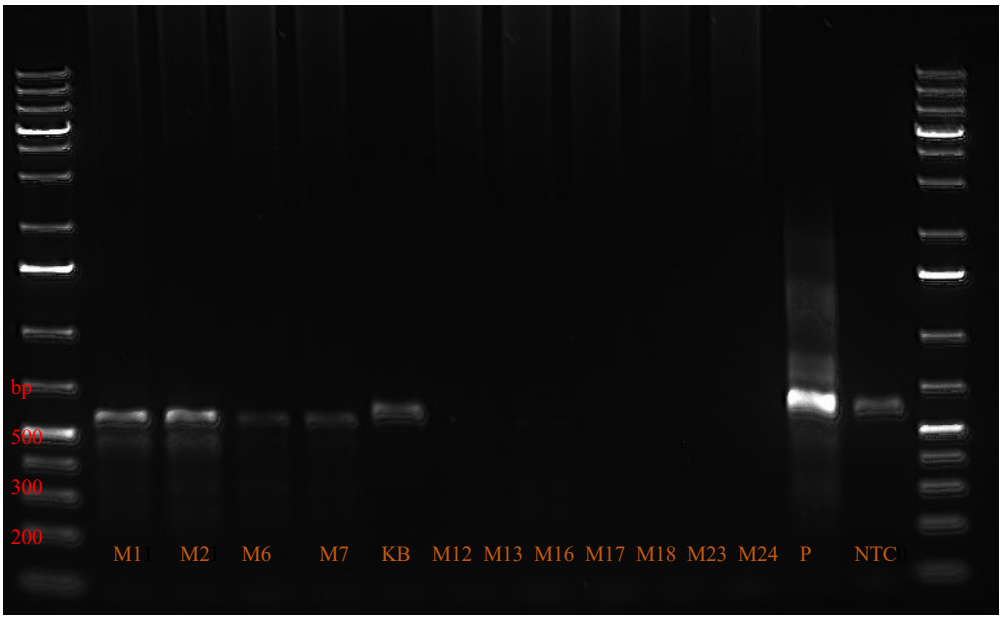


**Figure 3.2:** Agarose gel of V3+V4 region of 16S rDNA amplicons for skin mucus samples obtained with primers 338F and 805R for 38 cycles, for the rest conditions as described in Section 2.2, except added spermidine (0.1 mM). All skin mucus samples were diluted (1:10). Numbers indicate sample number (Appendix A). NTC : non-template control; P : positive control (produced from a DNA sample from a Vibrio strain) ; KB : negative control from DNA extraction kit; M1-M18 represents PCR products generated for skin mucus samples taken at sampling time t2; M23-M24: represents PCR products generated for skin mucus samples taken at sample time t1.

BSA, a PCR facilitator, was tested to examine if it had any effect on the inhibition in the PCR reaction with two template dilutions (1:100 and 1:1000) of the mucus DNA extracts. No effect of the BSA was observed, but the dilution of the mucus sample 1:100 affected the inhibition. The 1:100 dilution of the DNA extract produced the expected PCR product for all mucus samples. This implies that it was inhibitors presents in the DNA extract and not too little target DNA that is the problem.

Next, the effect of the PCR facilitator spermidine was investigated. An agarose gel was prepared with PCR products from skin mucus, as described in Section 2.2, with and without spermidine (0.1 mM) (Fig. 3.3). The gel indicated that PCR products with spermidine had a higher yield, but the non-template control with spermidine got as high yield as the mucus samples. Therefore, the PCR product in the skin mucus samples might have been dominated by contaminated DNA. The non-template control without spermidine did not have any PCR product, which implies that the spermidine was contaminated with DNA. Hence, spermidine was eliminated from the protocol.

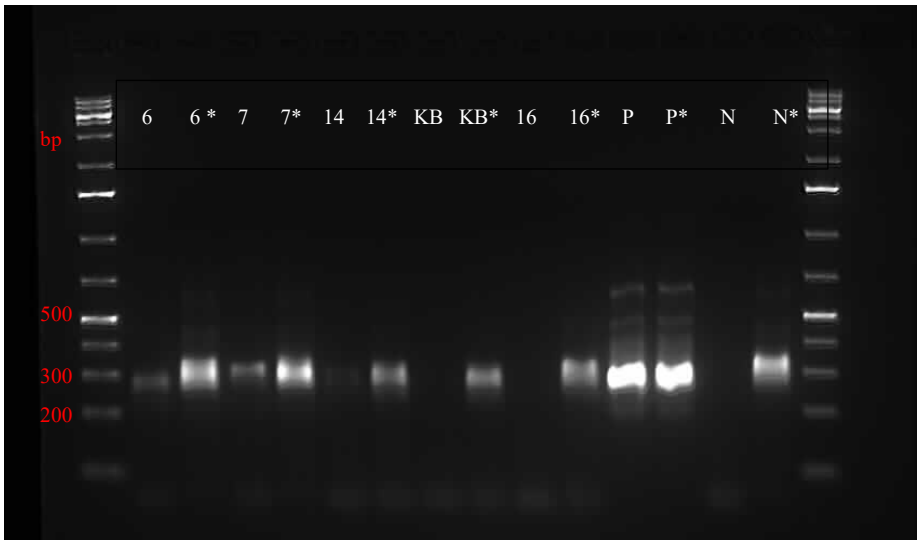


**Figure 3.3:** Agarose gel with PCR product of V3+V4 16S rDNA for skin mucus samples. PCR protocol used as described in Section 2.2. Mucus samples were diluted 1:100. Samples with * were added spermidine (0.1 mM). Numbers indicate sample number (Appendix A). KB: DNA extraction kit control; P : positive control (prepared of a pure bacterial strain); N : non-template control.

PCR amplicons of the V3 region of the 16S rRNA were used in DGGE analysis. The NTNU research group ACMS have previously experienced that amplifying the V3 region with primers

338F/518R (Tab. 2.3) from Atlantic salmon samples, the PCR product will be dominated of salmon DNA (18S rDNA). A solution is to use a nested PCR protocol (Bakke et al., 2011). Here we tested a strategy where the V3+V4 region of the 16S rRNA gene was amplified first, to eliminate the amplification of salmon DNA. The V3 region is amplified from this PCR product as a template.

By using the nested PCR protocol, the V3 rDNA was successfully amplified for most of the skin mucus and water samples (Fig. 3.4), and all amplicons had the expected length. There was some PCR product in the non-template control, but a higher PCR product yield was obtained in the samples. The PCR products were further used in DGGE analysis.

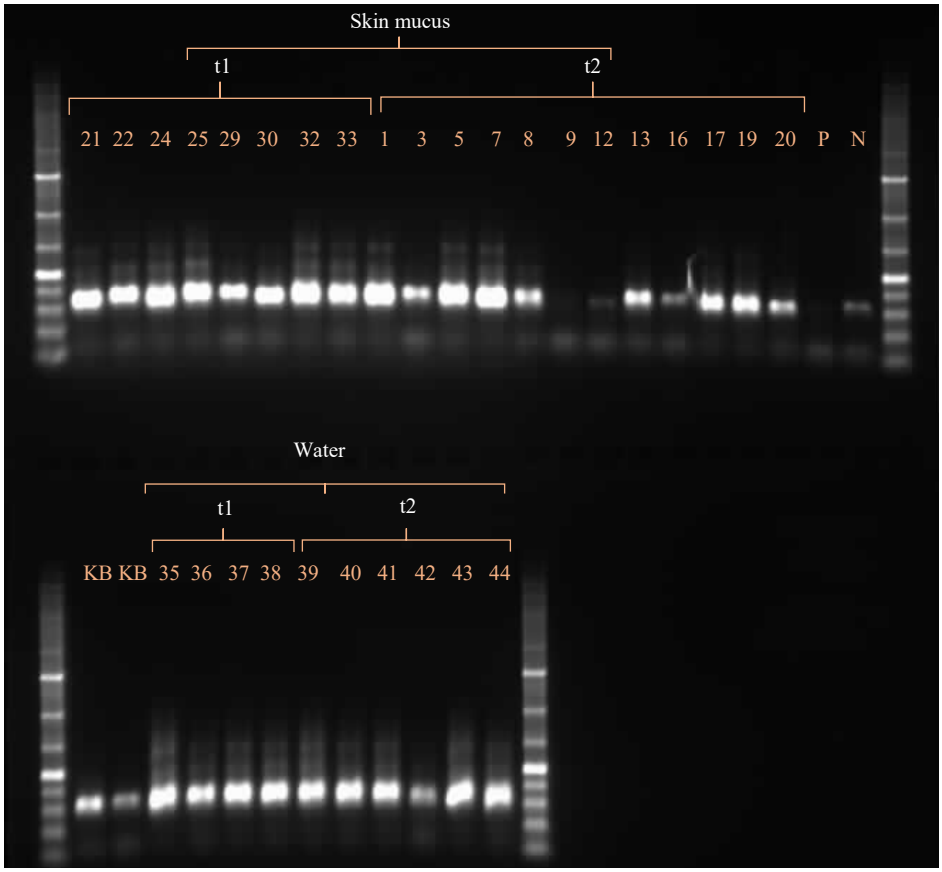


**Figure 3.4:** Agarose gel of V3 16S rDNA amplicons for skin mucus and water samples for t1 and t2. PCR protocol followed as described in section 2.2.1. External PCR was run for 28 cycles, while internal PCR was run for 25 cycles. All skin mucus samples were diluted 1:100. Numbers indicate sample id (Appendix A). KB : negative control from DNA extraction kit; P : positive control produced from a *Vibrio* strain; N : non-template control.

## 3.3 Effect of feed and lice infection on the composition of skin mucus microbiota

To examine if the functional feed affected the skin mucus microbiota, DGGE analysis was used. Mucus samples from t2, after lice infection of Atlantic salmon, were analysed by DGGE of 16S rDNA amplicons.

The DGGE gel indicated a difference in the microbial community composition of the skin microbiota between the two types of feeds (Fig. 3.5). There was a band that was unique in each treatment group (marked with a red frame in Fig. 3.5). Some bands, marked in green frames, were classified by Sanger sequencing (Fig. 3.5). Bands 1 and 2 (Fig. 3.5), were classified as *Oleispira*. This indicates that a strain of *Oleispira* is an abundant member of the skin microbiota to fish treated with the control feed. Band 4 and 6 seemed to be more abundant in skin mucus samples from the functional feed group (Fig. 3.5), and they were classified as *Streptococcus*. Hence, the indication that a strain of *Streptococcus* is an abundant member of skin microbiota to fish treated with the functional feed. Band 5 was classified to be *Propionibacterium*, which is known as a typical contamination of DNA extraction kit (Salter et al., 2014, Glassing et al., 2016). This band was present in most of the samples, and in the KB sample which is a control for the DNA extraction kit. Therefore, it implies that the *Propionibacterium* contaminated the DNA from the start of the process. No statistical analysis was performed on this DGGE gel. It is due to the smiling effect on the right side of the gel (Fig. 3.5), which made it hard to analyse the band patterns.

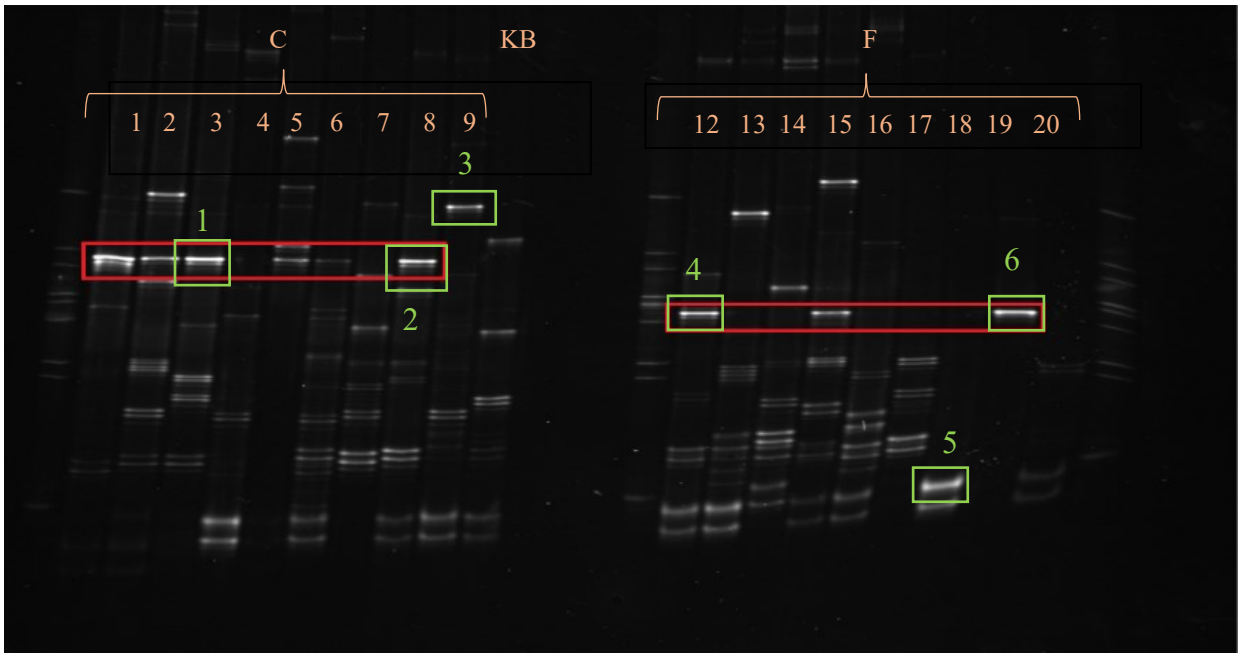


**Figure 3.5:** DGGE profile of bacterial 16S rDNA amplicons of the V3 region for skin mucus samples from fish after the infestation of sea lice. C: control group; F: functional feed. Band unique to the two feed groups are marked in red frames. The bands marked in green frames were reamplified and sequenced by Sanger sequencing for taxonomic assignment.

### 3.3.1 Characterisation of skin mucus microbiota by Illumina sequencing of 16S rDNA amplicons

Based on the DGGE-photo three samples from each treatment (Fig. 3.2) were further investigated with Illumina MiSeq sequencing of the V3 and V4 region of the 16S rRNA gene. The data were processed by using Usearch as described in Section 2.5.1. The sequences were clustered into operational taxonomic units (OTU) with similarity at 97 %. All OTUs not representing bacterial DNA, or OTUs probably representing contaminating DNA (identified by comparison to the community profile for the negative DNA extraction control, and the non-template control as described in Section 2.5) representing known contaminants were removed. This resulted in a total of 46 OTUs. Number of reads for each sample is presented in table 3.1.

**Table 3.1:** Number of reads per sample after quality filtering and removal of OTUs contaminating DNA, and non-bacterial DNA from the OTU-table. The control group (C) and functional feed group (F) is specified with sample numbers (Appendix A).

| Sample number | Number of reads |
|---|---|
| C3 | 3921 |
| C8 | 56 308 |
| C9 | 65 183 |
| F16 | 27 378 |
| F18 | 22 483 |
| F20 | 17 561 |

Sample number C3 had only 3921 reads (Tab. 3.1) before normalisation. However, the OTU table was normalised at 17 500 reads per sample as described in Section 2.5.1, to not lose too much data.

A taxonomic summary at the class level presents that Bacilli is almost exclusively present in the samples representing the experimental functional feed group (Fig 3.6), while the Deltaproteobacteria is more abundant in the control group samples. Gammaproteobacteria is present in all samples with differing abundance. Nevertheless, it is large variations between the individual samples.

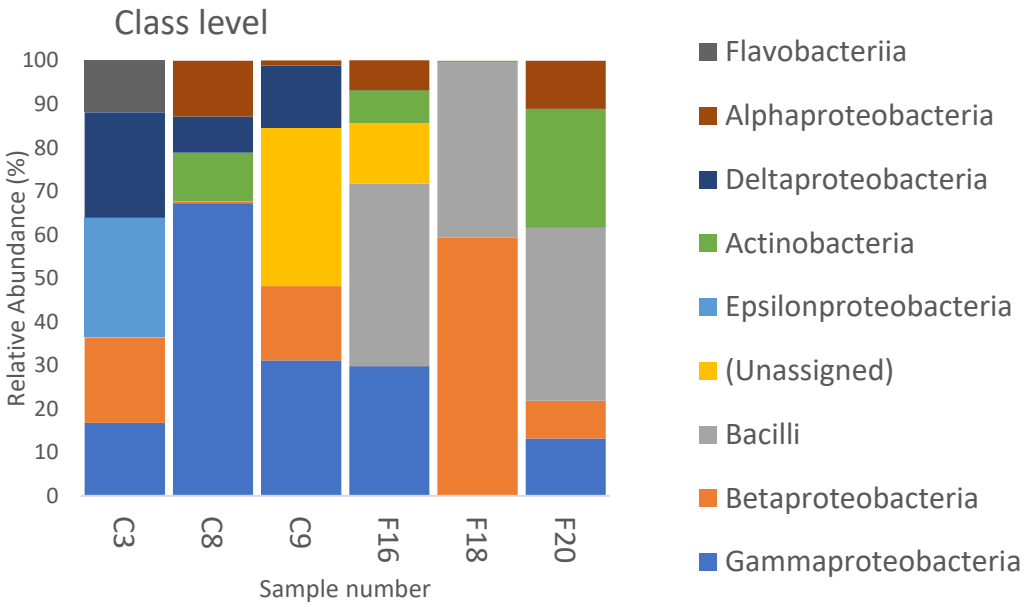


**Figure 3.6:** Taxonomic summary of microbial composition at class level in individual skin mucus samples after lice infection. Only taxa with abundance over 1 % is included. Samples are marked with sample numbers (Appendix A) and group. F : functional feed; C : control group.

To investigate the composition of each sample, the 10 most abundant OTUs were registered for each sample (Tab. 3.2). The composition of OTUs could indicate a trend of microbiota in the different feeding groups.

**Table 3.2:** The 10 most common OTUs in the individual skin mucus sample. If the sample did not have 10 OTUs, then all OTUs in the sample is presented. Taxonomy is specified according to a 0.8 for the Usearch Sintax confidence threshold (Edgar, 2016).

| C3: | OTU ID | Taxonomy | Number of reads |
|---|---|---|---|
| | OTU_46 | *Sulfurovum* (Eplisonproteobacteria) | 4816 |
| | OTU_4 | Desulfuromondales (Deltaproteobacteria) | 4236 |
| | OTU_54 | Neisseriaceae (Betaproteobacteria) | 3423 |
| | OTU_59 | Pasteurellaceae (Gammaproteobacteria) | 2941 |
| | OTU_62 | *Cloacibacterium* (Flavobacteriia) | 2084 |

| C8: | OTU ID | Taxonomy | Number of reads |
|---|---|---|---|
| | OTU_10 | *Oleispira* (Gammaproteobacteria) | 3081 |
| | OTU_14 | Alteromonadaceae (Gammaproteobacteria) | 2620 |
| | OTU_15 | Gammaproteobacteria | 2147 |
| | OTU_16 | *Marinobacter* (Gammaproteobacteria) | 2109 |
| | OTU_19 | Gammaproteobacteria | 1808 |
| | OTU_4 | Desulfuromonadales (Deltaproteobacteria) | 1455 |
| | OTU_23 | *Methylobacterium* (Alphaproteobacteria) | 1362 |
| | OTU_30 | *Micrococcus* (Actinobacteria) | 1114 |
| | OTU_34 | Rhodobacteraceae (Alphaproteobacteria) | 881 |
| | OTU_33 | Corynebacterium (Actinobacteria) | 830 |

| C9: | OTU ID | Taxonomy | Number of reads |
|---|---|---|---|
| | OTU_7 | Proteobacteria | 5101 |
| | OTU_8 | *Massilia* (Betaproteobacteria) | 2987 |
| | OTU_11 | *Pseudomonas* (Gammaproteobacteria) | 2730 |
| | OTU_4 | Desulfuromonadales (Deltaproteobacteria) | 2496 |
| | OTU_21 | *Shewanella* (Gammaproteobacteria) | 1616 |
| | OTU_25 | Bacteria | 1260 |

| | | |
|---|---|---|
| OTU_27 | *Pseudomonas* (Gammaproteobacteria) | 1093 |
| OTU_23 | *Methylobacterium* (Alphaproteobacteria) | 210 |
| OTU_223 | Burkholderiales (Betaproteobacteria) | 6 |
| OTU_150 | *Propionibacterium* (Actinobacteria) | 1 |

| F16: | OTU ID | Taxonomy | Number of reads |
|---|---|---|---|
| | OTU_6 | *Streptococcus* (Bacilli) | 7091 |
| | OTU_22 | Pasteurellaceae (Gammaproteobacteria) | 4168 |
| | OTU_31 | Bacteria | 2306 |
| | OTU_38 | *Phenylobacterium* (Alphaproteobacteria) | 1204 |
| | OTU_40 | Moraxellaceae (Gammaproteobacteria) | 1049 |
| | OTU_44 | *Gordonia* (Actinobacteria) | 702 |
| | OTU_47 | *Mycobacterium* (Actinobacteria) | 614 |
| | OTU_66 | Bacillus (Bacilli) | 238 |
| | OTU_83 | Bacteria | 123 |
| | OTU_150 | *Propionibacterium* (Actinobacteria) | 4 |

| F18: | OTU ID | Taxonomy | Number of reads |
|---|---|---|---|
| | OTU_9 | *Curvibacter* (Betaproteobacteria) | 8164 |
| | OTU_12 | *Staphylococcus* (Bacilli) | 7061 |
| | OTU_32 | *Tepidimonas* (Betaproteobacteria) | 2211 |
| | OTU_50 | *Corynebacterium* (Actinobacteria) | 59 |
| | OTU_150 | *Propionibacterium* (Actinobacteria) | 2 |
| | OTU_21 | *Shewanella* (Gammaproteobacteria) | 2 |
| | OTU_8 | *Massilia* (Betaproteobacteria) | 1 |

| F20: | OTU ID | Taxonomy | Number of reads |
|---|---|---|---|
| | OTU_29 | *Corynebacterium* (Actinobacteria) | 3849 |
| | OTU_36 | *Colwellia* (Gammaproteobacteria) | 2313 |
| | OTU_12 | *Staphylococcus* (Bacilli) | 2106 |
| | OTU_6 | *Streptococcus* (Bacilli) | 1891 |
| | OTU_39 | *Lactobacillus* (Bacilli) | 1758 |
| | OTU_42 | Bradyrhizobiaceae (Alphaproteobacteria) | 1229 |
| | OTU_43 | Comamonadaceae (Betaproteobacteria) | 1218 |
| | OTU_48 | *Gardnerella* (Actinobacteria) | 949 |
| | OTU_49 | *Lactobacillus* (Bacilli) | 823 |
| | OTU_56 | Rhizobiales (Alphaproteobacteria) | 702 |

It was observed major differences in OTUs present among all the samples in Tab. 3.2. Very few OTUs was consistent in all samples or the three replicates in the two feed groups. Desulfuromonadales (OTU_4) was the only OTU present in all of the control samples, while *Propionibacterium* (OTU_150) was the only OTU present in all of the skin mucus samples from fish fed the functional feed. These differences indicate major variations between the individual samples.

The OTUs 25, 31 and 83 were only classified at the domain level. These OTUs were examined using the RDP Classifier (Wang et al., 2007). OTU_25 was classified as Proteobacteria and OTU_31 as Actinobacteria with the confidence threshold of 0.5. OTU_83 was classified only as uncultured bacteria. The Bacilli in the skin mucus microbiota in the functional feed group is classified further as *Staphylococcus*, *Streptococcus*, *Lactobacillus* and Bacillus, where the *Staphylococcus* and the *Streptococcus* are most abundant. *Staphylococcus* and *Streptococcus* contribute together to 17.29 % of the differences in the mucus microbiota between the two feed groups (SIMPER, Tab. 3.3). Bacilli had very low abundance in the control group and was not found amongst the 10 most abundant OTUs in the control samples (Tab. 3.2). Despite great variation between individuals, it was remarkable that the control group had a relatively high abundance of a single OTU (OTU_4) which was classified as Desulfuromonadales. Gammaproteobacteria is very abundant in the control feed group, while the strains of Gammaproteobacteria varies a lot between the samples in the control group. A SIMPER analysis showed that three OTUs together could explain 25 % of the dissimilarities (Bray-Curtis) in the skin microbiota between the two treatment groups (Tab. 3.3).

**Table 3.3:** SIMPER analysis based on Bray-Curtis dissimilarities, which identifies the three OTUs that contribute to the difference between the two feed groups. The cumulative contribution of the OTUs to dissimilarity and the mean abundance of the OTUs in group C and F. OTU_12 was only classified at the order level.

| OTU ID | Taxonomy | Cumulative % | Mean C | Mean F |
|---|---|---|---|---|
| **OUT 12** | *Staphylococcus* | 8.73 | 0.33 | 3.06E+03 |
| **OTU_6** | *Streptococcus* | 17.29 | 0 | 2.99E+03 |
| **OTU_4** | Desulfuromonadales | 25.08 | 2.73E+03 | 0.667 |

The diversity within an individual sample is described with the α-diversity. The α-diversity of the community profiles were investigated with Shannon's diversity index and observed OTU richness. Shannon's diversity index reflects both richness and evenness of the community (Chao 1 was not determined because the data had been rarefied). Both the OTU richness and Shannon's diversity index indicates variations in the α-diversity of OTUs in the skin microbiota in both experimental groups (Fig. 3.7), but no clear difference between the two feed groups.

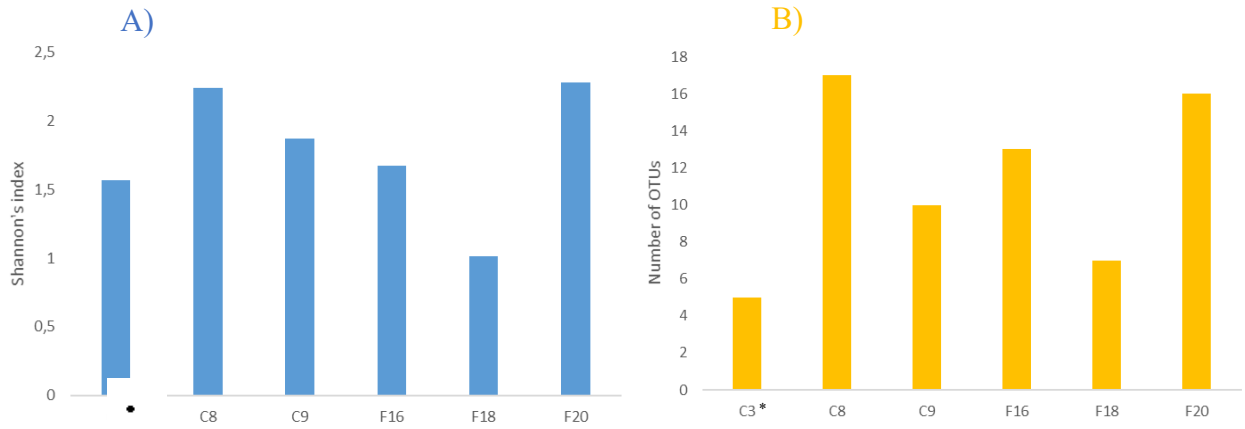


**Figure 3.7:** A) Shannon's diversity index and B) Observed OTU richness for individual skin mucus samples. Numbers indicate sample id (Appendix A). C : control feed; F: functional feed. (* indicate the sample with only 3921 reads that was rarefied to 17,5 K reads)

Furthermore, the β-diversity was analysed, and a principal coordinate analysis (PCoA) based on Bray-Curtis similarities was performed to compare the skin microbial community of the two feed groups (Fig. 3.9). The plot indicates a difference in the skin mucus microbiota between group C and F. A One-way PERMANOVA test ($p > 0.05$) showed that the difference was not significant. In the plot. There is more a considerable clustering in the control samples than in the samples from the functional feed group. This might indicate that there are higher variances in the functional feed group than in the control feed group.

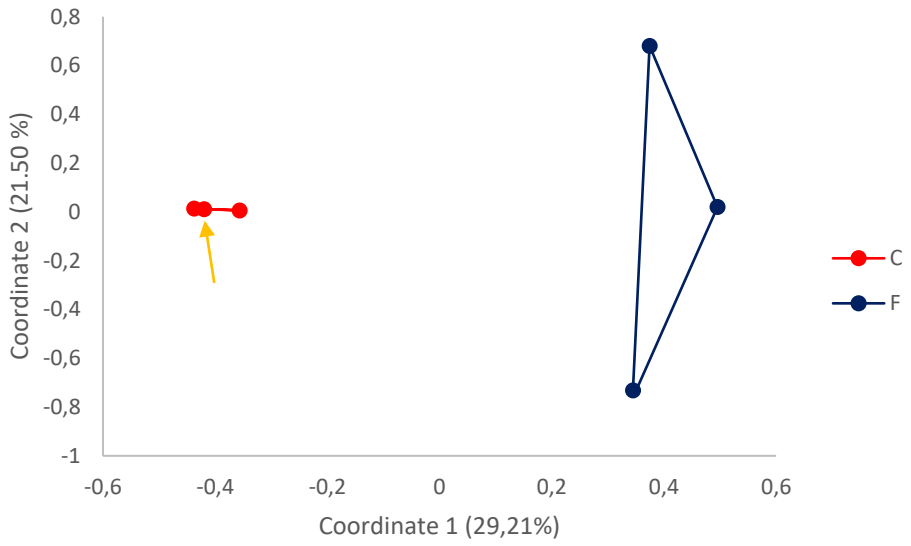


**Figure 3.8:** PCoA plot based on Bray-Curtis similarities of 16S rDNA amplicons from skin mucus samples. The yellow arrow marks the sample (C3) with a low number of reads. C : control feed group; F : functional feed group.

## 3.4 Effect of feed treatment on skin mucus microbial density

Quantitative PCR was conducted to quantify the number of copies of bacterial 16S rDNA in the skin mucus samples. This was used to estimate the density of bacteria per skin area and to investigate if the functional feed had any effect on the bacterial density. There were 9 individuals from each group, C and F, from the sampling time t2. Generally, the mucus samples seemed to have a reduced amplification efficiency than the standard curve (Fig 3.9). In the NTC and the germfree salmon tissue sample, there was little to no amplification.

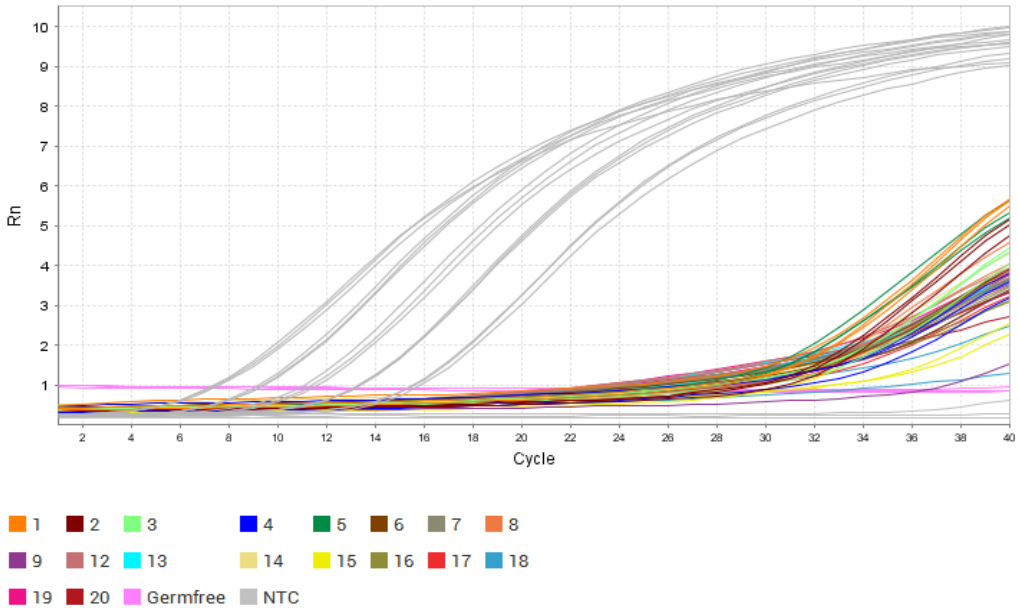


**Figure 3.9:** Amplification curve for skin mucus samples from qPCR, with number of cycles plotted against Rn. The Rn-value is the fluorescent signal from SYBR Green. The grey curves to the left, represents the standard curve. The pink and light grey samples down in the right corner are the triplicates of the non-template control (NTC) and a sample representing DNA extracted from germ-free salmon fry sample, respectively. Remaining samples represents the skin mucus samples from sampling time t2. Numbers represent the sample code in Appendix A.

DNA extracted from an isolated *Vibrio* strain was used to generate a standard curve. The standard curve was made by a five-fold dilution series from 0.04 ng $\mu L^{-1}$ to 6.4 x $10^{-5}$ ng $\mu L^{-1}$. The equivalent to the copy number of the PCR product was calculated as described in Section 2.4.1. The logarithm of the copy number was plotted against the average Ct-value for each triplicate, which resulted in a regression line with a slope of -2.95. This would indicate an amplification efficiency of 118 %. A PCR efficiency of over 100% is not possible and is probably due to an inhibiting effect at high template concentrations (further discussed in 4.3.2). Therefore, the highest DNA concentration (0.04 ng $\mu L^{-1}$) was excluded, resulting in a slope of -3.08 (Fig. 3.10), and the calculated amplification efficiency was 111 %.

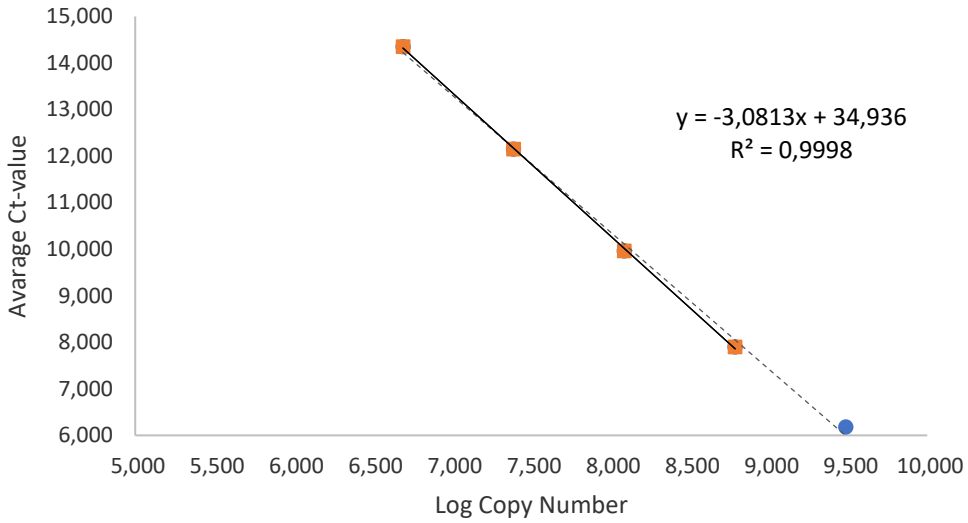


**Figure 3.11:** Standard curve excluding the sample with highest DNA concentration (0.04 ng $\mu L^{-1}$)(shown in black) achieved using a *Vibrio* strain, as a template in qPCR. The dotted line is presenting the curve if all DNA concentrations were included. The blue dot is representing the sample with a DNA concentration 0.04 ng $\mu L^{-1}$.

Copy number of 16S rDNA per $cm^2$ for each skin mucus sample was calculated. The samples representing the functional feed group had a higher average of 16S rDNA copies (Fig. 3.11). A F-test showed that the variance was unequal between the two groups ($p = 0.0017$), and a Welch t-test for unequal variance was used to examine if the average values were different between the two feed groups. The t-test showed a significant higher copy number for the skin mucus samples from the functional feed group, which implies that the bacterial density in the skin mucus was affected by the functional feed.

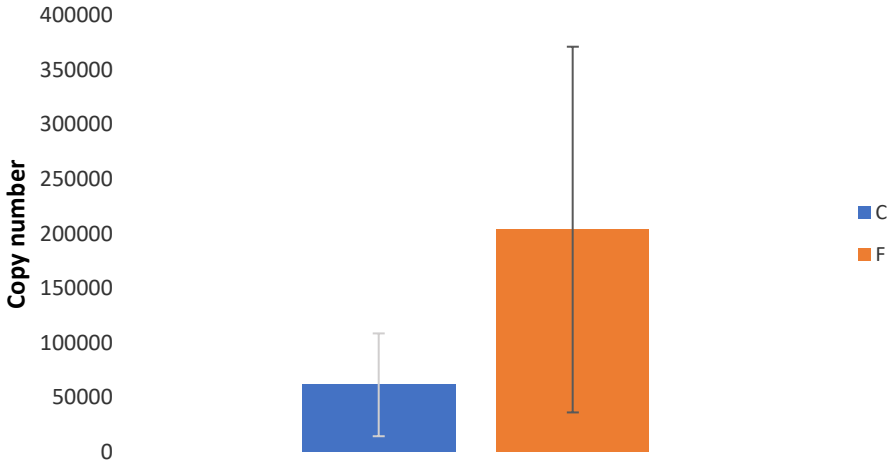


**Figure 3.10:** Average copy number per $cm^2$ skin based on qPCR data for 9 individuals for each feed group. Error bars represents standard deviation. C: control group; F : functional feed.

## 3.5 Comparison of water and skin mucus microbiota before and after lice infestation.

A second DGGE analysis was performed to investigate if the different types of feeds had an impact on the water microbiota, and to investigate if the skin microbiota was affected by the salmon lice infection. PCR products representing the V3 16S rRNA gene region were amplified by the use of a nested PCR protocol (Section 2.2.1). DGGE profiles of the water samples were similar to each other (Fig. 3.9), and all had one common dominating band. This band was classified as *Oleispira*. In the mucus samples from the control group at the second sample time t2, the same band was observed as in the water samples (Fig. 3.9). This band was also classified as *Oleispira*. Therefore, it is probably the same strain of *Oleispira*.

There are poor quality and low resolution in the bottom part in one of the DGGE gel (Fig. 3.12). Comparison of DGGE community profiles for skin mucus samples that were run on both gels (Fig. 3.5, 3.12), shows a strong band in several samples in the last DGGE gel (Fig. 3.12), which possibly consist of multiple DGGE-bands. This collection creates an artificial band, and it can consist of more than one bacterial strain.

Water microbiota has been shown earlier to be quite diverse. Therefore, it is remarkable that it was so few bands in the water samples. At sample time t1 there is two strong bands, but only one strong band at t2. This might indicate that *Oleispira* dominated the water microbiota at t2 (Fig. 3.9).

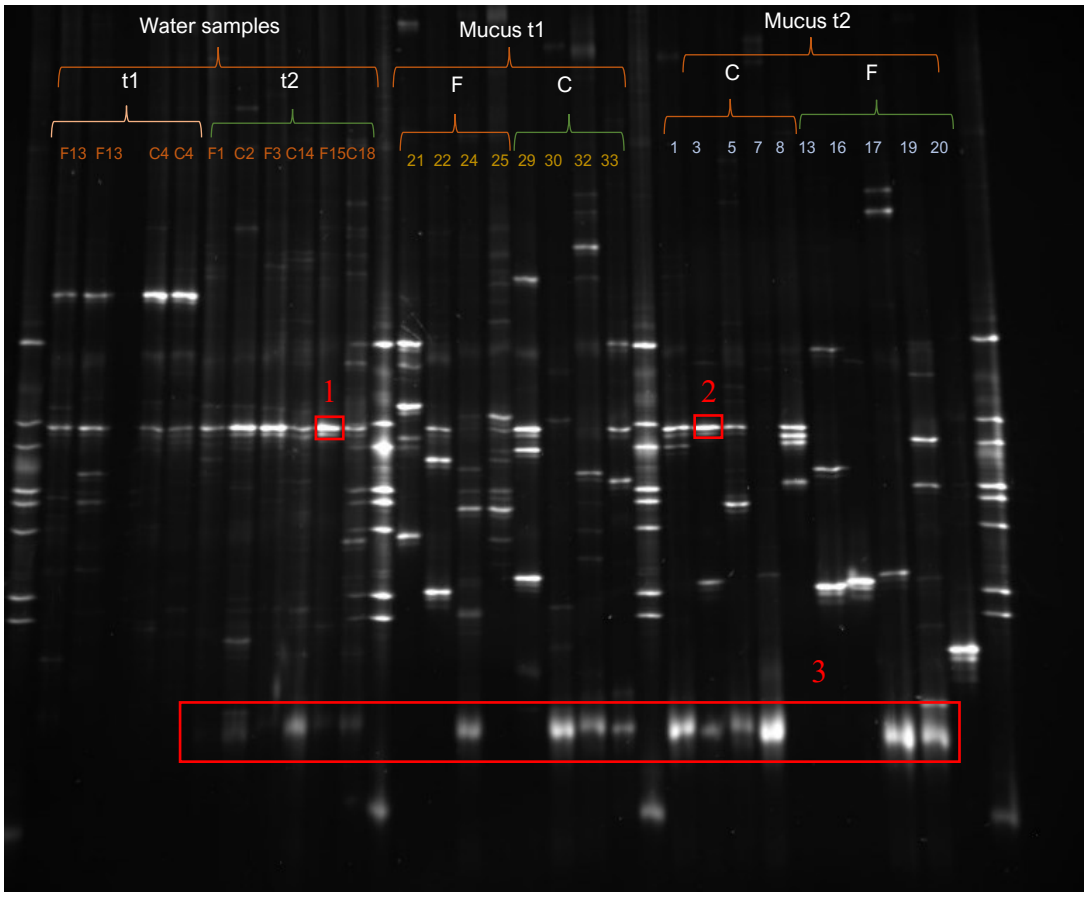


**Figure 3.12:** DGGE gel of PCR products representing the V3 16S rDNA in water and mucus samples at two sampling times (t1, t2) for the control group (C) and the functional feed (F). Numbers indicate sample numbers (Appendix A). Water samples from t1 (prior infestation consisted of two replicates of samples in one tank. The bands marked in red frames were classified by reamplification and Sanger sequencing (1,2), and represent poor quality in the gel (3) . t1: 10/9-18; t2: 1/10-18 and 2/10-18;

A PCoA ordination based on Bray-Curtis similarities was performed for the DGGE profiles (Fig. 3.10). The PCoA plot indicates that water microbiota is distinct from the skin mucus microbiota. A One-Way PERMANOVA (Bray-Curtis) showed a significant difference ($p = 0.0003$) between the water microbiota and the skin mucus microbiota. The water samples seem to cluster based on the sampling time and type of feed. Despite considerable variation between individuals, it seems to be a tendency that the mucus samples cluster depending on the type of feed and to a certain extent they cluster depending on the time of the sampling. In general, the PCoA plot indicates that the water microbiota seems to be unaffected by the different types of feed, and the mucus microbiota did not appear to be much influenced by the salmon lice infection.

Some mucus samples cluster on the right of the PCoA plot. The DGGE profiles for these samples were inspected and the strong band in the lower part of the gel was found to be in common (frame 3, Fig.3.12). As argued above, this band probably represent many distinct bands, and this may, therefore, represent an artificial similarity between the DGGE community profiles.

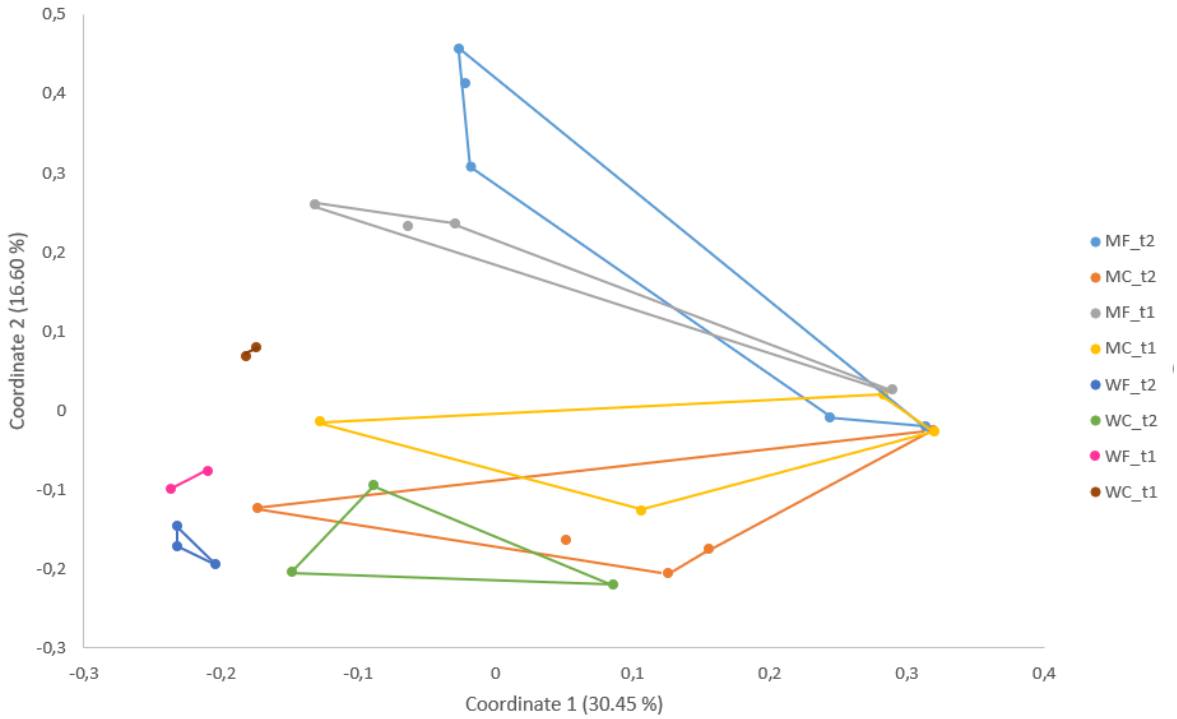


**Figure 3.13:** PCoA plot based on Bray-Curtis similarities for DGGE profiles of V3 region of 16S rDNA for water and mucus samples taken at t1and t2. M: mucus; W : water; C : control feed; F : functional feed.

A One-way PERMANOVA test indicated that the difference in skin mucus microbial communities between the two feeds were significant ($p = 0.0155$). This is another indication that the functional feed affected the skin mucus microbiota.

# 4 Discussion

## 4.1 Evaluation of methods

### 4.1.1 Amplification of Bacterial 16S rDNA

Skin microbiota is challenging to work with because amplifying bacterial DNA from DNA extracts of skin mucus samples from salmon has proven to be difficult. There could be three major reasons for these problems. First, there could be a considerable smaller amount of target bacterial DNA in comparison to the host DNA in the DNA extracts. This has previously been identified as a problem when studying microbiota in fish samples (Austin, 2006). Second, PCR inhibitors are probably present in the DNA extract. Third, homology with the salmon 18S rRNA gene with the broad-coverage bacterial PCR primers would cause co-amplification of the host DNA (Huys et al., 2008). There were problems to amplify bacterial 16S rDNA from the skin mucus samples (Fig. 3.2). After testing different PCR conditions, the amount of bacterial DNA was found to not be the cause of the problems.

Dilution of the DNA extract to 1:100 had a positive effect showing that inhibitors in the DNA extract was probably the cause of the PCR problems for the skin mucus samples. When the DNA extract is diluted, the inhibitors are also diluted and has less inhibiting effect. There were indications of DNA contaminations in the non-template PCR control during the PCR amplification. Spermidine was identified as a contributor to this contamination (Fig. 3.3), but it was not the exclusive source. Therefore, there could be contamination in the other PCR reagents. The PCR product in the NTC (Fig. 3.4) generally had a lower yield than the skin mucus and water samples. This implies that the bacterial DNA in the samples had been amplified, and not just the contaminated DNA. DNA contamination in the NTC can originate from several sources, e.g. carry-over contamination from the PCR reagents. All PCR reagents were aliquoted to reduce the contamination risk as much as possible, and all tubes and racks used were UV-radiated, and the PCR was set up on a sterile bench. To avoid co-amplification of salmon and PCR inhibition, the following criteria were used; dilution of the DNA extract 1:100, using bacterial-specific primers which target the V3 +V4 region of the bacterial 16S rRNA gene and precautions during the PCR set up to minimise the DNA contamination.

### 4.1.2 Analysis of microbial community composition and diversity with DGGE and Illumina sequencing

PCR amplification is a major source of bias in PCR based studies (Balázs et al., 2013). In addition that the copy number of 16S rDNA molecules in a bacterial species varies, and if a specie has multiple copies of the 16S rRNA gene, there would be more PCR product from this strain and the abundance would be overestimated. In the case of sequence divergence between the genomic rRNA gene copies, it could appear several bands on a DGGE gel from one strain or as bands being stronger than the other bacteria with the same abundance, and here the diversity would be overestimated (Malik et al., 2008). In Illumina sequencing of 16S rDNA amplicons, the same effect could occur, and there could be more reads for the relevant OTU. This would overestimate the abundance of the OTU due to the artificially high number of reads of the gene copies representing that OTU (Ibarbalz et al., 2014). This is not a problem considering the β-diversity due that all samples would be affected by the same bias. An alternative culture-independent method to study the microbial community composition, avoiding the PCR-amplification bias, is to use metagenomics. With metagenomics it is possible to perform 16S rDNA sequencing and functional-based analysis of the microbial metagenome in an environmental sample. First, the total DNA from an environmental sample is isolated and cloned (Riesenfeld et al., 2004). Then, the total DNA is sequenced to create a fragment-pool of sequences that represent the metagenome of the microbial community (Mardis, 2008). Metagenomics avoids the PCR bias, but it requires more advanced and complex analysis of the sequences (Jünemann et al., 2017).

The analysis of the DGGE gel with the software Gel2K included manually processing to separate and align the bands between samples. The software was not able to separate bands that were positioned close to each other nor if the sharpness of the gel photo was weak, and some bands were not identified at all. The deficits were manually corrected in the Gel2K software (Appendix D), and this could be a source of error. DGGE analysis is usually not a suitable method to classify microbial communities taxonomically, even though some taxonomical information could be generated. Bands that were reamplified from the DGGE gel were sequenced by Sanger sequencing as classified by RDP with a confidence threshold on 0.5. The classification results for the most dominant bands from DGGE corresponded with the OTUs found with the Illumina deep sequencing.

DGGE is a relatively easy and low-cost method to analyse the microbial community composition and get an overview of the diversity within the microbial community and

community dynamics by comparing DGGE profiles for several samples. The reproducibility of the DGGE is poor, and it is difficult to replicate the same denaturing gradient between gels (Muyzer and Smalla, 1998). Therefore, comparison of several gels should be avoided.

Some samples were included on both of the DGGE gels (Fig.3.5 and Fig. 3.12). The band patterns varied between the gels for the same samples, and the resolution was quite different, especially in the lower part of the gel. This indicates that the denaturing gradient on the two DGGE gels differed. The DGGE gel (Fig. 3.5) with only skin mucus samples from t2, was not analysed due to a smiling effect on the right side. The smiling effect was due to an air bubble trapped in the gel-chamber during the electrophoresis. Another drawback with DGGE is that the number of samples is restricted, due to the limited number of wells. Regardless of the drawbacks, DGGE is a suitable tool to compare community profiles, e.g. community dynamics.

Illumina sequencing has some advantages compared with DGGE. The resolution is much greater, more taxonomic information is provided, and the number of samples analysed together can be significantly higher. Here, the bacterial V3+V4 16S rDNA amplicons were sequenced with Illumina high throughput sequencing. Samples for three individuals from each feed treatment were selected based on the DGGE profile and if they had an acceptable yield of the V3+V4 16S rDNA PCR product (Fig. 3.5). This selection of samples would only characterise the skin microbiota for a few individuals in the two feed groups, and it is too few samples for statistical analysis. The reason for the few numbers of samples that were sequenced was due to economic reasons. Luckily, I got the opportunity to include my samples in a different sequencing project. This gave the possibility to investigate the composition of the skin mucus microbial community with at a more detailed level for a few representative samples.

Here we sequenced both the V3 and V4 variable regions of the 16S rRNA. It is optimal to choose two variable regions to get a higher resolution of the sequence analysis and greater taxonomic assignment (Hamady and Knight, 2009). Sample number 3 had only 3921 reads (Tab. 3.1) before normalisation of the OTU-table of 17 500 reads. The reason for this low number of reads could imply that this sample could consist of a lot of host DNA.

### 4.1.3 Quantification of bacterial 16S rDNA by qPCR

Quantitative PCR was conducted to estimate the density of bacteria in skin mucus from Atlantic salmon treated with the two types of feed, by quantifying the amount of 16S rDNA copies. This analysis method is integrating the amplification and monitoring of the DNA produced in each

PCR cycle by using fluorescence dye (Saleh-Lakha et al., 2005). The dye applied was the double-stranded DNA binding dye SYBR® Green. This dye binds both specific and non-specific PCR products.

Measures were performed to make the sampling of the skin more similar for each individual. By using a homemade plastic square with a 3x6 $cm^2$ square to cut out skin pieces of the same size, which the mucus was collected from by scraping, to make the area of skin from each fish more or less equal. The amount of mucus and host tissue collected probably differed between individual samples. This could affect the estimation of copy numbers of the 16S rDNA based on the qPCR data.

The non-template control should consist of 20 µL of the PCR master mix, but unfortunately, by mistake, only 5 µL was used in the qPCR. Without proper negative PCR control, it cannot be excluded that some of the qPCR signals represented contaminated DNA.

The estimated amplicon efficiency as determined from the standard curve was found to be above 100 %, which is not realistic (Fig. 3.10). The amplification efficiency in qPCR should range from 90 – 100 %. At 100 % the polymerase enzyme is working at its highest capacity. Inhibition of the polymerase due to high template concentration is usually the reason for overestimation of the efficiency, especially if high template amounts are added to the reagent mixture (Zupancic, 2019). Inhibition would result in lower Ct-values than the amount of template would indicate. When the efficiency plot flattens, the slope will get lower, and it would result in efficiency of over 100 % (Zupancic, 2019). Since the amplification efficiency was overestimated, then the amount of copy number of 16S rDNA would also be overestimated in the samples. The DNA concentration 0.04 ng $\mu L^{-1}$ was excluded from the standard curve to minimise the overestimation of the amplification efficiency. Still, the efficiency was above 100 % and probably the dilution series should be diluted to lower DNA concentrations for the most concentrated samples in the standard curve.

## 4.2 Salmon skin mucus microbiota

There are very few studies on the Atlantic salmon skin mucus microbiota, and to my knowledge, only 4 papers have been published on this topic (Llewellyn et al., 2017, Lokesh and Kiron, 2016, Minniti et al., 2017, Karlsen et al., 2017). The most abundant phylum in skin mucus microbiota is Proteobacteria (Lokesh and Kiron, 2016, Minniti et al., 2017). Proteobacteria was also observed as the most abundant phylum in the skin mucus samples in this project (Fig. 3.6).

Many of the bacterial strains found in the skin mucus samples (Appendix F) correspond to the microbiota found in the different studies of Llewellyn (2017), Lokesh and Kiron (2016) and Minniti (2017), but not all strains from the skin mucus samples were found in every study. Lokesh found the *Oleispira* was the cause of the high abundance of Proteobacteria (89 %), and *Oleispira* was found to be very abundant in a control sample (Tab. 3.2) and in all water samples (Fig. 3.12). *Methylobacterium* was only found in the skin mucus samples from fish fed the control feed, and this was an abundant genus in the studies performed by Minniti (2017). The *Methylobacterium* ssp. is previously found in healthy fish skin mucus for brook charr (Boutin et al., 2013) and has been found to produce an antimicrobial component β-hydroxybutyrates (Boutin et al., 2013, Halet et al., 2007). Since the *Methylobacterium* was not observed in the skin mucus samples from the functional feed group, probably it could not colonise the skin mucus for the fish fed the functional feed. Very few microbes associated with the salmon skin mucus have a known function (Lokesh and Kiron, 2016). Therefore, more research should be performed on the function and composition of the skin mucus microbiota.

The total of OTUs after processing of the data as described in 2.5.1, were 46 OTUs. In studies working with salmon skin mucus microbiota, numbers of OTUs observed was from 250-925 (Karlsen et al., 2017, Lokesh and Kiron, 2016), and this indicates that the OTU richness in this project is very low. For the control group, only one OTU was abundant in all three skin mucus samples (OTU_4, Desulfuromonadales, Appendix F). For the skin mucus samples from the functional feed group, also only one OTU was abundant in all three (OTU_150), and this OTU was classified as *Propionibacterium* which is a known contaminant (Salter et al., 2014, Glassing et al., 2016). These dissimilarities of the microbiota between the samples, even in the same experimental group shows how different the individual samples are and the diversity among all the samples, and could indicate the dynamic nature of the fish skin mucus.

## 4.3 Effect of experimental functional feed

The functional feed tested in this project was observed to not affect the salmon lice infection between the two feed groups. On the other hand, through analysis done in this thesis, the functional feed was found to have an effect on other aspects concerning the mucosal barrier of the Atlantic salmon. The effect of the functional feed on the amount of mucus produced, the composition of the skin microbiota and the bacterial density was investigated. The mucosal

layer is a dynamic surface both in quantity and presence of substances (Esteban and Cerezuela, 2015), and therefore some variance between the samples is expected.

The fish fed the functional feed was observed to have more mucus during the collection of samples (Fig. 3.1), but the difference between the groups was not significant (t-test). The amount of mucus measured for the two feed groups seems to differ. A thicker layer of mucus could make it harder for the salmon lice to attach to the skin. This could be what the producer expected from the feed.

### 4.3.1 Effect on the composition of the skin mucus microbiota

Differences were observed in the composition of the microbial community in the fish skin mucus between the two feed groups (Fig. 3.5, 3.6). In the DGGE analysis, unique bands were observed for each of the feed group. A band unique to the control group was classified as *Oleispira*, whereas the band unique to the functional feed group was classified as *Staphylococcus*. This was partially confirmed by the deep sequenced data. In the OTU-table (Appendix F), *Oleispira* was only found for one of the skin mucus samples from the control group, and *Staphylococcus* was found for 2 out of three individuals of the functional feed group. These differences suggest that the functional feed affected the microbial composition in the skin mucus. The genus *Bacillus* was found in one of the functional feed samples (F16, Tab. 3.2). *Bacillus* can produce antibiotics, and have antimicrobial activity and has wanted properties to be used in functional feeds (Paniagua-Michel, 2014). The antimicrobial activity could be a positive effect from the functional feed.

Bacilli were the most abundant class of bacteria related to the experimental functional feed, and in general there were major differences in the presence of OTUs in all samples. Desulfuromonadales (OTU_4) was among the most abundant in all samples from the control feed (Fig. 3.6), and it is a contributor to the dissimilarities between the groups due to its very low abundance in the skin mucus from the functional feed group. This absence in the skin mucus microbiota in the functional feed group could indicate that the Desulfuromonadales could not colonise the skin mucus. Desulfuromonadales is an anaerobic respiratory bacteria, which can transform $H_2S$ under anoxic conditions and can cause a toxic environment for animals in aquaculture (Lin et al., 2017). The effect of the functional feed has resulted in a selection of microbiota, which is interesting with the thoughts for future investigations.

Results from DGGE, Sanger and Illumina sequencing confirms that *Oleispira* was the most dominating genus in all water samples from t2 and present in water samples from t1, and was

found in skin mucus samples in the control group (Fig. 3.5, 3.12). Bacteria belonging to this genus have been documented to be present in both on Atlantic salmon skin in both freshwater and seawater (Lokesh and Kiron, 2016).

Components in fish feed have been shown to appear in the epidermal mucus within 12 hours and had a full effect within 2 days (Church et al., 2008). There is almost no documentation on how the microbiota in the fish skin mucus microbiota gets affected by the feed, but from results from this project, it could imply that the functional feed affected the skin mucus microbiota.

### 4.3.2 Effect on the bacterial density in skin mucus microbiota

When comparing the calculated copy number of the 16S rDNA obtained by qPCR for skin mucus samples between the two feed groups, there was a clear difference (Fig. 3.11). Even though the standard deviation in both groups was large, the difference is significant (Welch t-test). The copy numbers varied from 2 x $10^3$ to 4.3 x $10^5$ copies per $cm^2$ skin, and the skin microbiota from functional feed group had the highest number of copies. This indicates a clear effect of the functional feed on the skin mucus microbiota density.

A low number of copies indicates the low density of bacterial in the skin. In a previous study by Austin (2002) the reported bacterial load on the fish skin per $cm^2$ was from $10^2$ to ~ $10^4$ (CFU) bacteria which, according to the author, were low numbers. The numbers of copies per skin obtained in this project are in the same magnitude (Austin, 2006). Quantitative data is difficult to compare across studies since the molecular methods used can be different (Minniti et al., 2017). Sampling techniques may vary, and the different body parts on the fish have variance in the bacterial community (Chiarello et al., 2015), which makes it even more troublesome to compare the data.

The experimental functional feed gave the salmon a significantly higher density of bacterial load. The feed was designed to have an inhibiting effect on the salmon lice infection. Some of the other functional feeds on the marked, that claims to reduce salmon lice infestation mentioned in Section 1.5.3, aims to thicken the mucus layer to protect the fish. To my experience, no studies are found about that a higher density of bacterial load in the fish skin makes the skin mucus layer thicker, and then again, the fish more resistance against salmon lice infection. Here we observed a higher volume of mucus in the functional feed group, although the difference was shown not to be significant. Thus, the functional feed seems to thicken the mucus and bacterial load, even though it did not affect the salmon lice infection rate.

A new method that also aviods amplification bias when it comes to quantification of DNA, is digital droplet PCR (ddPCR). Digital droplet PCR use micro droplets aiming at amplifying one DNA molecule in each droplet, thus using the droplets as reactions chambers (Hanssen et al., 2017). Additionally, end-point measurements make possible for nucleic acid quantitation without dependency on the reaction efficiency (Taylor et al., 2017). The advantage with ddPCR is a direct and independent quantification of the target DNA, independent of amplification efficiency and without the need of a standard curve. This will produce more accurate and reproducible data, especially when inhibitors are present. There is no ddPCR equipment in the Department of Biotechnology and Food Science, therefore it was not conducted.

## 4.4 DGGE Analysis for comparison of water and skin microbiota, and skin microbiota before and after lice infestation

When comparing the water and mucus samples at the different sampling times, the DGGE and PCoA plot indicated that the functional feed affected the skin mucus microbiota. In earlier studies, water microbiota clearly differs from the skin mucus microbiota (Llewellyn et al., 2017). *Oleispira* was the most abundant genus in the water samples, and the only band that was classified (Fig. 3.12). It was remarkable how few bands there were on the DGGE gel (Fig. 3.12) for the water microbiota, which indicates very low diversity. This differs from earlier studies that show that water microbiota usually is much more divers than skin mucus microbiota (Minniti et al., 2017). It is even more remarkable that these bands seemed to be present in the fish skin microbiota since it has been reported that the skin mucus of healthy fish and the rearing water have a different composition of the microbiota (Merrifield and Rodiles, 2015). In the PCoA plot based on the DGGE gel (Fig. 3.13) the water samples cluster almost completely separate from the mucus samples, this indicates that in general there are differences between the water and skin mucus microbiota. If the water samples had been subjected to Illumina sequencing, more taxonomic information would be provided, and there would be higher resolution of the results. This could help to explain some of the low diversity observed from the DGGE gel.

Some of the skin mucus samples clustered together in the PCoA plot (Fig. 3.13) based on DGGE community profiles, although they belonged to a different group and sampling times. This was found to be due to a strong band in the lower part on the DGGE gel, red frame 3 (Fig. 3.12).

This part of the DGGE gel had poor resolution and could consist of multiple bands. This might have created an artificial similarity between the samples. Thus, this is an example of the lower reproducibility and resolution in DGGE analysis compared to deep sequencing of amplicons.

## 4.5 Future perspectives

In this project, only a few numbers of samples were subjected to Illumina amplicon sequencing. These results indicated the composition of the skin mucus microbial community. If all skin mucus samples were deep sequenced, more statistical analysis could have been performed. The major improvement of this project would be to have a lot more individuals in each feed group, and all samples should be subjected to Illumina amplicon sequencing. The water samples had very few bands on the DGGE gel, and by deep sequencing these samples could give more information about the composition of the water microbiota and maybe confirm the results obtained in the DGGE analysis.

Research in the aquaculture industry is an on-growing field and is constant developing. Still, little is known about the Atlantic salmon skin mucus microbiota and its function against pathogenic infections. As mentioned in Section 4.3.2, no research was found on the relationship between the thickness of the epidermal mucus and bacterial load. Microbes have a unique property to adapt and change its environment, and therefore it is important to understand the vital role of microbes in the skin mucus barrier.

When talking to people in the industry, very few of them have faith in that functional feed is the solution to fight the problems with salmon lice alone. Today, the industry uses several methods combined to get the most successful salmon lice treatment with both preventative and direct treatment methods. The effect of salmon lice treatment methods on the salmon's welfare and health is inadequate documented, but in general, it gives increased risk of injury and stress (Holan et al., 2017). Preventative measures, e.g. the functional feed could improve the salmon's skin mucus barrier instead. To get the feed more efficient, more specific research is needed to understand the relationship between the feed, mucus properties and microbiota community composition.

# 5 Conclusion

In this thesis, the feeding of Atlantic salmon with a functional feed was investigated, which was supposed to reduce the attachment of salmon lice, effected the mucus production, the composition of skin mucus microbiota and the bacterial load in the skin mucus. The major findings in this project were as follows:

- The functional feed did not reduce the amount of salmon lice attached to the Atlantic salmon.

- The amount of mucus produced appeared to be larger for the fish fed functional feed, although the differences between the fish fed functional feed and control feed was not significant.

- The composition of the skin microbiota differed between individuals fed the functional feed and control feed. Proteobacteria was the most abundant phylum in all samples, while Bacilli was the most abundant class in the fish fed functional feed with *Staphylococcus* and *Streptococcus* as most abundant genera. In the control group, Gammaproteobacteria was the most abundant class with *Oleispira* as the most abundant genus. Desulfuromonadales was only abundant in the skin mucosa from the fish fed the control feed and was not observed the skin mucus on fish fed with the functional feed. Thus, the functional feed appeared to influence the skin microbiota.

- The number of 16S rDNA copies per skin area was estimated by qPCR and differed significantly between the fish fed functional feed and control feed. This indicates that it was a higher amount of bacteria per $cm^2$ skin mucus for salmon fed the functional feed.

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# Appendix A - Skin mucus samples

**Table A.0.1:** Mucus samples taken at t1 infestation. Data from before salmon lice infection withdrawing of skin mucus from Atlantic salmon. Both weight (g) and length (cm) was measured from sampled individuals. There were only two tanks, with one of each type of feed.

| **Sample id** | **Functional feed** | | **Sample id** | **Control feed** | |
|---|---|---|---|---|---|
| | Length (cm) | Weight (g) | | Length (cm) | Weight (g) |
| **21** | 29.2 | 210.8 | **28** | 31.5 | 243.6 |
| **22** | 28.5 | 230.6 | **29** | 29.8 | 252.0 |
| **23** | 27.2 | 202.3 | **30** | 28.4 | 198.3 |
| **24** | 27.0 | 180.7 | **31** | 28.7 | 237.9 |
| **25** | 28.0 | 191.6 | **32** | 28.5 | 247.1 |
| **26** | 29.2 | 208.2 | **33** | 27.5 | 187.0 |
| **27** | 28.7 | 202.4 | **34** | 25.3 | 176.9 |

**Table A.0.2:** Mucus samples taken at t2. Data from after salmon lice infection withdrawing of skin mucus from Atlantic salmon. Both weight (g) and length (cm) was measured from sampled individuals. There were three replicas from three tanks with same type of feed.

| **Sample id** | **Control feed** | | | **Sample id** | **Functional feed** | | |
|---|---|---|---|---|---|---|---|
| | Length (cm) | Weight (g) | Cage no | | Length (cm) | Weight (g) | Cage no. |
| **1** | 33.5 | 349 | 14 | **12** | 33.6 | 324 | 1 |
| **2** | 31.0 | 285 | 14 | **13** | 33.0 | 378 | 1 |
| **3** | 31.7 | 321 | 14 | **14** | 31.5 | 338 | 1 |
| **4** | 32.2 | 341 | 2 | **15** | 31.8 | 342 | 15 |
| **5** | 33.7 | 372 | 2 | **16** | 34.6 | 300 | 15 |
| **6** | 32.6 | 392 | 2 | **17** | 31.8 | 430 | 15 |
| **7** | 34.7 | 430 | 18 | **18** | 31,7 | 347 | 3 |
| **8** | 33.2 | 330 | 18 | **19** | 32,0 | 376 | 3 |
| **9** | 36.4 | 452 | 18 | **20** | 30.6 | 307 | 3 |

## Appendix B - DNA Extraction Protocol

May 2017

# DNeasy® PowerSoil® Kit Handbook

For the isolation of microbial genomic DNA from all soil types

Sample to Insight

QIAGEN

# Protocol: Detailed

## Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves.
- 2 ml Collection Tubes are provided.

## Procedure

1. Add 0.25 g of soil sample to the PowerBead Tube provided. Gently vortex to mix.
   **Note:** After your sample has been loaded into the PowerBead Tube, the next step is a homogenization and lysis procedure. The PowerBead Tube contains a buffer that will (a) help disperse the soil particles, (b) begin to dissolve humic acids and (c) protect nucleic acids from degradation. Gentle vortexing mixes the components in the PowerBead Tube and begins to disperse the sample in the buffer.
2. If Solution C1 has precipitated, heat at 60°C until precipitate dissolves. Add 60 µl of Solution C1 to sample and invert several times or vortex briefly.
   **Note:** Solution C1 may be added to the PowerBead tube before adding soil sample. Solution C1 contains SDS and other disruption agents required for complete cell lysis. In addition to aiding cell lysis, SDS is an anionic detergent that breaks down fatty acids and lipids associated with the cell membrane of several organisms. If it gets cold, it will form a white precipitate in the bottle. Heating to 60°C will dissolve the SDS but will not harm it or the other disruption agents. Solution C1 can be used while it is still warm.
3. Secure PowerBead Tubes horizontally using a Vortex Adapter for 24 (1.5–2.0 ml) tubes (cat. no. 13000-V1-24).
4. Vortex at maximum speed for 10 min.
   **Note:** If using the 24-place Vortex Adapter for more than 12 preps, increase the vortex time by 5–10 min. Vortexing is critical for complete homogenization and cell lysis. Cells are lysed by a combination of chemical agents from steps 1–4 and mechanical shaking

introduced at this step. By randomly shaking the beads in the presence of disruption agents, collision of the beads with microbial cells will cause the cells to break open. Use of the Vortex Adapter will maximize homogenization, which can lead to higher DNA yields. Avoid using tape, which can become loose and result in reduced homogenization efficiency, inconsistent results and reduced yields.

5. Centrifuge tubes at 10,000 x *g* for 30 s.
6. Transfer the supernatant to a clean 2 ml Collection Tube.
   **Note:** Expect between 400–500 µl of supernatant. Supernatant may still contain some soil particles.
7. Add 250 µl of Solution C2 and vortex for 5 s. Incubate at 2–8°C for 5 min.
   **Note:** You can skip the 5 min incubation. However, if you have already validated the DNeasy PowerSoil extractions with this incubation we recommend you retain the step. Solution C2 is patented Inhibitor Removal Technology (IRT). It contains a reagent that can precipitate non-DNA organic and inorganic material including humic substances, cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.
8. Centrifuge the tubes at 10,000 x *g* for 1 min.
9. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml Collection Tube.
   **Note:** The pellet at this point contains non-DNA organic and inorganic material including humic acid, cell debris and proteins. For best DNA yields and quality, avoid transferring any of the pellet.
10. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.
    **Note:** You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with this incubation we recommend you retain the step. Solution C3 is patented Inhibitor Removal Technology (IRT) and is a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.
11. Centrifuge the tubes at 10,000 x *g* for 1 min.

12. Avoiding the pellet, transfer up to 750 µl of supernatant to a clean 2 ml Collection Tube.
    **Note:** The pellet at this point contains non-DNA organic and inorganic material including humic acid, cell debris and proteins. For best DNA yields and quality, avoid transferring any of the pellet.
13. Shake to mix Solution C4 and add 1200 µl to the supernatant. Vortex for 5 s.
    **Note:** Solution C4 is a high-concentration salt solution. Since DNA binds tightly to silica at high salt concentrations, this will adjust the DNA solution salt concentrations to allow binding of DNA, but not non-DNA organic and inorganic material that may still be present at low levels, to the MB Spin Columns.
14. Load 675 µl onto an MB Spin Column and centrifuge at 10,000 x *g* for 1 min. Discard flow-through.
    **Note:** DNA is selectively bound to the silica membrane in the MB Spin Column device in the high salt solution. Contaminants pass through the filter membrane, leaving only DNA bound to the membrane.
15. Repeat step 14 twice, until all of the sample has been processed.
16. Add 500 µl of Solution C5. Centrifuge for 30 s at 10,000 x *g*.
    **Note:** Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the MB Spin Column. This wash solution removes residual salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane.
17. Discard the-flow through. Centrifuge again for 1 min at 10,000 x *g*.
    **Note:** This flow-through fraction is non-DNA organic and inorganic waste removed from the silica MB Spin Column membrane by the ethanol wash solution. The second spin removes residual Solution C5 (ethanol wash solution). It is critical to remove all traces of wash solution because the ethanol in Solution C5 can interfere with many downstream DNA applications such as PCR, restriction digests and gel electrophoresis.
18. Carefully place the MB Spin Column into a clean 2 ml Collection Tube. Avoid splashing any Solution C5 onto the column.

19. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you can use sterile DNA-free PCR-grade water for this step (cat. no. 17000-10).
**Note:** Placing the Solution C6 (sterile elution buffer) in the center of the small white membrane will make sure the entire membrane is wet. This will result in a more efficient and complete release of the DNA from the silica MB Spin Column membrane. As Solution C6 passes through the silica membrane, DNA that was bound in the presence of high salt is selectively released by Solution C6 (10 mM Tris), which lacks salt.
20. Centrifuge at room temperature for 30 s at 10,000 x *g*. Discard the MB Spin Column. The DNA is now ready for downstream applications.
**Note:** We recommend storing DNA frozen (–20°C to –80°C) as Solution C6 does not contain EDTA. To concentrate DNA, see the Troubleshooting Guide.

# Appendix C - PCR Product Purification Protocol

Quick-Start Protocol

## QIAquick® PCR Purification Kit
## QIAquick® PCR & Gel Cleanup Kit

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in ● a provided 2 ml collection tube or into ▲ a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.
3. To bind DNA, apply the sample to the QIAquick column and ● centrifuge for 30–60 s or ▲ apply vacuum to the manifold until all the samples have passed through the column. ● Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 750 µl Buffer PE to the QIAquick column ● centrifuge for 30–60 s or ▲ apply vacuum. ● Discard flow-through and place the QIAquick column back into the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min and then centrifuge.
8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

# Appendix D - Gel2K

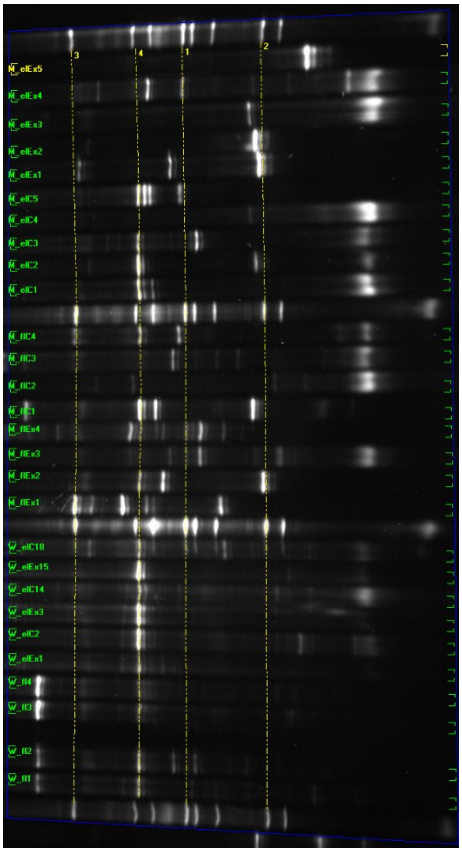

**Figure D.0.2:** DGGE profile in Gel2K. Four guidelines were manually set up to ease the marking of bands of skin mucus and water samples. The samples were from both before and after lice infection.

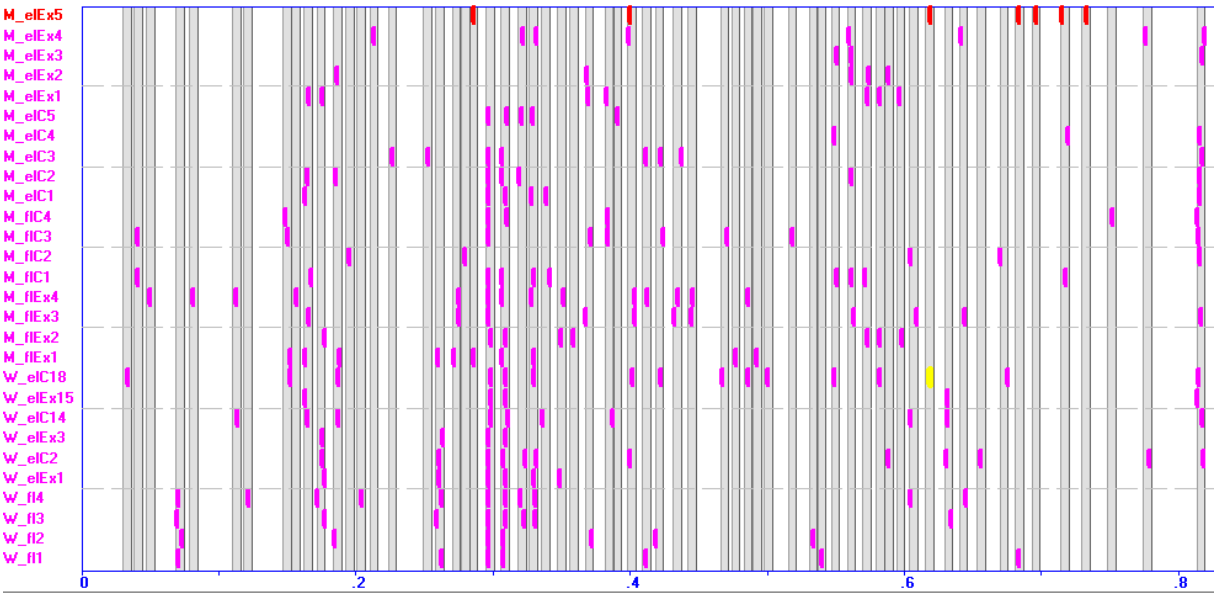


**Figure D.0.1**: Band patterns to the associate DGGE profile (Fig. D.0.2). The bands were manually aligned to separate and determine which bands belong together.

# Appendix E - Acrylamide solutions used for DGGE

**Acrylamid solution (0% denaturating):**

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

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

2.5 ml 50 x TAE (MilliQ water til 250 ml)

**Denaturating acrylamide solution (80% denaturating):**

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

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

2.5 ml 50 x TAE

84 g Urea

80 ml Deionized formamidel

# Appendix F - OTU-table

**Table 0.3:** OTU-table after normalisation to 17,5 K reads per skin mucus sample. The OTUs are sorted from the most abundant OTU to the least abundant OTU. The taxonomy is specified according to a 0.8 confidence threshold for the Usearch Sintax. The classification level and confidence threshold is shown in the brackets. C : control feed; F: functional feed.

| *#OTU ID* | *C3* | *C8* | *C9* | *F16* | *F18* | *F20* | *Taxonomy* |
|---|---|---|---|---|---|---|---|
| *OTU_7* | 0 | 3 | 5101 | 0 | 0 | 0 | Proteobacteria (p, 1.0) |
| *OTU_8* | 0 | 84 | 2987 | 0 | 1 | 0 | *Massilia* (g, 0.98) |
| *OTU_6* | 0 | 0 | 0 | 7091 | 0 | 1891 | *Streptococcus* (g, 1.0) |
| *OTU_9* | 0 | 0 | 0 | 0 | 8164 | 1 | *Curvibacter* (g, 1.0) |
| *OTU_11* | 0 | 1 | 2730 | 0 | 0 | 0 | *Pseudomonas* (g, 1.0) |
| *OTU_10* | 0 | 3081 | 0 | 0 | 0 | 0 | *Oleispira* (g, 0.9605) |
| *OTU_4* | 4236 | 1455 | 2496 | 1 | 0 | 1 | Desulfuromondales (o, 0.9801) |
| *OTU_12* | 0 | 1 | 0 | 0 | 7061 | 2106 | *Staphylococcus* (g, 1.0) |
| *OTU_14* | 0 | 2620 | 0 | 0 | 0 | 0 | Altermonadaceae (f, 0.8455) |
| *OTU_15* | 0 | 2147 | 0 | 0 | 0 | 0 | Gammaproteobacteria (c, 0.99) |
| *OTU_16* | 0 | 2109 | 0 | 0 | 0 | 0 | *Marinobacter* (g, 1.0) |
| *OTU_22* | 0 | 0 | 0 | 4168 | 0 | 0 | Pasteurellaceae (f, 1.0) |
| *OTU_21* | 0 | 0 | 1616 | 0 | 2 | 0 | *Shewanella* (g, 1.0) |
| *OTU_19* | 0 | 1808 | 0 | 0 | 0 | 0 | Gammaproteobacteria (c, 1.0) |
| *OTU_25* | 0 | 1 | 1260 | 0 | 0 | 0 | Bacteria (d, 1.0) |
| *OTU_23* | 0 | 1362 | 210 | 0 | 0 | 0 | Methylobacterium (g, 1.0) |
| *OTU_27* | 0 | 1 | 1093 | 0 | 0 | 0 | *Pseudomonas* (g, 1.0) |
| *OTU_29* | 0 | 0 | 0 | 0 | 0 | 3849 | *Corynebacterium* (g, 1.0) |
| *OTU_31* | 0 | 0 | 0 | 2306 | 0 | 0 | Bacteria (d, 1.0) |
| *OTU_30* | 0 | 1114 | 0 | 0 | 0 | 0 | *Micrococcus* (g, 0.97) |
| *OTU_32* | 0 | 0 | 0 | 1 | 2211 | 2 | *Tepidimonas* (g, 0.9801) |
| *OTU_34* | 0 | 881 | 0 | 0 | 0 | 0 | Rhodobacteraceae (f, 1.0) |
| *OTU_33* | 0 | 830 | 0 | 0 | 0 | 0 | Actinomycetales (o, 1.0) |
| *OTU_36* | 0 | 0 | 0 | 0 | 0 | 2313 | *Colwellia* (g, 1.0) |
| *OTU_38* | 0 | 0 | 0 | 1204 | 0 | 0 | *Phenylobacterium* (g, 0.9) |
| *OTU_39* | 0 | 0 | 0 | 0 | 0 | 1758 | *Lactobacillus* (g, 1.0) |
| *OTU_40* | 0 | 0 | 0 | 1049 | 0 | 0 | *Moraxellaceae* (f, 1.0) |
| *OTU_42* | 0 | 0 | 0 | 0 | 0 | 1229 | Bradyrhizobiaceae (f, 0.97) |
| *OTU_43* | 0 | 0 | 0 | 0 | 0 | 1218 | Comamonadaceae (f, 1.0) |
| *OTU_44* | 0 | 0 | 0 | 702 | 0 | 0 | *Gordonia* (g, 0.99) |
| *OTU_46* | 4816 | 0 | 0 | 0 | 0 | 0 | *Sulfurovum* (g, 1.0) |
| *OTU_47* | 0 | 0 | 0 | 614 | 0 | 0 | *Mycobacterium* (g, 1.0) |
| *OTU_48* | 0 | 0 | 0 | 0 | 0 | 949 | *Gardnerella* (g, 0.94) |
| *OTU_49* | 0 | 0 | 0 | 0 | 0 | 823 | *Lactobacillus* (g, 1.0) |
| *OTU_54* | 3423 | 0 | 0 | 0 | 0 | 0 | Neisseriaceae (o, 0.9108) |
| *OTU_56* | 0 | 0 | 0 | 0 | 0 | 702 | Rhizobiales (o, 0.86) |
| *OTU_59* | 2941 | 0 | 0 | 0 | 0 | 0 | Pasteurellaceae (f, 1.0) |
| *OTU_62* | 2084 | 0 | 0 | 0 | 0 | 0 | *Cloacibacterium* (g, 1.0) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *OTU_66* | 0 | 0 | 0 | 238 | 0 | 0 | *Bacillus* (g, 1.0) |
| *OTU_68* | 0 | 0 | 0 | 0 | 0 | 369 | *Pediococcus* (g, 1.0) |
| *OTU_73* | 0 | 0 | 0 | 0 | 0 | 289 | *Ralstonia* (g, 1.0) |
| *OTU_83* | 0 | 0 | 0 | 123 | 0 | 0 | Bacteria (d, 0.9) |
| *OTU_50* | 0 | 0 | 0 | 0 | 59 | 0 | *Corynebacterium* (g, 1.0) |
| *OTU_223* | 0 | 0 | 6 | 0 | 0 | 0 | Burkholderiales (o, 0.99) |
| *OTU_150* | 0 | 1 | 1 | 4 | 2 | 2 | *Propionibacterium* (g, 1.0) |
| *OTU_154* | 0 | 0 | 0 | 1 | 0 | 0 | Microbacteriaceae (f, 1.0) |

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