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Analytical challenges and characterization of skin and gut microbiota of Atlantic salmon fry in a commercial smolt production facility.

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Abstract

Although the Atlantic salmon is an economically important specie, little information exist on the composition of its gut and skin microbiota during early lifestages. Fish microbiota play an essential role in health of fish larvae. Initially, this study aimed at the characterization of the gut and water microbiota of salmon fries from a first feeding experiment in a research facility at Frøya. Despite our efforts to optimize the PCR protocol by applying different PCR facilitators, annealing temperatures, PCR primers, PCR cycles and DNA concentrations, we were not able to amplify 16S rDNA from this samples. This caused the study from the Frøya experiment to be discontinued. Nevertheless, processing and analysis of an Illumina sequencing data set for V4 16S rRNA gene amplicons was used to characterize the gut and skin microbiota of salmon fries of different age in three production batches sampled at three-time points (t1, t2, t3) from a commercial production farm at Follafoss (Salmar Settefisk AS). Each production batch represented fries of a distinct age group reared in different rearing systems: Production batch 1R was reared in RAS and represented the youngest fry (50 dph at the first sampling), while production batch 1A (97 dph at the first sampling time) and 5S (287 dph at the first sampling time) were reared in FTS and RAS, respectively. Sampling time represented the increase in age of fries in each production batch (first sampling time (t1): 22.02.2017, t2: 36 days after t1, t3: 63 days after t1). At the class level Betaproteobacteria, Actinobacteria and Gammaproteobacteria dominated the gut and skin microbiota. OTUs that contributed most dissimilarity between gut and skin microbiota included OTU-1(*Propionibacterium*), OTU-9 (*Zoogloea*), OUT-8 (*Delftia*), OTU-12 (*Vibrio*) and OTU-4 (*Brevinema*). *Propionibacterium*, *Zoogloea* and *Delftia* were more abundant in the skin microbiota while *Vibrio* and *Brevinema* were more abundant in the gut microbiota. The gut microbiota had a higher alpha diversity than the skin microbiota while the skin microbiota had a higher beta diversity than the gut microbiota. In the gut microbiota, older fries in the production batch 1A and 5S had a higher alpha diversity than youngest fries in production batch 1R. The skin microbiota seemed to be more influenced by the sampling time and production batch than the gut microbiota. Next, we investigated how the gut and skin microbiota varied between production batches and sampling times. The production batches seemed to influence the gut and skin microbiota more than the sampling time. The difference in skin and gut microbiota between production batches at each sampling time and between sampling time in each production batch was also studied. There was no significant difference in gut microbiota between production batches or their sampling time, although the PCoA plot suggested differences between sample groups. For the gut microbiota at t2, OTU-4 (*Brevinema*), OTU-12 (*Vibrio*), OTU-20 (*Moritella*) and OTU-1312 (*Eubacterium*) was far more abundant for the oldest fry in production batch 5S than in the younger fries within production batch 1R and 1A. Moreover, at t1 and t2, OTU-8 (*Delftia*), OTU-9 (*Zoogloea*), OTU-13 (*Acinetobacter*) and OTU-23 (*Comamonas*) were more abundant in the younger fires in production batch 1A and 1R than older fires in production batch 5S. The most abundant genera in the salmon skin microbiota were *Propionibacterium*, *Zoogloea* and *Bacillus*. There was no significant difference in skin microbiota between production batches or sampling time. This is the first study reporting a relatively high abundance of genus *Zoogloea* on the skin microbiota of a teleost. The result from our study indicates that age probably had more influence on the microbial composition of the gut and skin than the rearing systems used for the production batches. Furthermore, the gut and skin microbiota was more similar for the younger fries in production batch 1A and 1R than older fries in production batch 5S. Gut microbiota of fries in production batch 1A and 5S became more similar with increasing sampling time (t1-t2). The older fries in production batch 5S had the most variable skin microbiota among individuals at second sampling time (t2). Surprisingly, PcoA analysis indicated that both the gut and skin microbiota clustered together at t3, independent of the production batch. It was found that DNA from the samples at the third sampling time had been extracted by a separate DNA

extraction kit and probably were influenced by contaminating DNA. OTU representing *Propionibacterium* was highly abundant in these samples. This reason lead to the exclusion of results from the third sampling time when the investigated factors were discussed. This study indicates that age is a major determinant in the architecture of the gut and skin microbiota of developing salmon larvae.

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Abbreviations

1A	Fries in production batch 1A
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5S	Fries in production batch 5S
ANOVA	Analysis of variance
BP	Base pair
Br-Cs	Bray-Cutis similarity
DFF	Days after first feeding
DHA	Docosahexaenoic acid
DPH	Days post hatch
EPA	Eicosapentaenoic acid
FTS	Flow through system
LAB	Lactic acid bacteria
LC-PUFA	Long chain polyunsaturated fatty acid
NKC	Negative DNA extraction kit control
OTU	Operational taxonomic unit
PAST	Paleontological Statistics
PCoA	Principal coordinate analysis
PERMANOVA	Permutational multivariate analysis of variance
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
RAS	Recirculating aquaculture system
SIMPER	Similarity percentages
t1	First sampling time
t2	Second sampling time
t3	Third sampling time

1. Introduction

1.1 Techniques for investigation of microbial diversity

The study of teleost microbiome interaction has witnessed profound improvements these past decades due to the increase in availability of improved techniques unlocking the ever-complex nature of this believed ancient symbiotic interaction. Initial studies used culture dependent techniques. The culture based methods selectively allowed the growth of few microbial colonies, its result was inconsistent when compared to other modern culture independent methods; these inconsistencies were due to the non-culturability of some bacterial species in the so called non-selective media (Hugenholtz et al., 1998). Large fractions of the gut microbiota of Atlantic salmons have shown to be unculturable (Navarrete et al., 2009a). Culture independent technique have further improved the knowledge on the gut microbiome. They exploit the existence of the variable and conserved regions in the bacterial 16S rRNA gene to study microbial diversity and abundance. These techniques include Denaturing Gradient Gel Electrophoresis (DGGE) (Zhou et al., 2009), quantitative PCR (qPCR), terminal restriction fragment length polymorphism (tRFLP) (Llewellyn et al., 2014) and high throughput sequencing techniques such as Illumina sequencing and 454 pyrosequencing.

A major challenge in techniques used in the study of microbial community is experienced during the PCR of the target variable regions. These has been a major drawback for culture independent techniques that are PCR based. Exclusively amplifying target bacterial gene encounters problems which include; contamination, primer dimer formation, error during amplification and co-amplification of host sequence. The high sensitivity of the PCR has resulted in amplification of bacteria DNA from other sources apart from the host. This introduces contamination thereby affecting the fidelity of the sequenced data. Co-amplification of host mitochondrial (Ghyselinck et al., 2013) and nuclear DNA is also a limiting factor which might make up a large percentage of the sequenced data. In bacterial taxonomic studies, such data is not required for sequence processing and reduces the reliability of the study. Primer dimer occurs due to high primer concentration in which the hybridization of the forward and the reverse primer outcompetes the hybridization of the primer to the target gene. Surplus primers pose a problem during Illumina sequencing because

they outcompete the amplicons for hybridization to the flow cell (Michael et al., 2008). There has also been evidence of bias associated with OTU clustering when different primer set are used (Julien et al., 2015), these questions the reliability in combination and comparing microbial sequence data from variant sources using different primer set. Sequence error during amplification can affect the actual bacterial diversity (Kuczynski et al., 2011). There have also been problems associated with choice of PCR conditions such as number of cycle, annealing temperature and DNA concentration where they affect the actual abundance of most dominant taxa (Hongoh et al., 2003). Complementary techniques that are PCR independent and directly sequence the bacterial 16S rRNA has been suggested as a better strategy for resolving the demerits of PCR based techniques (Riccardo et al., 2016).

The Illumina sequencing reads and taxa composition is subject to the procedure of DNA extraction, primer selection (Kuczynski et al., 2012), sample type (Ghanbari et al., 2105), PCR amplification during the preparation of amplicon library (Siqueira et al., 2012) and storage condition of DNA extract (Hill et al., 2016). Its immense importance in host-gut microbiota studies is recognized in the wide range of taxonomic information it provides. This plethora of sequences has helped broaden the knowledge on microbial structure and diversity in the gut and skin of teleost. There are also drawbacks associated with overestimation of the microbial diversity in bacterial communities with high 16S rRNA copy number (Vetrovsky and Baldrain, 2013). The effect of varying copy number on diversity can be mitigated by normalization (Morgan et al., 2013). Furthermore, the Illumina sequencing is not reliable in functionality studies and ecological relevance involving host-microbiota (Delong and Pace, 2001). Despite the limited information on the functionality of various gut microbial colonizers, the gut microbial structure has been widely correlated to different potential functions (Ghanbari et al., 2015) such as immunity (Ramíres and Romero, 2017), lipid biosynthesis (Dailey et al., 2016), carbohydrate metabolism (Meiling et al.,2014), nutrition (Ye et al., 2014). Gnobiotic studies, transparent juveniles (Lescak and Millighan-Myhre, 2017) and the research in minimal gut genome would be vital in understanding mechanism of action of various microbial genera in isolation, in relation to the whole microbiome and identification of essential genes required for gut colonization (Qin et al., 2010). Advancement in metagenomics that tackle these challenges would be revolutionary and pivotal in relating functional relevance of a bacterial taxa to their presence in the gut (Dehler et al., 2017a) and standardize metagenomic techniques which would aid in proper comparative study.

1.2 16S rRNA as a marker gene of bacterial diversity

The 16S rRNA gene is a component of the ribosome that plays a vital role in protein synthesis. It comprises of nine hypervariable and nine conserved regions (Wang and Qian, 2009). The universality and conserved nature of the 16S rRNA gene has aided in its use as a benchmark for bacterial taxonomic and phylogenetic study (Case et al., 2007). The conserved region aids in the construction of universal primers that target specific variable regions (Vos et al., 2012). The product of these variable region accounts for the bacterial diversity. Although there have been controversies on which variable region that is most efficient in phylogenetic study, research by Yang et al., 2016 suggest the V4-V6 region offered a higher resolution in classifying bacterial taxa.

A major drawback with the use of these gene in OTU assignment is the variations in copy number in different bacterial species. These alters the actual abundance of the bacteria. Furthermore, some bacterial species 16S rRNA gene exhibit intragenomic heterogeneity, where the organism possesses variant copies of the 16S rRNA genes (Ray et al., 2010). This can introduce bias in the actual bacterial abundance by overestimating the actual bacterial specie present. Studies have shown that some variable regions are more prone to this bias than others. Sun et al., (2013) suggested that the V4 and V5 region are less prone to overestimation of bacterial taxa and the V1 and V6 experience the most bias. More so, the gene do not contain enough variability to consistently classify some bacteria at the specie and strain level (Pei et al., 2010; Caro and Ochman, 2015). The use of other genes such as RNA polymerase β subunit (rpoβ), DNA gyrase β subunit (gyrβ), Chaperonin 60 (cpn 60) have been suggested in aiding the 16S rRNA gene in bacterial taxonomic classification (Case et al., 2007; Schellenberg et al., 2009) but the information database for these genes are not well developed as the 16S rRNA. Furthermore, these alternatives have a single copy which resolves the problem of high copy number associated with the 16S rRNA gene (Coenye and Vandamme, 2003). This single copy phylogenetic marker does not have a widely conserved region that can aid in large taxonomic study, although it gives a better resolution when some specific taxa are targeted (Vos et al., 2012: Caro and Ochman, 2015). Deep sequencing techniques have been vital in classification of rare species (Rob et al., 2012). The 16S rRNA is also not reliable in studies that involve functionality and metabolic potential of the micro-organism, although attributing functions based on the 16S rRNA gene have been achieved using closely

related genomes. This method of functionality assignment is based on the strong correlation between functionality and phylogeny (Morgan et al., 2013).

1.3 Functions of teleost's gut and skin microbiota

Microbial communities colonize teleost skin, gut and gills (Wilson and Laurent, 2002; Austin, 2006; Ringo et al., 2007; Bogwald and Dalmo, 2014). They gain entry to the gut through the environment few days after hatching (Hansen and Olafsen, 1999) and further inoculate the alimentary canal via feed, water, microscopic algae (De Schryver and Vadstein, 2014) and equipment in aquaculture systems (Sharrer et al., 2005). In turn the bacteria released by the fish gut and skin might alter the microbial profile of the surrounding water. These microorganisms are majorly aerobic and anaerobic bacteria that can be autochthonous i.e. attach to the mucous membrane of the gut. Other groups of microorganisms are allochthonous which do not adhere to the mucous membrane (Beck and Peatman, 2015). The microbiota host interaction has shown to be beneficial and harmful to teleost (Vadstein et al., 1993; Vadstein et al., 2013). Interactions that can exist between these microbes and their microbial entry channels (skin, gut, gills) could be mutualistic, commensal or parasitic (Vestrum et al., 2018). Parasitic microbes are major cause of various teleost diseases (Vadstein et al., 1993; Vadstein et al., 2013). Contrarily, mutualistic interaction has been shown to be beneficial in the physiological development of fishes (Vestrum et al., 2018).

More so, microbiome homeostasis has been implicated in immunity, nutrition and physiological development. These has paved way for the interest in the research of the fish gut microbiome (Dehler et al., 2017a) with the aim of finding possible techniques to maintain healthy fishes and improve their economic viability (Merrifield et al., 2010). Presence of some indigenous gut microbes and augmented probiotic bacteria in feed and water system has been shown to possess some antimicrobial property against pathogenic bacteria (Llewellyn et al., 2014; Skjermo and Vadstein, 1999) through the production of antimicrobial peptides, organic acids, bacteriocins and free radicals (Beck and Peatman, 2015). Nutritionally these microbes provide the gut with proteolytic enzymes which aids in breakdown of nutrient and toxic agents, and they are also beneficial by producing essential vitamins that cannot be synthesized by the host (Hansen and Olafsen 1999; Xu and Gordon, 2003; Nayak, 2010). Host organ and tissue development is also

affected by these microbial communities, for example in the formation of a well-adapted alimentary canal (Stephens et al., 2016).

1.4 Factors that shape fish microbiota

Result from studies suggest that selection and changes in microbial composition in the gut of teleost is because of an interplay between the fish physiology, its physical environment, genetics and ecological factors (Wong and Rawls, 2012). Nutrition affect composition of intestinal microbes, this is seen in humans during early infancy. Exposure to breast milk favours the abundance of *Lactobacillus bifidus*, which its relative abundance in the host gut is later dominated by other microbial communities during weaning (Bergström et al., 2014). Similar scenario has been observed in Zebrafish where an adult like microbiome is attained after weaning and exposure to feed (Stephens et al., 2016). Varying feed composition expose teleost to gut surface antigens which predominantly select microbial taxa that would colonize the gut. Effect of feed on gut microbiome is seen in Atlantic salmon when exposed to soya bean based protein, which affected their gut microbial composition (Green et al., 2013). Contrary results were found in Cod larvae, where feed was not a major contributor to the gut microbial composition (Bakke et al., 2013). These discrepancies may be because of specie specific governing factors which exert a stronger selective pressure on the microbial profile of the host gut (Rasheeda et al., 2017) and skin. Studies has also shown that feed can support the proliferation of beneficial microbial communities such as lactic acid bacteria (LAB) that has been implicated in probiotic effects (Gómez and Balcázar, 2008), antibiotic resistance and inhibit proliferation of pathogenic bacteria (Do Vale Pereira et al., 2017) . These LABs can also be an essential probiont in the surrounding environment of aquaculture system where they prevent the proliferation of disease causing opportunistic bacteria (Gatesoupe, 1999). Adverse effect of some feeds has also been recorded in the gut of teleost, as seen in the administration of soya bean meal in fish feed which led to intestinal enteritis in some salmonids (Bakke-Mckellep et al., 2000; Krodaghl et al., 2015; Booman et al., 2018) and reduction of LAB on administration of vegetable meal on sea bass (Torrecillas et al., 2017a). Trophic level of a teleost can also impact gut microbial composition, where herbivorous fishes has shown to possess a higher microbial complexity (Ward et al., 2009) compared to their carnivorous and omnivorous counterpart.

A vital component of most aquafeed formulate used in rearing farmed carnivorous fishes and hatchling of herbivorous fishes (Andrew, 2010) is the dietary lipids which can be sourced from plants and animals. Most teleost also require a rich protein source which is provided by fish meal and lately by modified soy protein. The fish meal is produced from dry and milled fish and make up 60% - 72% of most farm feed (Seafish, 2016) and about 18% in Atlantic salmon feed (Ytrestøyl et al., 2015). Research on plant based protein sources such as soybean (Zhao et al., 2016), cottonseed (Anderson et al., 2016) has also been conducted. Dietary lipids have been found to be essential in immunity and growth of Pacific white shrimp (Zhang et al., 2014). A major source of such lipids is the fish oil which is made from compressed cooked fish (Seafish, 2016), it makes up about 11% of Norwegian salmon feed (Ytrestøyl et al., 2015). Fish oil contains the essential long chain polyunsaturated fatty acid (LC-PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that are vital in brain development, vision, synthesis of cell membrane and eicosanoids (Glencross et al., 2009) such as prostaglandins, leukotrienes and thromboxane. Prostaglandins have been implicated in inflammatory responses. Despite the concentration of different PUFA in oil sources (Meiling et al., 2014) the importance of EPA and DHA cannot be undermined because these essential lipids are produced in limited amount by the host (Tocher, 2015), therefore augmentation in feed is a necessity for maximal growth performance and survival (Meiling et al., 2014).

In some case studies, replacement of some fraction of fish oil with other lipid sources did not reduce growth performance (Geay et al., 2011; Messina et al., 2013; Torrecillas et al., 2017a), suggesting there is a minimal dosage required by different teleost species (Rosenlund et al., 2016; Sissener et al., 2016). Fractional replacement of fish meal without affecting growth efficiency has also been accomplished (Glencross et al., 2010). Although minimal inclusion of essential oil in aquafeed has been difficult to achieve (Hardy et al., 2010), new formulations for certain life-stages of farmed teleost allow use of low concentrate of fish oil (Seafish, 2016). Total replacement of fish oil by plant sources without minimal essential fatty acid additives have shown to be detrimental to the growth efficiency (Le Boucher et al., 2013). Dietary lipids have the potential to alter microbial composition in vertebrates, in turn the microbiota regulate the metabolism of these lipids (Daniel et al., 2013; Semova et al., 2012: Yu et al., 2014). Due to the high demand of fish oil in aquafeed, more focus has been geared towards plant based alternatives (Ghanbari et al., 2015). Despite an inclusion of plant based sources in alleviating the burden of high market demand on

fish oil, there is still an increase in demand because of the drastic growth in the aquaculture industry (Hardy et al., 2010). In as much as plant based diet have been beneficial in their long chain saturated fatty acid and in the combat against pathogens in fishes like rainbow trout (Ingerslev et al., 2014a), there have been reports of their adverse effect in the use of vegetable oil where they reduced feeding efficiency in European sea bass (Torrecillas et al., 2017b) this has been associated with the presence of anti-nutrient (Francis et al., 2001; Gatlin et al., 2007). Metagenomic studies using the pyrosequencing technique showed that plant alternatives such as vegetable oil can cause a significant shift in the gut microbiota (Carda-Diéguez et al., 2013).

Beneficial interaction between the bacteria in the environment and the gut is essential for the survival of fishes in their habitat (Wu et al., 2012). The microbial composition of the teleost environment differs from the gut microbiota. This portrays the selectivity in gut microbe colonization (Bevins and Salzman, 2011). This could be explained by the hypothesis postulated by shimdt et al. (2015), who suggested that the environment consist of a consortium of different microbial taxa that are selectively filtered by host physiology, environmental factors such as salinity and temperature. These selective factors allow a fraction of the environmental microbes to assemble in the gut. The microbial community in the gut of teleost can reflect their environmental microbiota, as witnessed in the zebrafish which showed that the environment can be a determinant in the selective colonization of some microbial taxa, this is exhibited in the relative abundance of such bacteria in the environment occurring at similar frequency as the gut (Semova et al., 2012). Although there are differences in microbial composition in different geographically separated habitat, they have been shown to have no significant impact on gut microbiome of Atlantic salmons (Llewellyn et al., 2016), suggesting the teleost like Zebra fish and rainbow trout (Roeselers et al., 2011; Wong et al., 2013) may possess a core microbiome that is susceptible to other external cues other than their local environment. Other studies are contradictory, where despite the presence of a core microbiota significant changes in the gut microbiome of Atlantic salmon Parr were observed when bred in different rearing water (Dehler et al., 2017a). The core microbiota has been associated with protection of the gut against pathogenic bacteria (Loudon et al., 2014). Furthermore, saltwater and freshwater fishes have been shown to possess variant gut microbial composition (Lozupone and Knight, 2007). Some teleost in fresh water system, reflects more of the environmental microbiota and are less susceptible to the impact of feed (Lyons et al., 2017b).

Farmed Atlantic salmons have been selectively bred over the years for their economical valuable traits (Glover et al., 2009; Debes and Hutchings, 2014). Studies have identified sixty Single Nucleotide Polymorphisms(SNPs), that genetically differentiate aquaculture Atlantic salmons from their wild counterpart (Kaarlsson et al., 2011). Further evidence in their genetic differences has been shown in the detrimental effect of introgressive hybridization in the wild salmons, where genes are introduced by the farmed escapees to the wild groups over subsequent generations, these introduce traits to the wild group that make them less adaptive to their environment. Farmed fish are more susceptible to diseases, possibly due to practices that alter the microbiome on their mucosal surfaces (Minniti et al., 2017). Wild and farmed fishes host variant microbial community in their gut (Ramíres and Romero, 2017) which is attributed to host specific function of different microbial taxa.

Other studies have linked the presence of core microbial community in the gut to the effect of host selective pressure irrespective of the genetic background (Roeselers et al., 2011). Although the extent the host genome impacts the gut microbiota has been a controversial issue (Llewellyn et al., 2014), studies suggest host can supersede the environment as a major determinant of gut colonization (Smith et al., 2015). In other cases, clustering of gut microbiota can be because of combined effect of the feed and host (Li et al., 2017) or solely feed as seen in identical twins in mammals (Muegge et al., 2011; Zoetendal et al., 2001). Presence of high inter-individual variation in gut microbiota between same genetic group (Spanggaard et al., 2000) further complicates the study of the role of genetics, this has led to proposition of the possibility of a highly complex and stochastic process of gut colonization (Star et al., 2013).

Microbial composition in the gut of fishes can be affected by life-stage (Navarrete et al., 2009b), where various stage of teleost development favours different gut microbiome due to random and deterministic processes which could predominate at early and later developmental stages respectively (Yan et al., 2012). At the early life-stages, fishes are more prone to diseases and usually have a higher mortality (Pilar et al., 2000). In other to increase productivity, more research has been focused towards early lifestages of wild and farmed salmonids. Host related factors are prominent in selection of gut microbes during the adult stage (Forberg et al., 2016; Adam et al., 2015). This occurs in salmonids during their developmental and migratory phases they undergo

distinct shift in gut microbiota to adapt to changes in their new environment (Schmidt et al., 2015). Furthermore, gradual loss of bacterial diversity and increased interindividual variation in teleost has been associated with alimentary canal development (Bakke et al., 2015; Yan et al., 2016; Zac et al., 2016). This differ from gut microbiota of humans which possess a high interindividual variation and lower diversity during infancy that reduces and increases respectively with age (Avershina et al., 2014). In some fish species, complexity of feed could have a counter effect by increasing the bacterial diversity with age (Sullam et al., 2015).

Although most research has focused on the relationship between gut microbial profile of Atlantic salmon with various physiological conditions, and various techniques to manipulate the gut microbiota. Inadequate information still exists on the microbial composition of the gut and skin of Atlantic salmon fries during their early lifestages and how age and different aquaculture system affect fries gut and skin microbiota dynamics. This would be vital in understanding the driving forces in the gut colonization of Atlantic salmons during their early development, broaden our knowledge on previous studies which used techniques like DGGE and pyrosequencing. Furthermore, this study would highlight the major bacterial taxa of healthy Atlantic salmon fries, which can help in deciphering conditions of dysbiosis that might be associated with a disease or stress.

1.5 Developmental stages of Atlantic Salmons

Atlantic salmons spend a fraction of their lives in fresh water and the remaining fraction in the sea. The life-cycle of the Atlantic salmon begins when a matured male and female salmon from the sea return to freshwater (Atlantic salmon federation, 2017). The female spawns her eggs on a selected nest in the cool streams and the male fertilize the egg with his milt (Klemetsen et al., 2013). The female help protect the fertilized eggs from predators by covering them with gravel from the riverbed. The survival of these fertilized eggs is also dependent on the water temperature. Fertilized eggs in the riverbed develop into Alevin, which utilize the yolk sac as a food source.

The Alevin develops into fry and leave the riverbed to the upper level of the stream to feed and get air for buoyancy against water currents. Atlantic salmon fry which is about 2 to 3 inches in length grows and gain new stripes and spots which serve as a camouflage from predators (Atlantic salmon federation, 2017). At this stage of development, the salmon is called a parr, they remain in the fresh water for about 1 to 3 more years before undergoing internal physiological changes that equip them with osmoregulatory ability required for survival in the sea (Finstad and jonsson, 2001). The process of these internal changes is called smolting. Smolting develops the parr into smolt and marks the transition into the adult stage of the Atlantic salmons. The smolt migrates into the sea where they feed on fishes like herring (Thorstad et al., 2012). They mature and gain weight in the sea which would serve as an energy reserve when they return to the freshwater to spawn. Salmons that have lived in the sea for up to a year are called grilse while adults that have spawned in freshwater are called kelts.

1.6 Aquaculture systems for Atlantic Salmons

According the Norwegian aquaculture analysis 2016, aquaculture of Atlantic salmon occurs broadly in three stages, which include; egg and spawn production, smolt production and sea farming. The aquaculture systems mimic production system in the wild but with advancements that allow increase in productivity. Unlike production in the wild in which the female and male must migrate to freshwater to spawn, most aquaculture system start the production of adult fishes using already fertilized eggs which are commercially produced from harvesting eggs and milt from broodstock fish, there are also hatcheries-smolt companies which also specialize in production of smolts from eggs or fry. The smolt can act as starter for salmon production. In aquaculture systems it takes about 6 to 12 months from egg to smolt stage (The Norwegian aquaculture analysis, 2016).

Newly hatched fish fries are subjected to formulated feed under a well monitored system. Juvenile salmons can be bred in flow through systems (FTS) or recirculating aquaculture systems (RAS). These systems are usually located close to a freshwater reservoir (FAO, 2018) such as lakes and rivers. Smolting is achieved by supplying the parr with a mixture of a freshwater and water from the sea (Bergheim and Fivelstad, 2014) together with an artificial temperature and light system (Fitzgerald et al., 2009). Small size smolt (50g -70g) are produced in the flow through

system as compared to the larger sizes (140g – 170g) (Joesen, 2008) in RAS which are usually preferred because they reduce the risk of disease that may occur due to longer periods spent at the sea.

The RAS differ from the flow through system in different areas. Apart from operating at a lower flow rate, it provides better water quality and water treatment system that control temperature, pH, oxygen injection and carbon(iv)oxide removal. These parameters determine the nature of the bacterial community that colonize the water in these systems (Attramadal et al., 2011a). This in turn can affect the microbial community that colonize the gut and skin of the fish. Initially in Norway, 90% aquaculture system utilized a flow through system in the production of juvenile salmons, with a few percentage utilizing the RAS (Bergheim et al., 2009). Over the years more of the intensified FTS and RAS system has been adopted because they drastically reduce disease and mortality (Blancheton et al., 2012; Bergheim and Fivelstad, 2014). FTS can be intensified by inclusion of oxygenation, temperature, light and partial recirculating systems. Disease and mortality is a major drawback of the conventional FTS. In intensive RAS strict pathogenic control is achieved by ozonization and UV radiation. Studies has also shown that RAS system that possess UV radiation or ozonization support a more stable community than the FTS (Attramadal et al., 2011b).

A stable water microbial community in replicate tanks help control the surrounding environment of these fishes. This aids in the study of changes in the gut and skin microbiota when subjected to a host of factors. Ozone also oxidizes organic matter, removes odour and colour (Krumins et al., 2001). Furthermore, the RAS system is not subject to seasonal changes to water level as seen in FTS (Bergheim et al., 2009), this help maximize production. Although the RAS has a strong pathogen control system (Attramadal et al., 2011a), there are rare cases of disease outbreak in the RAS. A major cause of these outbreaks in the RAS is the presence of biofilms which may persist and cause diseases to the fishes when opportunistic bacteria are released (Blancheton et al., 2012). Other reasons for outbreaks may be because of high organic matter content, elevated retention time and a high population density (Sharrer and Summerfelt, 2007). The final stage of salmon production is the transfer of the smolt to the sea where they spend 14 to 24 months and attain maturity (The Norwegian aquaculture analysis, 2016). To maximize productivity, producers transfer the smolt to rectangular or circular sea cages (FAO, 2018).

1.7 Hypothesis and aims

The initial aim of this project was to study the effect of feed and host genetics on the gut microbiota through the development of Atlantic salmon fries from day 14 until 95 days after onset of exogenous feeding in an experimental recirculating aquaculture system at Frøya. The hypothesis of this work was that genetics of the fish will have a bigger effect on the gut microbiota than the feed, and that the effect of feed will decrease with age during exogenous feeding. Due to problems with getting PCR products for the bacterial 16S rRNA gene from these samples.

A second aim was introduced which was to optimize a PCR protocol for amplification of bacterial 16S rRDNA from skin and gut samples of the salmon fry. The optimization of the PCR protocol did not succeed with establishing a reliable PCR product after amplification of the 16S-rRNA gene from the Frøya samples (see Results). we had to give up on this project.

The focus of the master project was therefore changed to processing and analysing an Illumina amplicon sequencing data set (already available in the research group) for skin and gut microbiota of salmon fry in a commercial facility at Follafoss (SalMar Settefisk AS). Samples had been taken at three-time points from three production batches (1R, 1A, 5S). Each production batch represent fry of distinct age reared in a type of aquaculture system while the sampling time represent increase in age of fries in each production batch.

The third aim of this master project was therefore to characterise the gut and skin microbiota of Atlantic salmon fries from the commercial facility at Follafoss. Furthermore, the study examined the effect of age and production system on the gut and skin microbiota of salmon fries. The study will also help answer the following research hypothesis:

1 Skin and gut microbiota should differ since these organs provide different nutrient rich environments for different bacterial taxa.

2 Fries in different production batches should house different skin and gut microbial community because both rearing system and fry age differed between production batches. The separate rearing systems could influence the water microbial communities which might in turn affect the gut and skin microbiota.

3. The differences in gut and skin microbiota across production batches should increase with age due to host associated selective factors which would be expected to increase with age.

2. Materials and Methods

2.1 Biological material for the Frøya and Follafoss experiment

The fish samples analysed in this project, originated from two facilities. The first was a first feeding experiment with salmon fry in a research facility at Frøya. Gut samples were collected from wild and farmed Salmon fries at 14, 35, 65 and 93 (d.p.h) days post hatch. The second experiment was in a commercial smolt production facility (SalMar Settefisk AS) at Follafoss. Gut and skin samples from farmed salmon fries were collected from fries in three production batches (1R, 1A, 5S) at three-time points (t1, t2, t3).

2.2 The Frøya experiment

2.2.1 Experimental design

The Frøya experiment was conducted to study the role which feed and host genetics play in the composition of the gut microbiota of Atlantic salmon at their early lifestage. Two genetic groups of the fish were studied. One was the farmed strain while the other represented the wild strain. For each genetic group, fries were distributed in six tanks, and three different diets were given to the fish (two replicated tanks for each diet). The three diets were identical except for the lipid content. The lipids were vegetable oil, fish oil and phospholipid. Details of the constituents of these oil-enriched feeds and their level of abundance is shown in Appendix 5. In this study samples were only taken form tanks fed with vegetable oil and fish oil. A schematic presentation of the sampling regime is shown in Fig 2.1.

2.2.2 Sampling

For each genetic group, gut and water samples were taken at 14, 35, 65 and 93 days post hatch (d.p.h). At each sampling time, water samples were collected from both replicate tanks (i.e. Two water samples from each replicate tank) for each diet regime and ten gut samples were collected from each tank. Water was sampled by filtrating 50 mL water through a 0.22µm Dynaguard filter. The filters were stored at -20°C. The fish were anaesthetized and the gut was dissected out and

transferred to a 2mL Cryo tube and stored at - 20^{0}C. A schematic presentation of sampling regime is shown in Fig 2.1.

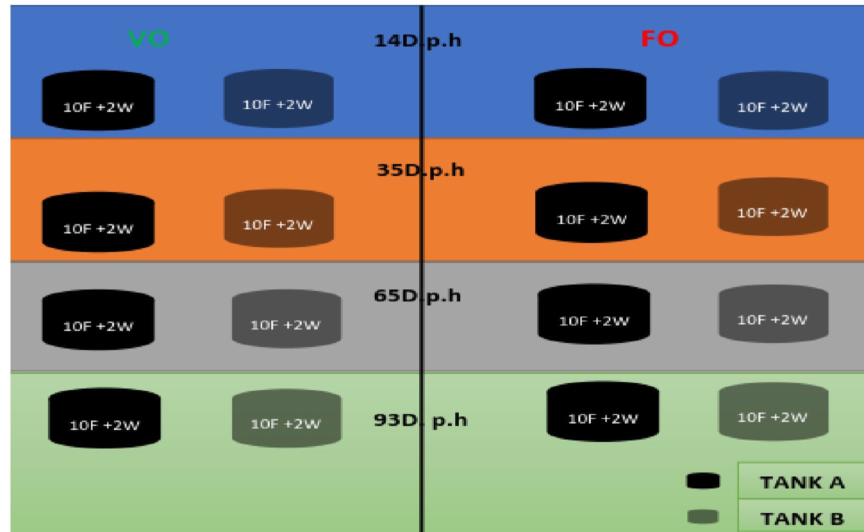

FIGURE 2.1: Schematic representation of the sampling regime from the Frøya experiment. Gut and water samples were collected at four-time points and numbers given for each time represent the number of samples taken. F: Fish, W: Water samples, VO: Diet with vegetable oil, FO: Diet with fish oil, D.p.h: Days post hatch.

2.3 Fish rearing and sampling at the commercial smolt production farm (SalMar Settefisk AS) at Follafoss

In order to study the importance different production batches and developmental stage have on the gut and skin microbiota of Atlantic salmon fry, fish was sampled in a commercial smolt production farm. Fries were sampled from three production batches (1A, 1R and 5S). Production batch 1A, 1R and 5S represented fish with different hatch date (distinct age group) and underwent their first feeding 47, 49 and 61 days after their hatch date respectively. Gut and skin samples were collected from 15 fishes in each production batch at three-time points (22-02-17 (sampling time t1), 30-03-17 (sampling time t2), and 26-04-17 (sampling time t3). Although the sample collection was on same days for all production batches, the sampling time based on the days post hatch (d.p.h) and in days after first feeding (d.f.f) differed for each production batch. At the first sampling time (t1) the age of the fries in production batch 1R, 1A and 5S were 50, 97 and 287dph

respectively. The age of fries in various production batches at other sampling time is shown in Fig 2.2. Production batch 1R and 5S representing the youngest and oldest fish fries were bred in a Recirculating aquaculture system (RAS) while fries in production batch 1A were reared in a flow through system (FTS). Details of the rearing condition and feeding rate in both aquaculture system are shown in Appendix 7. Schematic representation of the sampling design is shown in Fig 2.2.

2.3.1 Sampling

Initially, a total of 45 gut and 45 skin samples of *Salmo salar* fries were collected for the analysis of their gut and skin microbial profile. For each time point and sample type (gut or skin), samples were collected from 5 individuals for each production batch. i.e. 15 fish at three sampling time for each production batch. The gut and skin samples were analysed individually except for production batch 1R at the first sampling time (t1; representing the youngest fish), for which the skin and gut samples taken from the same individuals was pooled as one gut and one skin sample respectively because of the small amount of sample material (Fig 2.2). Therefore, a total of 40 individual samples and 1 pooled sample (5 individual) were analysed for both gut and skin samples. The fish were killed by administration of anaesthetics (Benzoak fat) and the weight of the fish was recorded. The stomach was opened with a scissors. The intestine was taken out using a pipette and the content of the intestine was scraped into a cryotube. Mucus samples from the skin of same individuals were collected by use of a scalpel. For the production batch (1R) with the smallest fish, the tails were cut off just beneath the anus prior to the sampling of the skin mucus, to avoid interference with gut bacteria. The mucus samples were also collected in cryotubes. Collected gut and skin samples were preserved by storing at -80ºC before further analysis.

FIGURE 2.2: Schematic representation of the sampling design. Forty-five salmon fries were used in the study. Fifteen fish each were distributed in three production batches (production batch 1R, 5S, 1A). Gut and skin samples were collected at three-time points (t1, t2, t3). d.p.h: Days post hatch, d.f.f: Days after first feeding, G: Gut sample, S: Skin sample, A_W: Average weight of fish.

2.4 Analytical techniques

2.4.1 DNA extraction

Total DNA were extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). DNA was extracted from the samples as described by the manufacturers protocol except for the addition of an extra step to lyse gram-positive bacteria. Detailed explanation of the extraction procedure is shown in Appendix 1. Prior to PCR amplification, DNA concentration was measured using the NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, US). The values of the measured DNA concentrations are shown in Appendix 3 for all DNA extracts.

2.4.2 DNA precipitation

For some PCR reactions used during the attempt to optimize a PCR protocol, DNA extracts were concentrated by precipitation. A volume of sodium acetate corresponding to 1/10 of the DNA sample volume were added to the DNA samples and 96% ethanol corresponding to 2 times DNA sample volume was added to the initial DNA samples. The mixture was incubated at room temperature for at least 15 minutes and centrifuged at >14,000 × g for 30 minutes at room temperature. The supernatant was discarded and the DNA pellet was washed with 70% ethanol. The supernatant was discarded after another round of centrifugation. Precipitated DNA pellet was dissolved in water in a volume corresponding to half the starting volume of DNA sample. Details of the procedure is given in Appendix 2.

2.4.3 PCR amplification

Variable regions of the 16S rRNA were amplified by polymerase chain reaction (PCR). The choice of primers was dependent on the target region of the bacterial 16S rRNA gene (Table 2.1). PCR conditions were similar for all amplifications but differed in their number of cycles and annealing temperature (Table 2). PCR reactions were performed with 5.0 U/µl of Phusion hot start polymerase (Qiagen, Hilden, Germany) and the accompanying reaction buffer (7.5 mM $MgCl_2$). Furthermore, the reaction included 10.0 µM of each primer, a final $MgCl_2$ concentration of 50.0 mM, 10.0 mM of each dNTP, 10mM of spermidine, Milli Q water and 2.0 µl of the DNA extracts was used as template. Nevertheless, some PCR conducted in this study had other template concentrations. These modifications are reported with the result section. The volume of the master mix composition used in a single PCR reaction is shown in Appendix 8.

Table 2.1: Primers, their sequences and the variable region of the 16S rRNA gene that they target. Illumina adapter sequences are not shown.

Primer	Sequence (5'-3')	16S rRNA Target gene
ILL 515F	GTGCCAGCMGCCGCGGTAA	**V4 Region**
ILL 805R	GGACTACHVGGGTWTCTAAT	
ILL 338F	ACTYCTACGGRAGGCWGC	**V3 Region**
ILL 532R	ATTACCGCGGCTGCTGG	
Eub8F	AGAGTTTGATCMTGGCTCAG	**V1- V3 Region**
ILL 532R	ATTACCGCGGCTGCTGG	

Table 2.2: Temperature cycling program used in PCR for amplifying variable regions of the 16S rRNA gene.

Step	Duration	Temperature	
Denaturation	1 minute	98°C	
Denaturation	15 seconds	98°C	X cycles
Annealing	20 seconds	X^{o}C	
Elongation	20 seconds	72°C	
Elongation	5 minutes	72°C	
Cooling	1 minute	10°C	
Storage	∞	4°C	

Annealing temperature where (X) varied between PCRs. The annealing temperature in ^{o}C (53 °^{o}C, 55 °^{o}C, 56°C, 58°C, 60°C or 63°C) is reported with the results. Number of cycles where (X) varied between PCRs, X cycles (36cycles, 38cycles) are also reported with the result.

2.4.4 Gel electrophoresis

Agarose gel electrophoresis was conducted for examining the yield and size of the PCR products using 1% agarose gel (Lonza) and × 1 TAE buffer (Appendix 3). Agarose was melted in the TAE buffer by boiling in a microwave oven and cooled before addition of 5µl of GelRed (Qiagen, Hilden, Germany) per 100ml of 1% agarose solution. The mix was poured into the gel chamber with the combs attached for well formation. After solidification of the gel, the electrophoresis chamber was filled with × 1 TEA buffer (which acts as the electrolyte). A 1kb DNA ladder (Thermo Scientific, Waltham, MA, US) was used as a gene ruler and 5µl of each PCR product was mixed with 1µl of 6× loading dye (Thermo Scientific, Waltham, MA, US) before applying the samples to the wells. The agarose gel electrophoresis was run around 1 hour at 140volts and the DNA bands were visualized by a GelDoc (SynGene) using the GeneSnap software (SynGene). For some PCR products, electrophoresis was conducted with 1.5% agarose gel at 1hour 30 minutes to properly differentiate DNA fragments of similar sizes.

2.4.5 Preparation of a 16S rRNA amplicon library for Illumina sequencing

Since the attempts to amplify bacterial 16S rDNA from the Frøya samples did not succeed, it was determined that I should analyse a data set for salmon fry gut and skin samples already available in the group. The amplicon library was prepared by Mia Tiller Mjøs as described below. A total of 75 PCR products representing 38 skins and 37 gut samples from the commercial smolt production farm at Follafoss, were included in the amplicon library that was subjected to Illumina sequencing. The samples are given in Appendix 6. Amplicons from gut and skin- mucus samples produced by the ILL515F and ILL805R primers at annealing temperature of 55°C and at 38 cycles were used in the PCR reaction. Before preparing the amplicon library sample for Illumina sequencing, the quality and yield of the PCR products were examined by running an agarose gel electrophoresis. To obtain equal amounts of each amplicon, the amplicons were normalized and purified by using a 96-well Sequelprep Normalization Plate (Invitrogen, USA) according to the manufacturers protocol (Appendix 9).

After the first normalization step, a second round of PCR amplifications was conducted to add sequence index tags to the purified amplicons. This was done to mark each amplicon with a unique index sequence, to allow for pooling of amplicons and subsequent sorting of sequence

reads according to samples. The indexing PCR was performed using the Nexetera XT index kit (Illumina) according to the manufacturers guide except for the temperature cycling program (Appendix 11). Each indexing PCR was conducted in a total volume of 25µl. The composition of the reaction mix is given in Table 2.3. The PCR temperature cycling conditions are given in Table 2.4.

Table 2.3: Composition of the PCR mix used during indexing of each sample

Component	Volume (µl)
dH_2O	11.68
5x Phusion buffer HF (7.5 mM $MgCl_2$)	5.00
dNTP (10mM each)	0.63
Phusion Hot Start DNA Polymerase (5U/µl)	0.19
Index 1	2.50
Index 2	2.50
Normalized template	2.50

The Nextera indexes contained 8 x 12 unique sequence indexes which were combined (as parts of the forward and reverse PCR primers) to get a unique combination for each amplicon.

Table 2.4: Temperature cycling program used during indexing PCR

Step	Duration	Temperature	
Denaturation	1 minute	98°C	
Denaturation	15 seconds	98°C	8 cycles
Annealing	20 seconds	50°C	8 cycles
Elongation	20 seconds	72°C	8 cycles
Elongation	5 minutes	72°C	
Cooling	1 minute	10°C	
Storage	∞	4°C	

In the next stage, a gel electrophoresis was carried out to verify sufficient yield of the new indexed PCR product. A second normalization was then done with Sequelprep Normalization Plate (Invitrogen, US) to purify and normalize the indexed amplicons. After the second normalization, the indexed amplicons were pooled by combining 20 µl eluate from each sample into a tube. An AmiconUltra 0.5 Centrifugal Filter (Merck Millipore, Ireland) was used to concentrate the pooled sample, according to the protocol provided by the manufacturers (Appendix 10). The concentration of the resulting amplicon library was measured by a NanoDrop ND-1000 spectrophotometer (ThermoFischer Scientific, Waltham, MA, US). The indexed samples were sequenced on one Illumina MiSeq lane using VX reagents for paired end reads at the Norwegian Sequencing Centre (NSC).

2.5 Bioinformatic analysis

The sequence reads provided by the NSC were demultiplexed according to their index tags to create fastq files (one for each sample). The fastq files containing the forward or reverse bacterial reads of all sample type were processed by the USERACH pipeline (version 9.2; http://www.drive5.com /usearch/). The forward and reverse reads were merged into paired reads, and the primer sequences were excluded by using the "mergepairs" function (Edgar, 2010). A 230 bp threshold was set to exclude co-amplified salmon mitochondrial 12S rRNA sequences (214bp), which was close in length to the amplified V4 region (298bp) of the bacterial 16S rRNA gene. Quality filtering of the merged reads were performed using the "fastq_filter" command with an expected error threshold of 1 (Edgar, 2010). This command converted the fastq files into fasta files. The sequences were pooled, sorted according to size, sample origin, and dereplicated. The dereplication aided in the identification of centroid sequences. Singletons (sequence reads observed only once in the entire data set) and chimeric sequences were also removed during the OTU clustering, which was performed using the command "Cluster_otus". OTU clustering was performed at 97% similarity using the UPARSE OTU algorithm (Edgar, 2013). Taxonomic assignment was performed using the "sintax" command (Edgar, 2016) with a confidence value threshold of 0.8 and RDP reference data set (version 15). The assigned OTU was aligned with the sample and the sample were mapped to the taxonomic classification.

After the processing of sequence reads in the USEARCH pipeline, an OTU table in "tab" format with taxonomic assignments was obtained. The total number of reads for each sample was calculated. The taxonomy for OTUs which were classified only at high taxonomic levels were examined further using the RDP Classifying tool (Wang et al., 2007) and NCBI Nucleotide BLAST (Altschul et al., 1997). This was done to identify and exclude OTUs that represented mitochondrial, chloroplast, fungal or salmon gene sequences. Samples whose total reads was below 1000 were also excluded from further analysis. Normalization of the proportion of each OTU was calculated by dividing the number of reads for the OTU on the total number of reads for the sample. The OTU table was normalized for analysis of beta diversity.

2.6 Statistical analysis

Analysis of alpha and beta diversity were performed using the Paleontological statistics (PAST) package version 3.19 (Hammer et al., 2001). The non-normalized version of the OTU table, containing the OTU count was used to calculate the alpha diversity of the bacterial community in skin and gut sample. The alpha diversity was evaluated by calculating the observed OTU richness, Shannon's diversity and Chao-1 index (estimated OTU richness). Significant difference in alpha diversity among groups of samples were tested by both t-test and one – way ANOVA. Similar statistical test was used to examine differences in the skin and gut community. Compared groups were considered significantly different when $p < 0.05$.

Beta diversity was examined using Bray-Curtis similarity calculated from the normalized OTU table. Principal coordinates analysis (PCoA) based on Bray -Curtis distance (Beals, 1984) were performed to compare microbial community profiles between groups of samples. Permutation multivariate analysis of variance (PERMANOVA) at a default of 9999 permutations was used to test if differences in bacterial community profiles were significantly different between groups of samples (Anderson, 2001). A group was considered significantly different from another when the Bonferroni-corrected p value was less than 0.05. Similarity percentage (SIMPER) analysis was conducted to determine OTUs responsible for the most dissimilarity between group comparisons (Clarke, 1993). The average numbers reported in the text in the Results section are reported with the standard deviation.

3. Results

3.1 Attempts to optimize a PCR protocol for the Frøya experiment

The initial aim of the project was to study the dynamics of the gut microbiota of Atlantic salmons at various time point (day 14 - day 93), under the influence of different feed on two genetically different groups. This was achieved by amplifying and sequencing DNA from the gut and water sample in the farmed and wild genetic group. The study was meant to compare and expand on the initial work done by Øygarden, (2017) on influence of genetics and the environment on the skin and gut microbiota of Atlantic salmon fry. Although it was a necessity that the study used similar methodology to set a baseline for proper comparative study, trial of the method used by Øygarden, 2017 wasn't satisfactory. This might be associated with the influence contaminating DNA had on the PCR products (Ingrid Bakke, personal communication,). Therefore, this prompted a need to improve PCR protocols.

PCR was performed to attempt the amplification of the V4 region of the bacterial 16SrRNA gene at an annealing temperature of 53ºC and 36 cycles (Fig 3.1). The gel showed that there was low amount of PCR product which was visualized as weak bands on the gel. In addition to the PCR product of expected size, there was a product of smaller size on the gel. The smaller size product was a second band below the expected product (Fig 3.1). Therefore, we tried to increase the annealing temperature to 55ºC and increase the number of cycles to 38 (Fig 3.2). This should result in higher stringency, and increase the specificity, and more cycles should give more product. Different template concentrations were also included in order to see which concentration maximizes product formation (Fig3.2). Lower amount of templates may also reduce the concentration of PCR inhibitors.

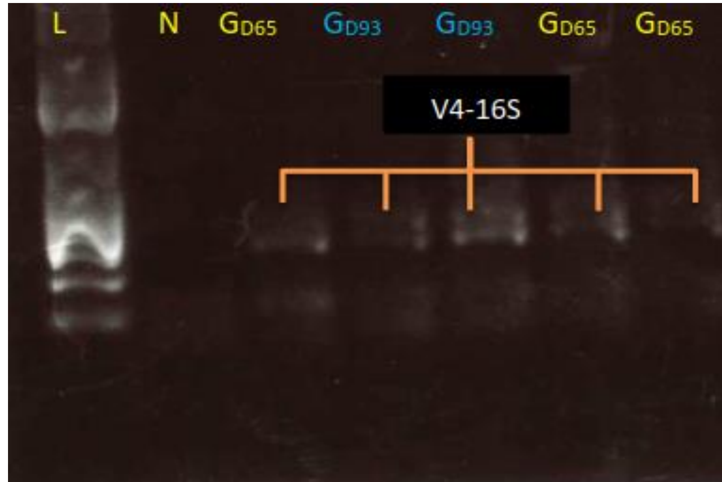

Figure 3.1: Agarose gel showing PCR products representing the V4 region of the bacterial 16S rRNA gene. PCR was performed with annealing temperature 53°C, 36 cycles and with primer ILL515F and ILL805R. Lanes are labelled as follows: L: DNA ladder, N: Non-template control, G_{D65}: gut DNA from day 65 post hatch, G_{D93}: gut DNA from day 93 post hatch, V4-16S: Amplified V4 16srRNA region. The expected DNA size of the amplified V4 16SrRNA region is about 390bp (adapter + bacteria DNA).

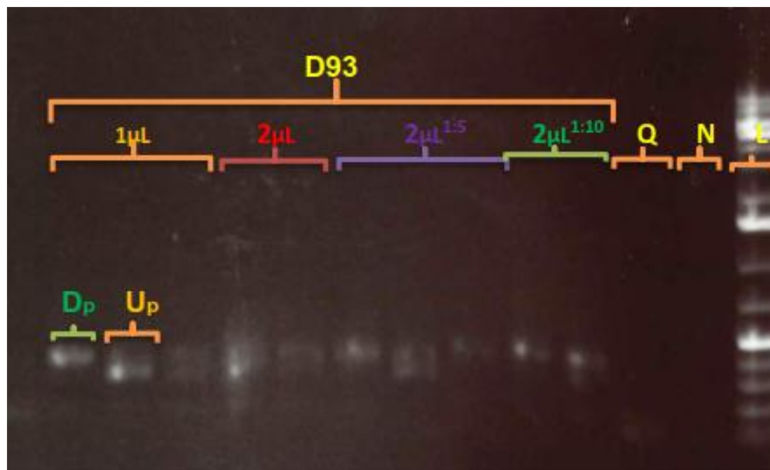

Figure 3.2: Agarose gel showing PCR products representing the V4 region of the bacterial 16S rRNA gene. PCR was performed at annealing temperature of 55 °C, 38 cycles and with the primers Ill-515F and Ill-805R. Lanes are labelled as follows: 1µL and 2µL represent PCR products obtained from 1µL and 2µL gut DNA respectively, 2µL $^{1:5}$ are PCR products from 1:5 dilution of 2µL gut DNA, 2µL$^{1:10}$ are PCR products from 1:10 dilution of 2µL gut DNA, Dp: expected product (390 base pairs), Up: Undesired product, Q: Negative kit control, N: Non-template control, L: DNA ladder. 1µL, 2µL, 2µL$^{1.5}$, 2µL$^{1:10}$ are gut samples from day 93 post hatch.

Some samples produced the expected amplicon size which is about 390 base pairs (Fig 3.2). Nevertheless, the gut samples were poorly amplified and produced weak products. There were also samples that generated smaller amplicon size than expected (Fig 3.2).

These challenges were not encountered in the PCR of DNA from water samples (Fig 3.3) which gave the expected product with high yield using the same protocol (annealing temperature 55ºC, 38 cycles). The non-template control and negative DNA extraction kit control (i.e. a control for which sterile filtered MilliQ water was used with the DNA extraction kit) resulted in PCR products, although they were weaker than the amplified water samples. Problems with contamination (Fig 3.3, Fig 3.4) in the non-template control was a reoccurring issue which was mitigated by readjusting some procedures during the post and pre-PCR steps and carefully distributing the PCR reagents in aliquots, these countermeasures helped prevent cross-contamination that may occur among users. Despite the success with the water samples, the result from the PCR of the gut samples at annealing temperature of 55ºC and 53ºC were insufficient for sequencing.

Next, we opted for precipitation of the extracted DNA before amplification. The motivation behind this approach was to concentrate and purify the extracted DNA because extracted DNA from the gut of salmons especially during their early life-stages are usually of minute concentration and have been associated with anti-PCR factors which might have originated from the fish.

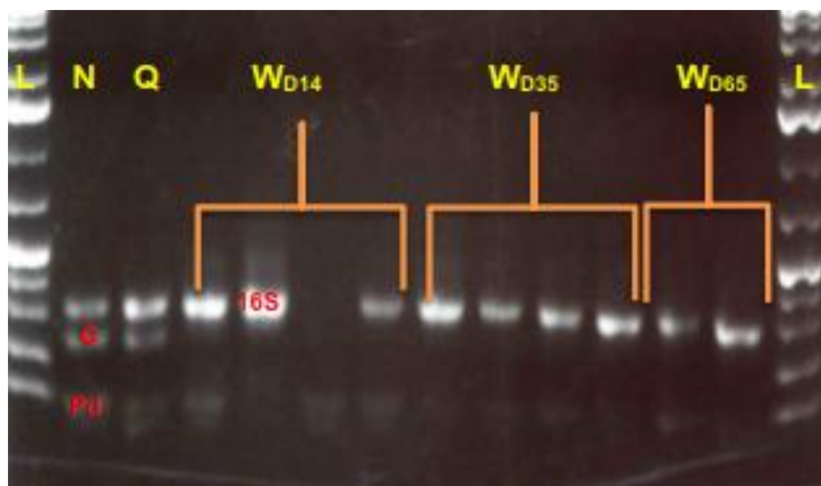

Figure 3.3: Agarose gel showing PCR products representing the V4 region of the bacterial 16S rRNA gene. PCR was performed at annealing temperature 55°C, 38 cycles and with the primers ILL515F and ILL805R. Lanes are labelled as

follows: L: DNA ladder, N: Non-template control, Q: Negative kit control, W_{D14}: bacterial DNA from water sample at day 14, W_{D35}: bacterial DNA from water sample at day 35, W_{D65}: bacterial DNA from water sample at day 65, c: contamination, Pd: Primer dimer. The expected DNA size of the amplified V4 16S rRNA region is about 390bp (adapter + bacteria DNA).

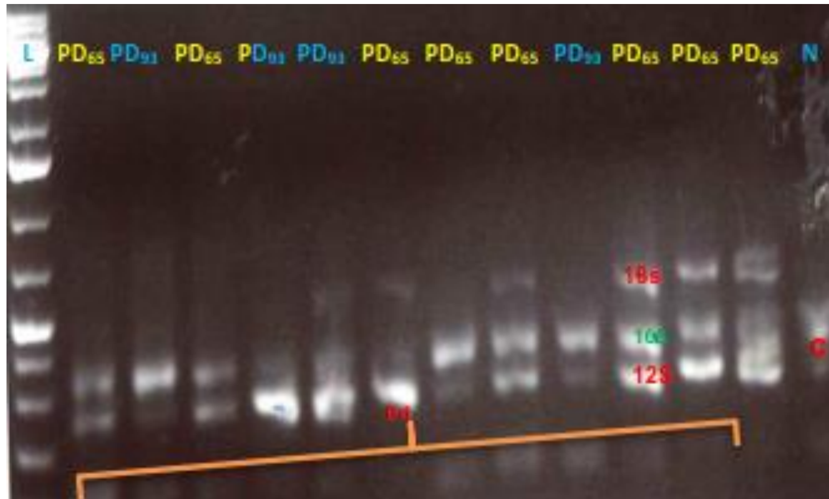

Figure 3.4: Agarose gel showing PCR products of amplified bacterial V4-16S rRNA region after precipitation of DNA extract. PCR of precipitated DNA was done at annealing temperature of 55°C, 38cycles with the primers ILL515F and ILL805R. Lanes are labelled as follows; PD_{93}: Precipitated DNA from gut at day 93, PD_{65}: Precipitated DNA from gut at day 65, L: DNA ladder, N: Non-template control, Pd: Primer dimer, C: contamination. 18S, 16S and 12S represent the amplified region of the 18SrRNA, V4 16SrRNA region and amplified region of the 12SrRNA respectively. The expected DNA size of the amplified V4 16SrRNA region is about 390bp (adapter + bacteria DNA).

Precipitation of the extracted DNA prior to amplification improved product yield, but still produced multiple PCR products. This suggests non-specific amplification of the V4 16S rRNA region. Illumina sequencing of PCR amplicons with similar banding pattern obtained for salmon samples in another project in the research group, showed that the salmon 18S rRNA (Fig 3.4) and mitochondrial 12S rRNA genes (Fig 3.4) were coamplified with the desired bacterial V4 16S rRNA gene product (Fig 3.4). This was due to the sequence homology between the primers and these eukaryotic rRNA genes. Sequencing of an amplicon product with undesired coamplified salmon gene regions could mask the actual microbial composition, thereby affecting the alpha and beta diversity after sequence processing and analysis. This prompted the trial of other primers.

A PCR with the primers Ill-338F and 532R was conducted (Fig 3.5). The forward primer has little sequence homology to the salmon rRNA gene. For the reverse primer, only a version without the

Illumina adapter sequences, needed for downstream amplicon sequencing, was available in the lab. The 338F 532R primer set amplifies the V3 region of the bacterial 16SrRNA gene.

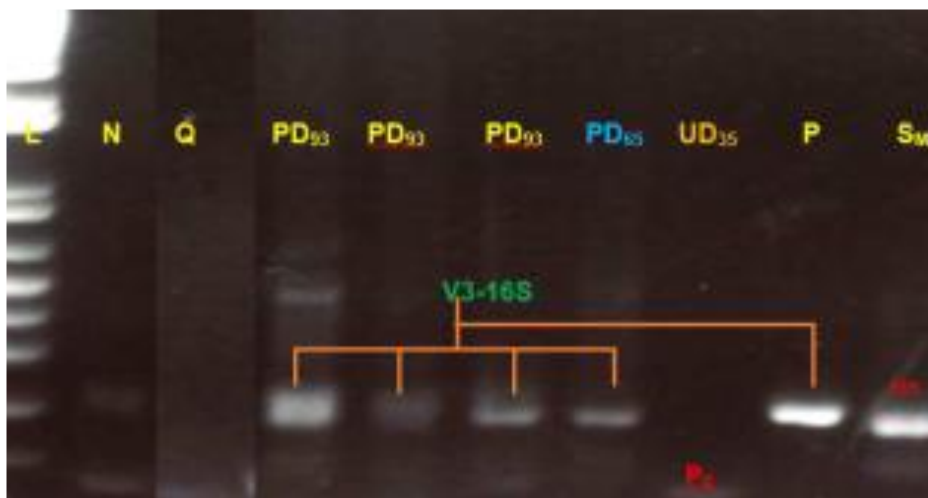

Figure 3.5: Agarose gel showing PCR product obtained with the primer ILL 338F and 532R with the following PCR condition; annealing temperature 55°C with 36 cycles. Lanes are labelled as follows: L: DNA ladder, N: Non-template control, Q: Negative kit control, PD_{93}: Precipitated DNA from gut at day 93, PD_{65}: Precipitated DNA from gut at day 65, UD_{35}: unprecipitated DNA from gut at day 35, P: Positive control, S_M: Salmon muscle, Pd: Primer dimer, V3-16S: Amplified V3 16S rRNA gene, 18S: Salmon 18S rRNA gene.

To investigate the potential co-amplification of salmon gene sequences, we also included a PCR reaction with template DNA obtained from salmon muscle, which was assumed to be bacteria free. Amplification of the homologous region of the salmon 18S rRNA gene using the Ill-338F and 532R primer was expected to produce DNA fragment of around 204bp-208bp. This was close to the size of the amplified bacterial V3-16S rRNA region (around 230 bp), and care was taken to differentiate both amplified regions by using higher agarose concentration (1.5%) and running the gel for a longer time than usual. A positive control representing bacterial DNA extracted from a *Vibrio* culture was also included. The PCR products obtained for positive control and the salmon muscle sample served as a guide to evaluate whether we amplified the bacterial V3-16S rRNA region or salmon 18S rRNA gene from the salmon fry gut samples. The absence of product formation in amplified unprecipitated DNA (Fig 3.5) further reinforced the importance of precipitation of the samples before amplification of the bacterial DNA. Agarose gel analysis of the

precipitated DNA indicated that the V3 region of the 16S rRNA was successfully amplified from the salmon gut samples (Fig 3.5).

To enable the sequencing of the bacterial V3 region, new PCR was conducted with the same PCR protocol and similar primer set as in Fig 3.5, but with Illumina adapter sequences were attached to both the forward and reverse primers i.e. ILL338F and ILL532R. Agarose gel of the amplified V3 region using the ILL338F and ILL532R (Fig 3.6) revealed no product formation, and instead a strong band representing primer dimer product (Fig 3.6) was observed for all samples. The expected product was only observed in the positive control alongside its primer dimer. With this result, gradient PCR was conducted to reduce primer dimer formation and optimize product.

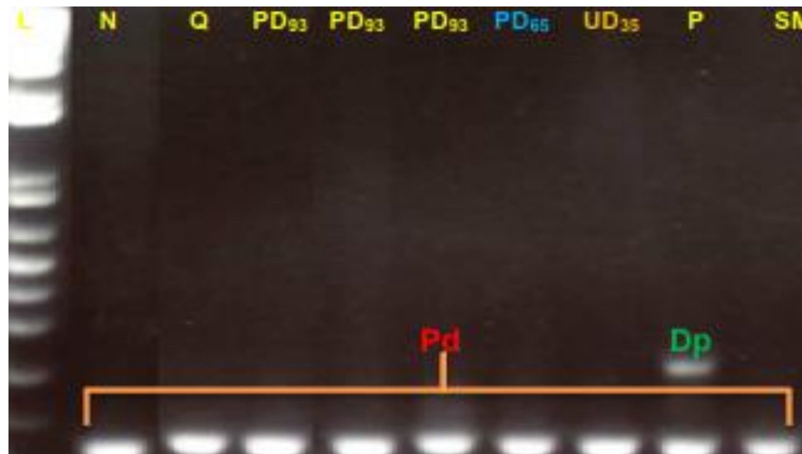

Figure 3.6: Agarose gel showing PCR product obtained with the primer ILL 338F and ILL532R with the following PCR condition; annealing temperature 55°C with 36 cycles. Lanes are labelled as follows: L: DNA ladder, N: Non- template control, Q: Negative kit control, PD_{93}: Precipitated DNA from gut at day 93, PD_{65}: Precipitated DNA from gut at day 65, UD_{35}: unprecipitated DNA from gut at day 35, P: Positive control, S_M: Salmon muscle, Pd: Primer dimer, Dp: Expected product.

Gradient PCR was conducted with increasing annealing temperature to reduce the primer dimer formation. The annealing temperature was set at 56ºC, 58ºC, 60ºC and 63ºC respectively (Fig 3.7). Analysis of the agarose gel shows that a strong band representing primer dimer formation was observed at annealing temperatures of 56ºC, 58ºC and 63ºC. The primer dimer bands were weakest at annealing temperature of 60ºC. Despite the presence of primer dimers in the positive control at all annealing temperature, the expected product was also observed (Fig 3.7). The

expected product had strong bands at 63°C while positive controls at other temperatures had little PCR product. Although an increase in annealing temperature has been shown to improve amplification specificity, gradient PCR conducted to optimize product formation did not amplify the V3 region.

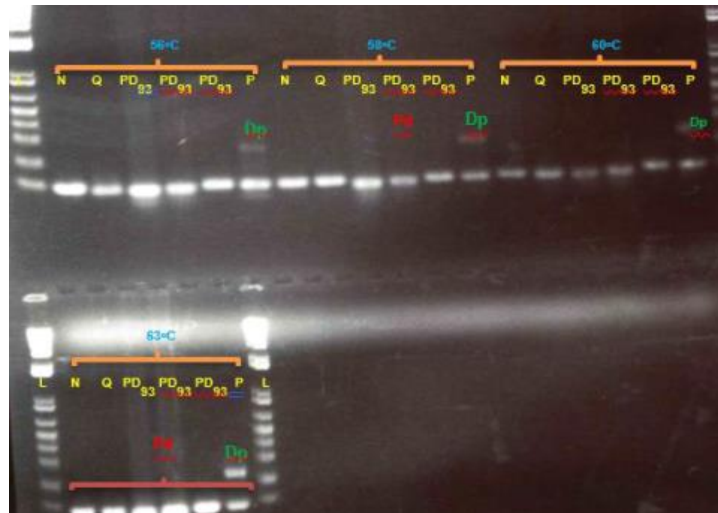

Figure 3.7: Agarose gel showing PCR product obtained with the primer ILL 338F and ILL 532R at different PCR annealing temperature of 55°C, 58°C, 60°C and 63°C all ran at 36 cycles. Lanes are labelled as follows: L: DNA ladder, N: Negative control, Q: Negative kit control, PD_{93}: Precipitated DNA from gut at day 93, Pd: Primer dimer. Dp: expected product.

The inability to explicitly amplify the V3 region with the Illumina adapted primers led to the trial of new primers; non-Illumina adapted forward primer (Eub8F) and Illumina attached 532R primers (ILL 532R). The Eub8F and 532R primers amplifies the V1- V3 region of the bacterial 16S rRNA gene which is about 530bp in length. Amplification of the V1- V3 region at 55°C annealing temperature with 36 cycles generated the expected product (Fig 3.8) and other unknown coamplified regions. The positive control and some precipitated gut samples from day 65 (Fig 3.8) generated strong bands while the gut samples from day 93 (Fig 3.8) produced weak product.

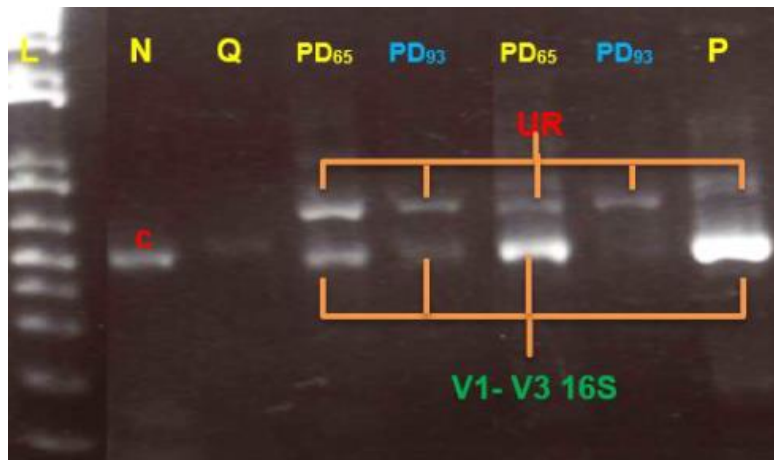

Figure 3.8: Agarose gel showing PCR product obtained with the primer EUB8F and ILL 532R at PCR condition: annealing temperature of 55°C,38 cycles. Lanes are labelled as follows: L: DNA ladder, N: Negative control, Q: Negative kit control, $PD_{93:}$ Precipitated DNA from gut at day 93, $PD_{65:}$ Precipitated DNA from gut at day 65, V1-V3 16S: Amplified V1-V3 16S rRNA gene, UR: Unknown coamplified sequence. C: contamination.

Because I was not able to get reliable PCR product from the Frøya gut samples, I continued my thesis by analysis of the samples from the Follafoss experiment. For these samples, we got reliable PCR amplification.

3.2 Illumina sequencing effort and quality of sequencing data for salmon fry gut and skin samples from Follafoss Settefisk AS

The sequencing of amplicon generated 8,047,599 paired sequence reads. The data set was processed using the Usearch pipeline, as described in 2.5. Paired reads with incomplete or no alignment made up 8.7% of the reads. An average of 69,898 paired reads per sample were shorter than 230bp and filtered out of the data set. After the exclusion of these reads, 2,595,610 reads were retained. Quality filtering of the resultant merged and trimmed reads at an E max value of 1, resulted in removal of 34,977 reads. Furthermore, 0.4% and 11% of the filtered reads were chimeric sequences and singleton respectively, and were excluded from the data set.

After this, 2,273,776 reads were clustered into 1,565 OTUs and an OTU table was generated. The gut samples accounted for approximately 70% of the reads while 27% and 3% of the reads were respectively from skin and negative DNA extraction kit control (NKC; obtained by applying sterile filtered water as input in the DNA extraction). OTUs that could not be taxonomically assigned were further examined to exclude non-bacterial OTUs. Analysis of OTUs with NCBI Blast (Altschul et al.,1997) and RDP classifier (Ribosomal data project Wang et al., 2007) led to the exclusion of 13 non-bacterial (fungal, chloroplast and mitochondrial) OTUs in the gut and skin samples. These OTUs were found to represent mainly fungal, mitochondrial, and chloroplast rRNA genes. After the removal of the non-bacterial OTUs, 1547 OTUs were left for all samples, and the total number of reads for all samples was 1,720,264. The gut samples accounted for approximately 63% and the skin accounted for 33% of the reads. The number of reads for individual samples is shown in Appendix 6.

Samples (1 gut and 8 skin) with less than 1000 reads were excluded from further analysis (Appendix 6). Finally, the average number of reads for gut and skin samples were 33780 ± 18964 (Mean ± SD) and 21739 ± 23621 (Mean ± SD) respectively. Table 3.1 shows the average reads of gut and skin samples for the three production batches at the three-sampling time points.

Table 3.1 Average numbers of sequence reads for sample groups. Values represent mean ± standard deviation. Production batches with a single sample are represented by only their mean. The number of samples is given in parenthesis for each reported average number of reads.

Sampling time / Production batch	t1 Gut	t2 Gut	t3 Gut	t1 Skin	t2 Skin	t3 Skin
1A	12967 ± 11931 (4s)	39652 ± 6717 (4s)	41841 ± 8589 (4s)	51162 ± 29855 (4s)	7072 ± 2713 (2s)	13820 ± 5528 (4s)
1R	4819 (1s)	3579 ± 2885 (3s)	41277 ± 13580 (4s)	1495 (1s)	6540 ± 70840 (4s)	21725 ± 26065 (4s)
5S	34703 ± 16062 (4s)	45960 ± 16565 (4s)	49955 ± 14427 (4s)	69251 (1s)	13751.0 ± 17899 (3s)	22031 ± 14484 (3s)

3.3 Gut and skin microbiota associated with Atlantic salmon fries at Follafoss Settefisk AS

3.3.1 Alpha diversity of gut and skin microbiota

The alpha diversity of the skin and gut samples was determined by estimating the observed OTU richness, Shannon's diversity and Chao-1 index (estimated OTU richness). The total Chao-1 index and observed OTU richness for the gut samples was 1418 ± 48 and 1281 ± 42, respectively, while that of the skin sample was 1001 ± 54 and 849 ± 53. These values suggest the sequencing effort covered 90% and 85% of the estimated richness for individual gut and skin samples respectively (Fig 3.9). For each production batch, the average observed OTU richness and Chao-1 index increased overtime (Fig 3.9), except for the skin production batch 1R (Fig 3.9). Difference in OTU richness between sampling times (ANOVA, $p > 0.05$) for either gut or skin samples was not statistically significant except for skin samples from production batch 1R, whose decrease was significant (ANOVA, $p = 0.00001$). The skin sample had a higher inter-individual variation in

richness within production batches than their gut counterparts. The gut microbiota in production batch 5S and 1A had significant higher richness (observed OTU richness and Chao-1 index) than production batch 1R (t-test, $p < 0.05$). Also in skin samples, the difference in richness between skin samples in production batch 1A and 1R was significant (t-test, $p < 0.05$). These observations propose that OTU richness in gut and skin samples increased with age. Furthermore, the observed OTU richness and Chao-1 index for the gut was higher than the skin samples. The difference was statistically significant for observed OTU richness (t-test, $p = 0.01$) and Chao-1 index (t-test, $p = 0.02$). This advocates that the gut of fries houses a more species richer microbial community than the skin.

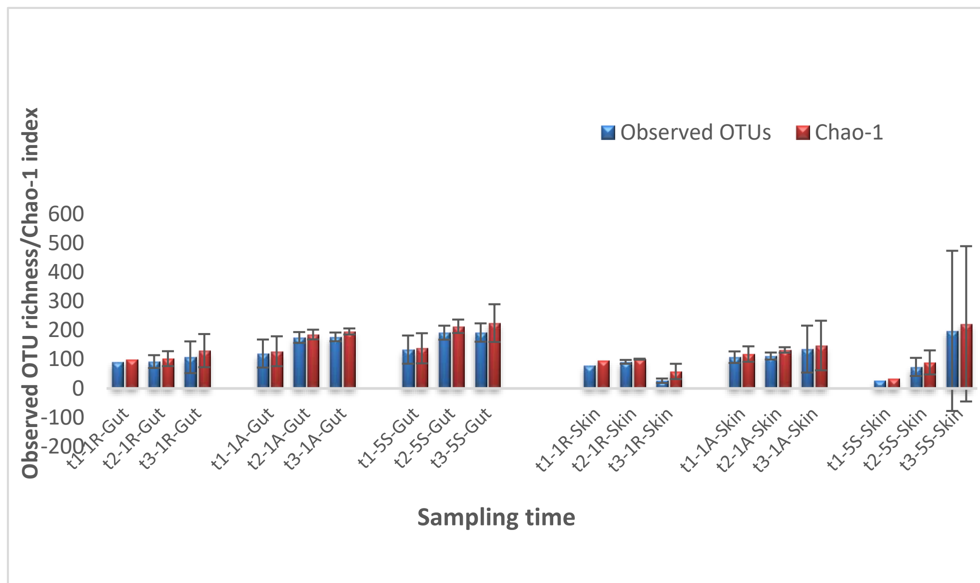

Figure 3.9: Average observed OTU richness and the Chao-1 index of the gut and skin microbiota from the three production batches (1R, 1A, 5S) at the three sampling times (t1, t2, t3). The error bars represent the standard deviation. Bars with no error bars represent production batches that had one gut or skin sample.

Analysis of the Shannon's diversity which takes in into account both the richness and evenness shows that the Shannon's diversity of the skin and gut samples in various production batches varied over time (Fig 3.10), although there was no clear trend. The Shannon's diversity of the gut samples was significantly higher than the skin samples (t- test, $p = 0.04$). This entails that the gut microbiota of the Atlantic salmon fries possessed a higher alpha diversity than their skin microbiota.

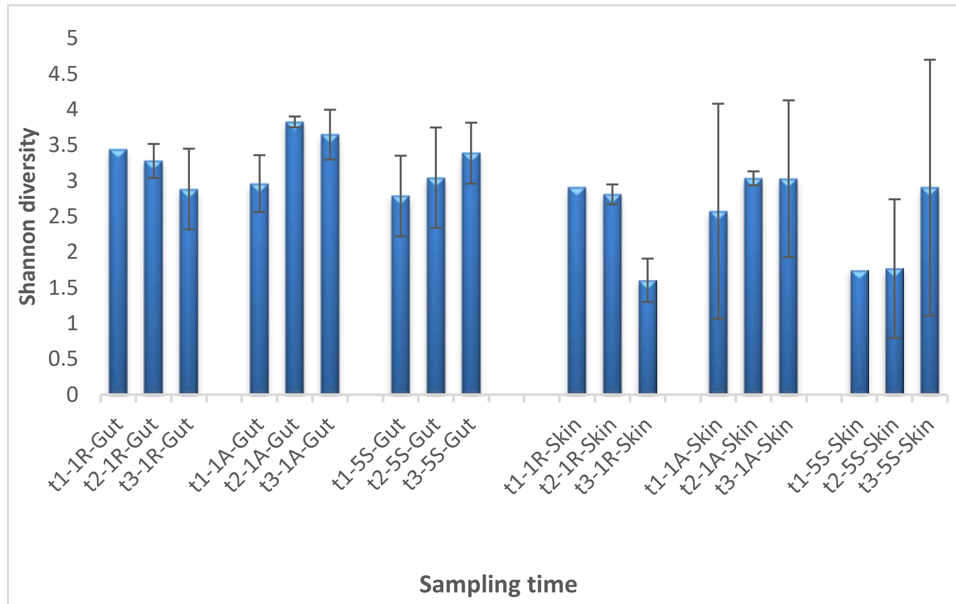

Figure 3.10: Average Shannon's diversity of the gut and skin microbiota from three production batches (1R, 1A, 5S) at three sampling times (t1, t2, t3). The error bars represent the standard deviation and bars with no error bars are production batches that contain one gut or skin sample.

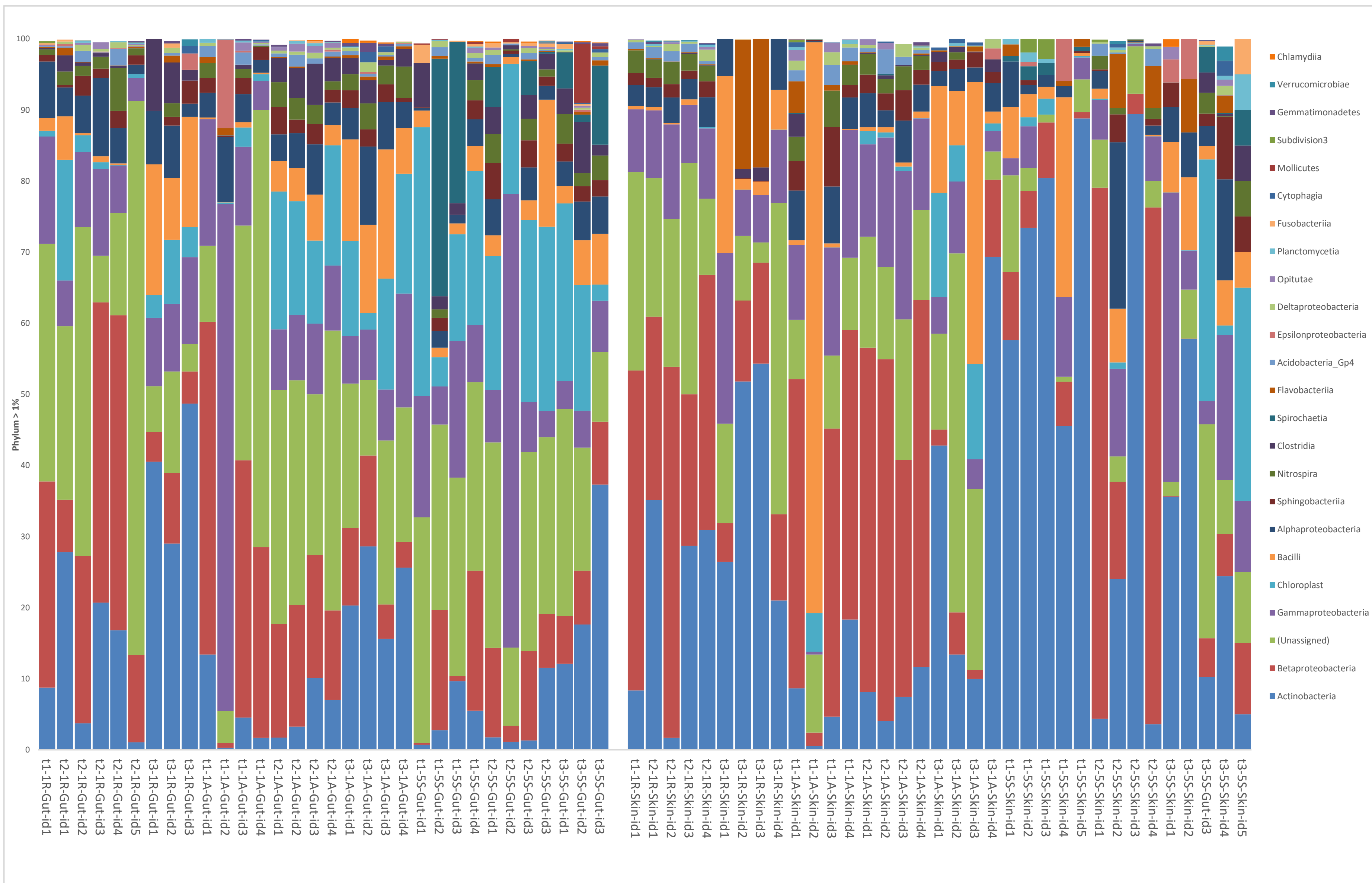

Figure 3.11: Relative abundance of the bacterial taxa at the class level in all individual gut and skin samples. Bacterial class with less than 1% abundance in all samples were excluded. The figure is based on the original OTU table constructed in Usearch, without removal of the 13 most abundant non-bacterial (fungal, chloroplast and mitochondrial). t1: first sampling time, t2: second sampling time, t3: third sampling time, 1R: RAS production batch with youngest fries, 1A: fries in FTS production batch, 5S: RAS production batch with oldest fries, id: individual.

3.3.2 *Composition of skin and gut microbiota*

The gut and skin samples were dominated by 24 bacterial phyla. Of these phyla, 21 were shared between both samples, although their relative abundances differed in the gut and skin samples. At the class level the gut and skin microbiota were clearly different and changed over time (Fig 3.11). Although Betaproteobacteria, Actinobacteria, Alphaproteobacteria and Bacilli were more abundant in the skin sample than the gut sample, only difference in Actinobacteria abundance was significant (t-test, $p = 0.002$). Phyla Gammaproteobacteria and Fusobacteria appeared to be more abundant in the gut than the skin samples, however this difference was not statistically significant (t-test; $p > 0.5$).

Gut samples were dominated by the phyla Proteobacteria (32.81%), Actinobacteria (13.34%) and Firmicutes (7.84%). Betaproteobacteria (14.96%) was the most abundant bacterial class in the gut samples followed by Actinobacteria (13.44%) and Gammaproteobacteria (12.86%). Gut samples in all production batches had a temporal increase and decrease in abundance of Actinobacteria and Betaproteobacteria respectively. At the genus level the gut microbiota was mostly dominated by *Propionibacterium* (11.93%) followed by *Zoogloea* (5.19%) and *Brevinema* (2.73%). There was also presence of OTUs classified as chloroplasts which was more abundant in the gut samples than the skin samples. This could be attributed to the plant constituent of the fish feed, and co-amplification of chloroplast rDNA with the bacterial 16S rRNA gene from microbes on the skin and gut samples. The unassigned OTUs, which were abundant in all samples, comprised majorly of OTUs probably representing fungal and mitochondrial rRNA genes. These OTUs and the chloroplast OTUs were excluded from the OTU table prior to further statistical analysis as described in 2.5.

The phylum Proteobacteria (37.90%), Actinobacteria (30.79%) and Firmicutes (8.28%%) were also the most abundant phyla in the skin. At the class level the skin samples were dominated by Actinobacteria (30.20%), Betaproteobacteria (29.99%) and Gammaproteobacteria (10.16%) (Fig 3.11). Lastly, the genera *Propionibacterium* (24.51%), *Zoogloea* (8.90%) and *Bacillus* (3.63%) dominated the skin microbiota of salmon fries. The skin microbiota underwent temporal changes in the microbial composition. Skin samples from younger fries in production batch (1A and 1R) experienced an increase in abundance of Actinobacteria, but both Betaproteobacteria *and*

Nitrospira decreased overtime. Skin samples from oldest fries in production batch 5S showed a different trend, where abundance of both Actinobacteria and Betaproteobacteria decreased but the relative abundance of Nitrospira increased with time.

3.3.3 Comparison of skin and gut microbiota

A PCoA plot based on Bray-Curtis similarity was generated (Fig 3.12) to compare all gut and skin microbial communities. The first two axes explained 43.80% of the variation in the data. The pattern of variation in the ordination plot showed that some gut samples clustered somewhat differently from that of the skin samples, although there was overlaps between sample groups. The difference in community composition between the gut and skin samples was shown to be statistically significant (PERMANOVA, p = 0.0098).

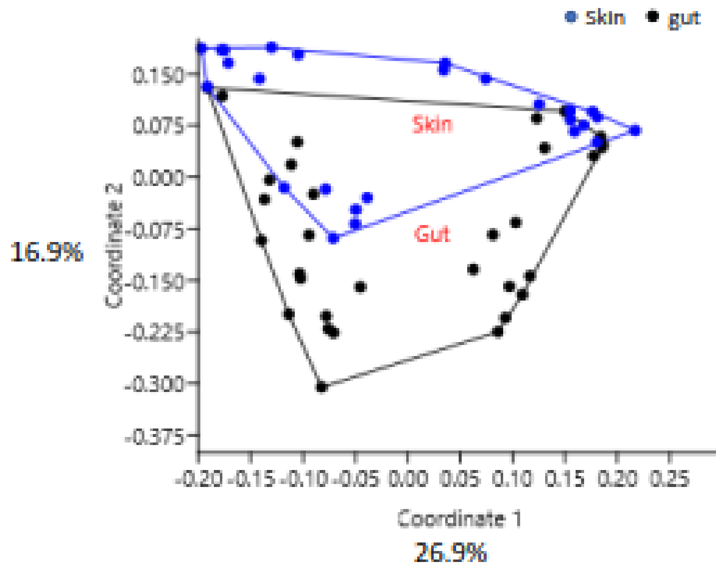

Figure 3.12: A PCoA plot showing the variation in skin and gut microbiota among samples irrespective of production batch (1R, 1A, 5S) and sampling time (t1, t2, t3). The ordination was based on Bray-Curtis distances. Percentages on axis indicate how much of the total variance that were explained by the two coordinates.

The Bray – Curtis values further establishes that the skin samples (Br-C = 0.26 ± 0.23) has a higher beta diversity than the gut samples (Br-C = 0.30 ± 0.19) which was statistically significant (t-test, p = 0.020). The large standard deviation of the between group comparison shows that individual gut and skin Bray-Curtis similarity differs extensively (Fig 3.13). In the diagram, there is also high

standard deviation for comparisons within groups. This indicates that there is large variation in community composition among individuals.

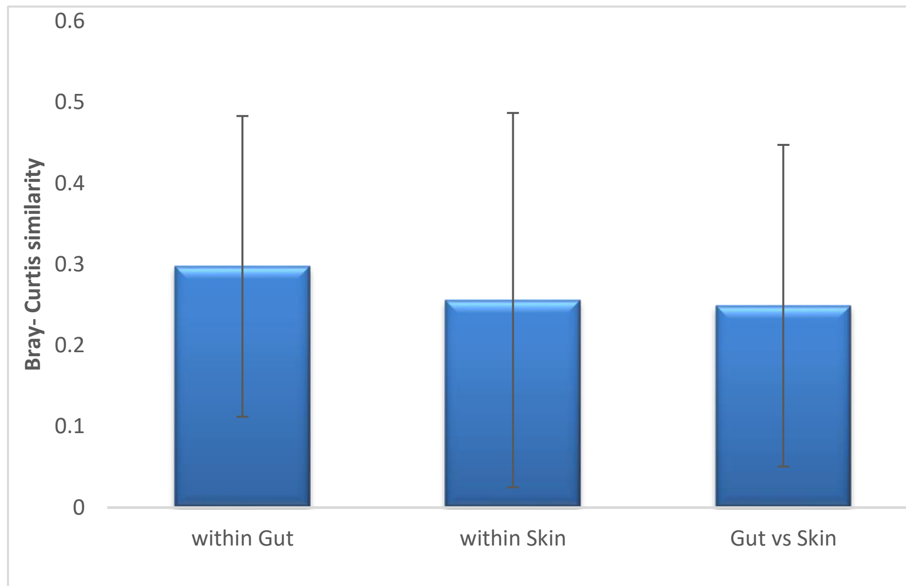

Figure 3.13: Average Bray-Curtis similarities for comparison of gut and skin communities among samples, within and between sample groups. Error bars represent standard deviation of mean Bray-Curtis value.

The OTU that contributed to most of the difference between the gut and skin community profiles was identified by the similarity percentage (SIMPER). Sixteen OTUs were responsible for 50% of the Bray -Curtis distance between the skin and the gut samples. The OTU that explained most of the Bray-Curtis distance (13.38%) between gut and skin communities was OTU-1, representing *Propionibacterium* (Class: Actinobacteria). This OTU was more abundant in the skin (22.80%) than the gut samples (15.50%). The OTU that contributed to the second most difference (7.46%) between the gut and skin microbiota was OTU-9, representing the genus *Zoogloea.* This OTU was also more abundant in the skin (10.40%) than the gut (6.70%) microbiota. OTU-12 and OTU-14 representing *Vibrio* and *Brevinema* were more abundant in the gut microbiota (3.88%), (4.04%) than the skin microbiota (1.16%), (5.46E-03%). Table 3.2 reports the 5 OTUs contributing most to the dissimilarities between gut and skin samples.

Table 3.2: OTUs that contributed most to the dissimilarity between the gut and skin microbiota. Analysis was made by SIMPER which also estimated the mean abundance of these OTUs in the gut and skin samples**.**

OTU ID	Taxa (Genera)	Taxa (Class)	Amount of dissimilarity (%)	Mean abundance in gut (%)	Mean abundance in skin (%)
OTU -1	*Propionibacterium*	Actinobacteria	13.38	15.50	22.80
OUT- 9	*Zoogloea*	Betaproteobacteria	7.46	6.70	10.40
OTU -8	*Delftia*	Betaproteobacteria	4.89	6.15	7.46
OTU -12	*Vibrio*	Gammaproteobacteria	2.99	3.88	1.16
OUT-4	*Brevinema*	Spirochaetia	2.69	4.04	5.46E-03

3.4 The Gut Microbiota

3.4.1 *Comparison of gut microbiota between production batches*

Gut microbiota from different production batches were compared in a PcoA plot (Fig 3.14a-d). There was a large degree of overlaps between groups (Fig 3.14a). A one-way PERMANOVA test showed that there was a no significant difference for comparisons between production batches (PERMANOVA, $p < 0.05$) at any sampling time (t1, t2, t3). However, when doing PcoA analysis, the gut microbiota differs between production batches at each sampling times (Fig 3.14b-d). These observations might indicate that the gut microbiota of salmon fries could be influenced by the production batches (1R, 1A, 5S).

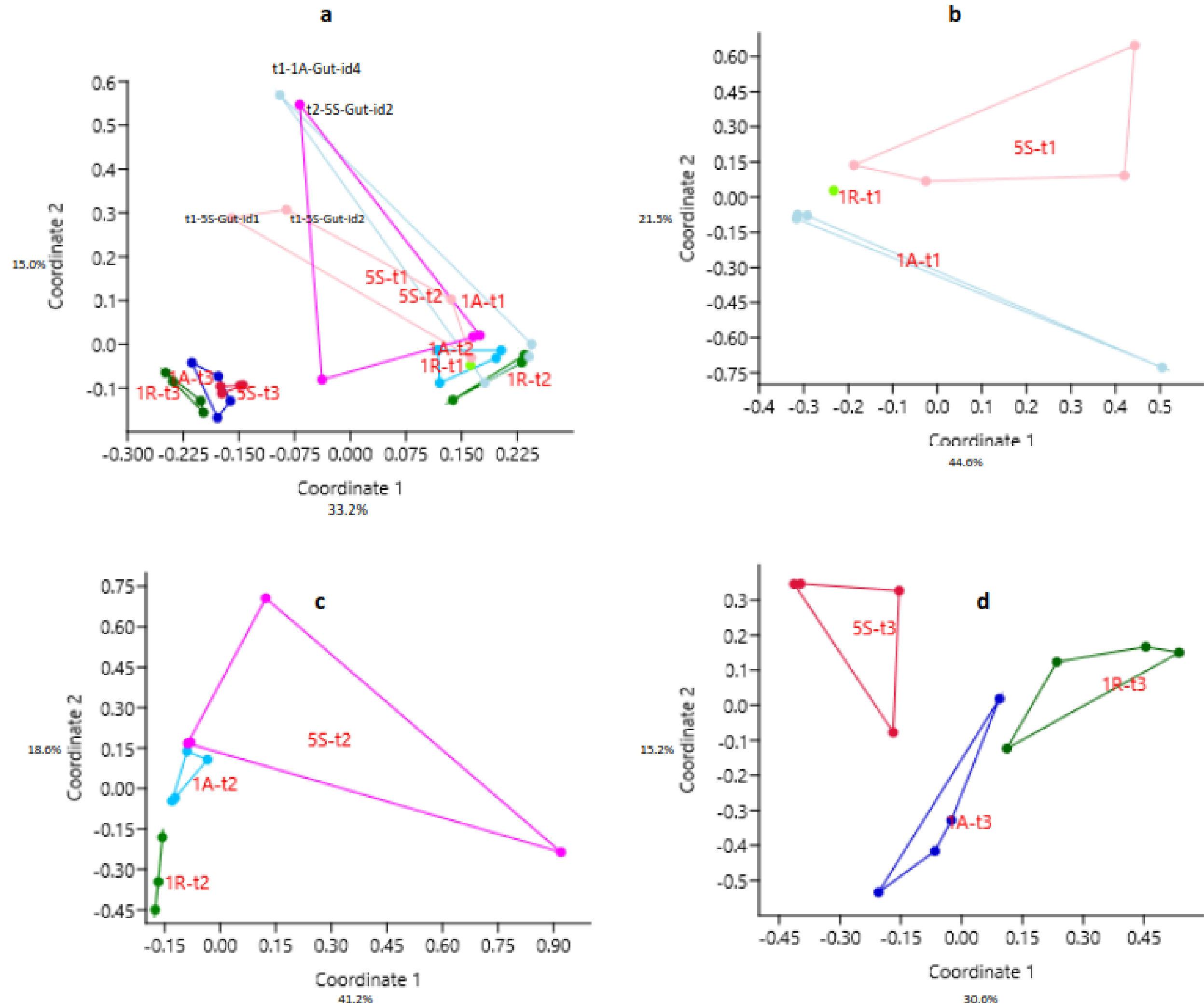

Figure 3.14: PCoA plots showing the variation in individual's gut microbiota between three production batches (1R, 1A, 5S) at three sampling times (t1, t2, t3). Green circles: Fries in production batch 1R. Blue circles: Fries in production batch 1A. Red circles: Fries in production batch 5S. Darker versions of the above-mentioned colours represent fries in same production batch but at a new sampling time. **a:** For all sampling time (t1, t2, t3) the variation in individual's gut microbiota between three production batches (1R, 1A, 5S) **b:** Variation in individual's gut microbiota between three production batches (1R, 1A, 5S) at the first sampling time (t1). **c:** Variation in individual's gut microbiota between three production batches (1R, 1A, 5S) at the second sampling time (t2). **d:** Variation in individual's gut microbiota between three production batches (1R, 1A, 5S) at the third sampling time (t3). The ordination plot was based on Bray-Curtis distances. Percentages on axis indicate how much of the total variance that were explained by the two coordinates.

The average Bray-Curtis similarity (Br-Cs) compared gut microbial communities within and between production batches at three sampling times (Fig 3. 15). At the first and second sampling time, gut microbiota of younger fries was more similar between production batch 1A and 1R (t1: Br-Cs= 0.45 ± 0.29, t2: Br-Cs= 0.55 ± 0.03) and distinct from fries in production batch 5S. Also, at both t1 and t2 there was lower interindividual similarity in gut microbiota for older fries in production batch 5S (t1: Br-Cs= 0.24 ± 0.16, t2: Br-Cs= 0.36 ± 0.31). Nevertheless, at the third sampling time (t3) a different trend was observed, where fries gut microbiota in all production batches became more similar, irrespective of distinct age group (1R: Br-Cs= 0.51 ± 0.07, 1A: Br-Cs= 0.49 ± 0.05, 5S: Br-Cs= 0.55 ± 0.19).

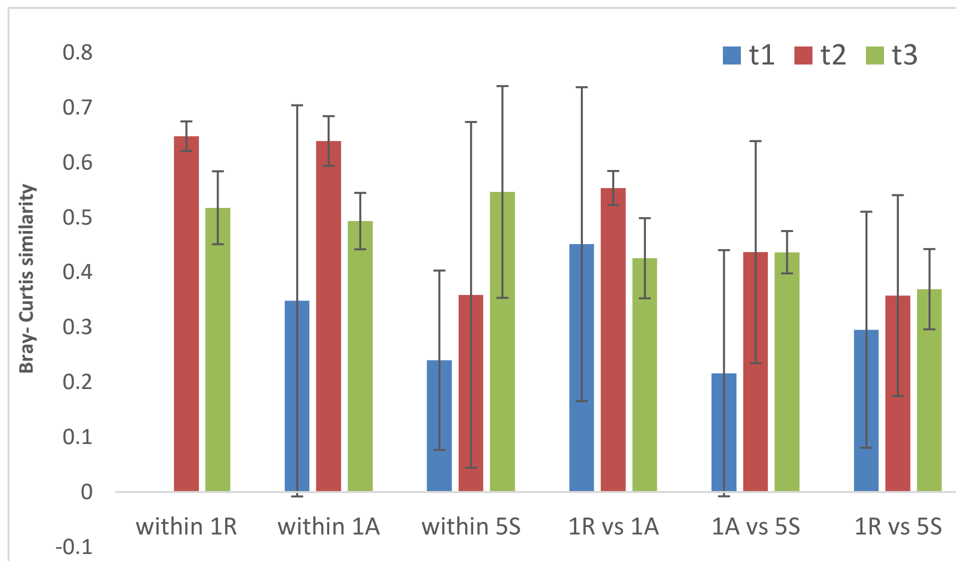

Figure 3.15 Average Bray-Curtis similarity for comparisons of gut microbiota within and between the three production batches (1R, 1A, 5S) at each sampling time (t1, t2, t3). Error bars represent standard deviation of mean. Label with no bar represent production batches with only one sample.

At the first sampling time (t1), SIMPER analysis showed that OTU-4 was responsible for the most difference (11.54%) between fries in all production batches (1R, 1A, 5S). OTU-4 which represents the genus *Brevinema* (Class: Spirochaetia) was far less abundant for the younger fries in the production batch 1R (0.02%) and 1A (0%) than the older fries in production batch 5S (20.30%). Similar trend was also observed at the third sampling time where OTU-4 accounted for the second most dissimilarity and was more abundant in the oldest fries with production batch 5S (7.75%) than younger fries in production batch 1A (2.74E-3%) and 1R (0%). Also, OTU-8, which represent genus *Delftia* (Betaproteobacteria), was responsible for the second most dissimilarity (8.44%) between gut microbiota in all production batches at t1. This OTU was more abundant in the younger fries with production batch 1R (17.0%) than the older fries in production batch 1A (15.30%) and 5S (4.48%).

At the second sampling time (t2) OTU-9, which represent the genus *Zoogloea* (Class: Betaproteobacteria), was responsible for the most dissimilarity (8.63%) in gut microbiota between production batches. This OTU was more abundant in the youngest fries in production batch 1R (20.6%) than the older fries in production batch 1A (9.4%) and 5S (5.4%). In figure 3.14, there are some samples (t1-1A-Gut-id4, t1-5S-Gut-id1, t1-5S-Gut-id2, t2-5S-Gut-id2) that appears to differ from the others. These outliers were found to have a higher abundance of OTU-12 (genus *Vibrio*) than the rest of the gut samples. Same OTU was responsible for the second most dissimilarity (6.51%) in gut microbiota between production batches at the second sampling time (t2). This OTU was more abundant in the oldest fires with production batch 5S (9.20%) than their younger counterpart in production batch 1A (1.57%) and 1R (0.1%). At the third sampling time (t3) OTU-1 representing *Propionibacterium* (Class: Actinobacteria) accounted for the most difference (10.86%) in skin microbiota between production batches. OTU-1 was more abundant in the youngest fries with production batch 1R (40.90%) than older fries in production batch 1A (26.80%) and 5S (22.40%). Furthermore, at t1 and t2, OTU-20 (*Moritella*, Gammaproteobacteria) and OTU-1312 (*Eubacterium, Bacilli*) were more abundant in the older fries with production batch 5S (OTU-20; t1:3.11%, t2:8.84%) (OTU-1312; t1:4.23%, t2:3.08%) than in younger fries with production batch 1A (OTU-20; t1:0%, t2:0.001%) (OTU-1312; t1:0.03%, t2:1.13%) and 1R (OTU-20; t1:0.13%, t2:0%) (OTU-1312; t1:0%, t2: 0.15%). Table 3.3 reports the 5 OTUs contributing most dissimilarity in gut microbiota between production batches at each sampling time.

Table 3.3: OTUs that contributed most to the dissimilarity in gut microbiota between three production batches (1R, 1A, 5S) at each sampling time. Analysis was made by SIMPER which also estimated the mean abundance of these OTUs in each production batch.

OTU ID	Taxa (Genera)	Taxa (Class)	Amount of dissimilarity (%)	Mean abundance in production batch 1R (%)	Mean abundance in production batch 1A (%)	Mean abundance in production batch 5S (%)
First sampling time (t1)						
OTU-4	*Brevinema*	Spirochaetia	8.44	0.02	0	20.3
OTU-8	*Delftia*	Betaproteobacteria	6.18	17.0	15.30	4.48
OTU-12	*Vibrio*	Gammaproteobacteria	5.43	0.27	10.70	4.31
OTU-9	*Zoogloea*	Betaproteobacteria	4.66	9.75	13.30	5.65
OTU-14	*Photobacterium*	Gammaproteobacteria	4.35	4.25	0.06	10.10
Second sampling time (t2)						
OTU-9	*Zoogloea*	Betaproteobacteria	8.63	20.60	9.40	5.40
OTU-12	*Vibrio*	Gammaproteobacteria	6.51	0.10	1.57	9.20
OTU-1	*Propionibacterium*	Actinobacteria	6.43	8.86	7.06	6.03
OTU-20	*Moritella*	Gammaproteobacteria	5.61	0	1.32E-03	8.84
OTU-5	*Staphylococcus*	Bacilli	4.81	0.04	0.94	7.03
Third sampling time (t3)						
OTU-1	*Propionibacterium*	Actinobacteria	10.86	40.90	26.80	22.40
OTU-4	*Brevinema*	Spirochaetia	4.38	0	2.74E-03	7.75
OTU-5	*Staphylococcus*	Bacilli	3.95	7.99	1.21	1.72
OTU-7	*Nitrospira*	Nitrospira	2.52	1.33	4.34	5.15
OTU-1312	*Eubacterium*	Bacilli	2.19	0.17	1.64	3.52

3.4.2 Effect of sampling time on the gut microbiota

To examine the temporal dynamics of the gut microbiota in salmon fry, a PCoA plot based on Bray-Curtis metrics was generated to map gut samples in each production batch at increasing sampling time (Fig 3.16a-d). For each production batch, gut samples at the first sampling time (t1) and second sampling time (t2) were relatively similar while gut samples at t3 clustered quite distinctly from other sampling times (Fig 3.16b-d). In each production batch, no significant difference existed between sampling times (t1, t2, t3) (PERMANOVA, $p > 0.05$). The PCoA plot Indicates that for all production batches, the gut microbiota was different at sampling time 3.

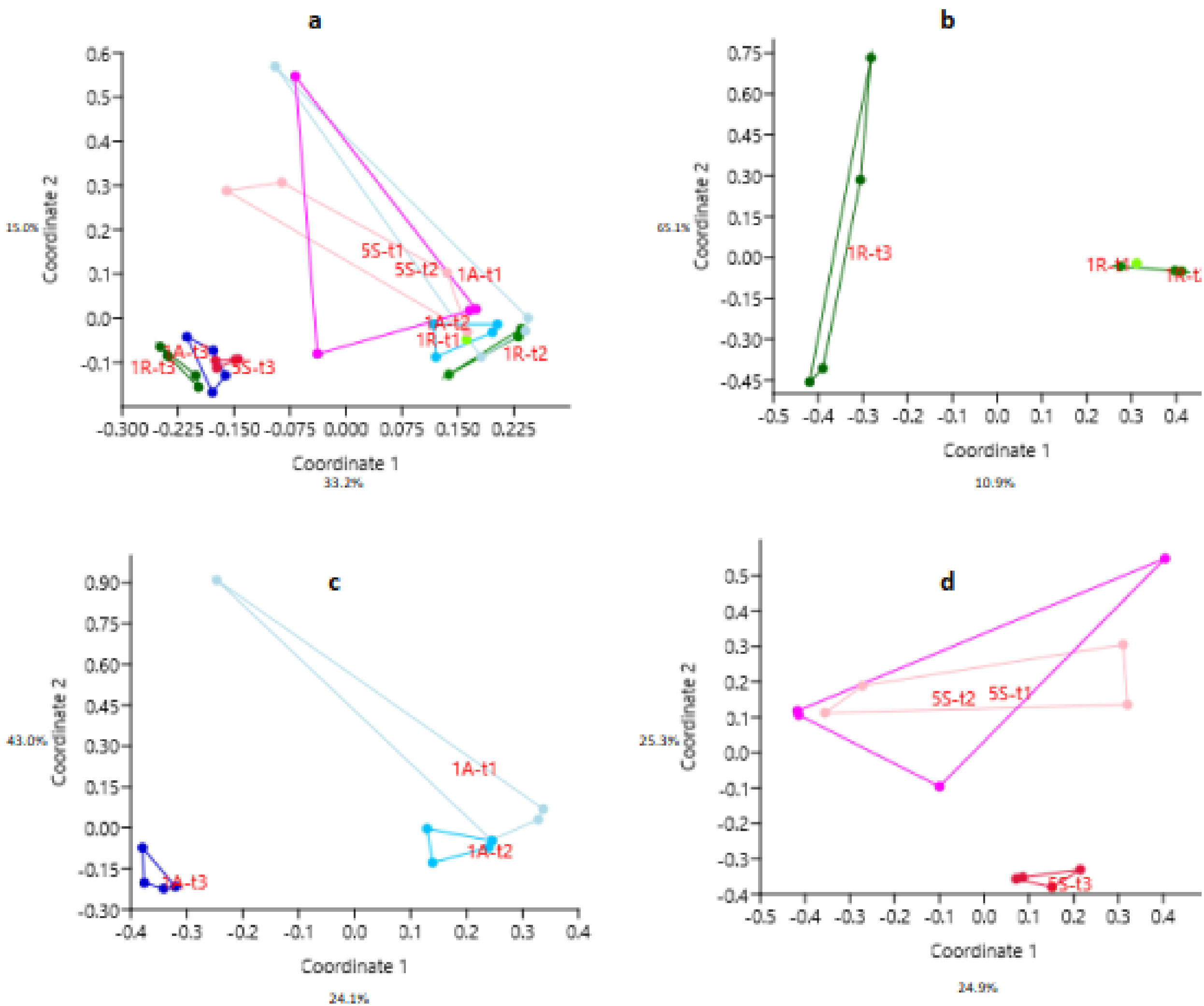

Figure 3.16: PCoA plots showing the variation in individual's gut microbiota between three sampling time (t1, t2, t3) for each production batch (1R, 1A, 5S). Green circles: Fries in production batch 1R. Blue circles: Fries in production batch

1A. Red circles: Fries in production batch 5S. Darker versions of the above-mentioned colours represent fries in same production batch but at a new sampling time. **a:** For all production batches (1R, 1A, 5S), variation in individual's gut microbiota between three sampling times (t1, t2, t3). **b:** Variation in individual's gut microbiota between three sampling time (t1, t2, t3) for fries in production batch 1R (t1). **c:** Variation in individual's gut microbiota between three sampling time (t1, t2, t3) for fries in production batch 1A. **d:** Variation in individual's gut microbiota between three sampling time (t1, t2, t3) for fries in production batch 5S. The ordination plot was based on Bray-Curtis distances. Percentages on axis indicate how much of the total variance that were explained by the two coordinates.

There was no clear trend in the within sampling time comparison for fries in production batch 1R and 1A but for fries in production batch 5S, the similarity in gut microbiota among individuals appeared to increase with increasing sampling time (Fig 3.17) (t1: Br-Cs = 0.24 ± 0.16, t2: Br-Cs = 0.36 ± 0.31, t3: Br-Cs = 0.55 ± 0.19). In the between sampling time comparison, both fries in production batch 1R and 1A, had the most similarity in gut microbiota between t1 and t2 (1R; t1 vs t2: Br-Cs = 0.54 ± 0.02, 1A; t1 vs t2: Br-Cs = 0.39 ± 0.21) (Fig 3.17). This observation agrees with the PCoA plot were fries in each production batch were relatively more similar at t1 and t2.

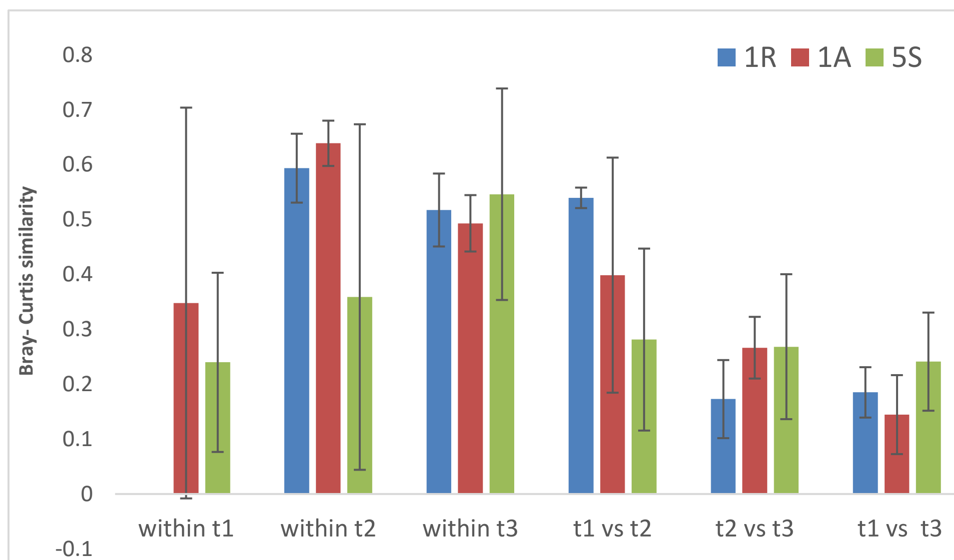

Figure 3.17: Average Bray-Curtis similarity for comparisons of gut microbiota within and between the three-sampling time (t1, t2, t3) in each production batch (1R, 1A, 5S). Error bars represent standard deviation of mean. Label with no bar represent production batches with only one sample.

For the younger fries with production batch 1R and 1A, SIMPER analysis implicates OTU-1 (*Propionibacterium*, Actinobacteria) as the major contributor to dissimilarity (1R: 18.10%, 1A: 10.64%) between the three sampling times in each production batch. *Propionibacterium* was much more abundant at sampling time t3 (1R: 40.90%, 1A: 26.8%) than at sampling time t2 (1R:

8.86%, 1A: 7.06%) and t1 (1R: 8.96%, 1A: 5,36%). OTU-4 (*Brevinema,* Spirochaetia) was most abundant in the oldest fries in production batch 5S. This OTU was more abundant at t1 (20.30%) than t2 (4.35%) and t3 (7.75%). Table 3.4 reports the 5 OTUs contributing most to dissimilarity between three sampling times for each production batch.

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Table 3.4: OTUs that contributed to most to the dissimilarity in gut microbiota between three sampling time (t1, t2, t3) for fries in each production batch. Analysis was made by SIMPER which also estimated the mean abundance of these OTUs at each sampling time.

Production batch 1R						
OTU ID	Taxa (Genera)	Taxa (Class)	Amount of dissimilarity (%)	Mean abundance in sampling time t1 (%)	Mean abundance in sampling time t2 (%)	Mean abundance in sampling time t3 (%)
OTU-1	*Propionibacterium*	Actinobacteria	18.10	8.96	8.86	40.90
OTU-9	*Zoogloea*	Betaproteobacteria	10.64	9.75	20.60	0.55
OTU-8	*Delftia*	Betaproteobacteria	5.63	17.0	9.23	2.37
OTU-5	*Staphylococcus*	Bacilli	4.34	1.0	0.04	7.99
OTU-405	*Acidovorax*	Betaproteobacteria	3.05	6.85	5.03	0.27
Production batch 1A						
OTU-1	*Propionibacterium*	Actinobacteria	10.64	5.36	7.06	26.80
OTU-9	*Zoogloea*	Betaproteobacteria	6.73	13.30	9.40	1.09
OTU-8	*Delftia*	Betaproteobacteria	6.67	15.30	5.29	2.68
OTU-12	*Vibrio*	Gammaproteobacteria	5.87	10.70	1.57	2.85
OTU-13	*Acinetobacter*	Gammaproteobacteria	1.95	3.39	3.72	0.35
Production batch 5S						
OTU_4	*Brevinema*	Spirochaetia	10.35	20.30	4.35	7.75
OTU_1	*Propionibacterium*	Actinobacteria	8.91	6.61	6.03	22.40
OTU_12	*Vibrio*	Gammaproteobacteria	5.66	4.31	9.20	0.46
OTU_20	*Moritella*	Gammaproteobacteria	5.01	3.11	8.84	0.10
OTU_14	*Photobacterium*	Gammaproteobacteria	4.51	10.10	1.71	1.86

3.5 The Skin Microbiota

3.5.1 Comparison of skin microbiota between production batches

A PCoA plot based on Bray-Curtis metrics was used for comparing the skin microbiota of samples from three production batches (1R, 1A, 5S) at each sampling time (Fig 3.18a-d). The plot showed that at the first and second sampling time, skin samples from the younger fries in production batch 1R and 1A clustered closely together and were distinct from the oldest fries in production batch 5S (Fig 3.18b-c). There was no significant difference (PERMANOVA, P > 0.05) in skin microbiota between production batches at each sampling time.

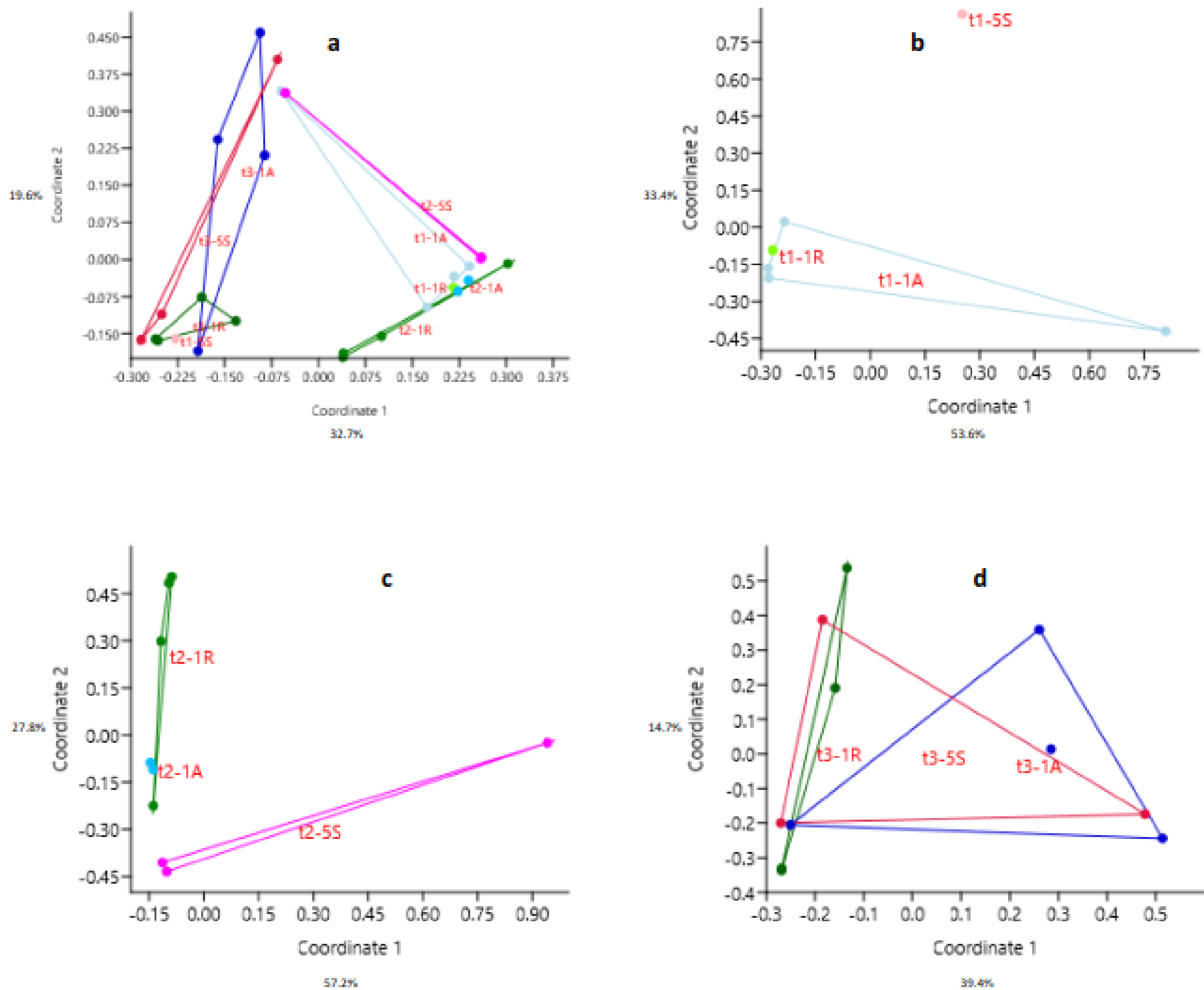

Figure 3.18: PCoA plots showing the variation in individual's skin microbiota between three production batches (1R, 1A, 5S) at three sampling times (t1, t2, t3). Green circles: Fries in production batch 1R. Blue circles: Fries in production batch 1A. Red circles: Fries in production batch 5S. Darker versions of the above-mentioned colours represent fries in same production batch but at a new sampling time. **a:** For all sampling time (t1, t2, t3) the variation in individual's skin microbiota between three production batches (1R, 1A, 5S). **b:** Variation in individual's gut microbiotas for three production batches (1R, 1A, 5S) at the first sampling time (t1). **c:** Variation in individual's gut microbiotas for three production batches (1R, 1A, 5S) at the second sampling time (t2). **d:** Variation in individual's gut microbiotas for three production batches (1R, 1A, 5S) at the third sampling time (t3). The ordination plot was based on Bray-Curtis distances. Percentages on axis indicate how much of the total variance that were explained by the two coordinates.

At t1 and t2, skin microbiota was most similar between the younger fries in production batch 1R and 1A (Fig 3.19) t1 (1R vs 1A: Br-Cs = 0.45 ± 0.29), t2 (1R vs 1A: Br-Cs = 0.55 ± 0.03). Similar trend was observed in the PCoA plot at the first and second sampling time where the skin microbiota of the younger fries in production batch 1R and 1A clustered closely and were distinct from the oldest fries in production batch 5S (Fig 3.17b-c). The third sampling time (t3) had the highest similarity between fries in production batch 1R and 5S (Fig 3.19).

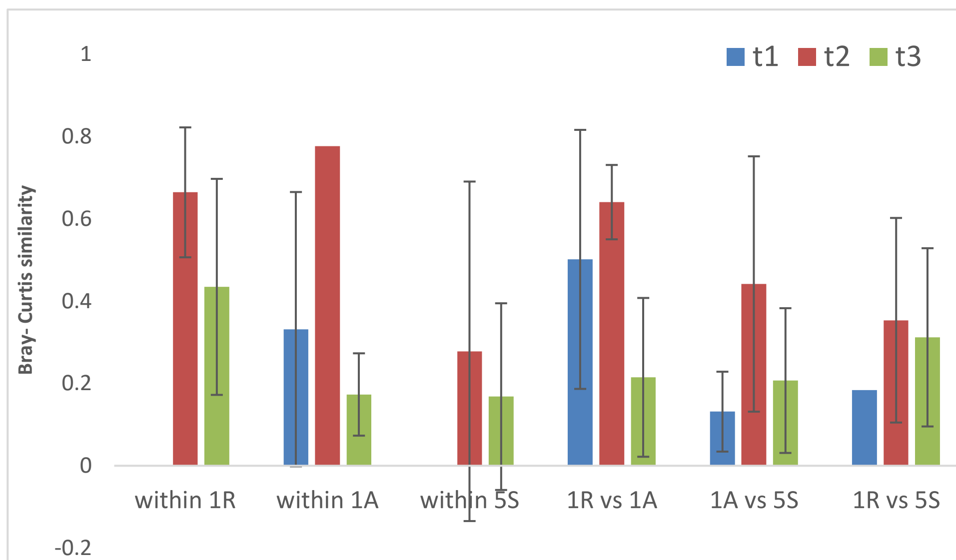

Figure 3.19: Average Bray-Curtis similarity for comparisons of skin microbiota within and between three production batches (1R, 1A, 5S) at each sampling time (t1, t2, t3). Error bars represent standard deviation of mean. Labels with no bar represent production batches with only one sample. Bars without an error bar represent production batches with two samples.

At t1, OTU-1 (*Propionibacterium,* Actinobacteria) contributed most to the dissimilarity (16.51%) in the skin microbiota among fries in all production batches. This OTU was more abundant in the oldest fries in production batch 5S (44.30%) than the younger fries in production batch 1A (7.48%)

and 1R (10.30%). Also at t1, OTU-6 (*Bacillus,* Bacilli) contributed second most to the dissimilarity (15.23%) in skin microbiota between all production batches. The relative abundance of this 0TU was higher for fries in production batch (1A) (23.90%) than in fries in production batch 1R (0.07%) and 5S (0%). At the second sampling time (t2), OTU-10 (*Streptomyces,* Actinobacteria), accounted for the most difference (19.07%) among fries in all production batches. OTU-10 was only abundant in fries in production batch 5S (29.60%) but absent in fries in production batch 1A and 1R. At t3, OTU-1 was responsible for the most difference (18.81%) among fries in all production batches and was more abundant in skin microbiota of fries in production 1R (43.80%) than fries in production batch 1A (24.90%) and 5S (32.70%). Also at t3, OTU-42 (*Bacillus,* Bacilli) accounted for the second most dissimilarity among fries in all production batches and was more abundant in fries in production batch 1A (10.60%) than fries in production batch 5S (0.01%) and 1R (0%). Table 3.5 reports the 5 OTUs contributing most to the dissimilarity in skin microbiota between production batches at each sampling time.

Table 3.5: OTUs that contributed most to the dissimilarity in skin microbiota between production batches (1R, 1A, 5S) at each sampling time. Analysis was made by SIMPER which also estimated the mean abundance of these OTUs in each production batch.

OTU ID	Taxa (Genera)	Taxa (Class)	Amount of dissimilarity (%)	Mean abundance in production batch 1R (%)	Mean abundance in production batch 1A (%)	Mean abundance in production batch 5S (%)
First sampling time (t1)						
OTU-1	*Propionibacterium*	Actinobacteria	16.51	10.30	7.48	44.30
OTU-6	*Bacillus*	Bacilli	15.23	0.07	23.90	0
OTU-5	*Staphylococcus*	Bacilli	9.92	0.40	0.26	25.0
OTU-8	*Delftia*	Betaproteobacteria	8.53	25.40	10.40	6.25
OTU-9	*Zoogloea*	Betaproteobacteria	7.02	17.3	9.86	0
Second sampling time (t2)						
OTU-10	*Streptomyces*	Actinobacteria	19.07	0	0	29.60
OTU-1	*Propionibacterium*	Actinobacteria	18.64	27.0	10.40	2.26
OTU-9	*Zoogloea*	Betaproteobacteria	15.37	18.40	27.70	27.40
OTU-8	*Delftia*	Betaproteobacteria	6.56	7.64	10.80	11.60
OTU-26	*Pseudoxanthomonas*	Gammaproteobacteria	2.06	2.88	3.35	0.05
Third sampling time (t3)						
OTU-1	*Propionibacterium*	Actinobacteria	18.81	43.80	24.90	32.70
OTU-42	*Bacillus*	Bacilli	4.90	0	10.60	0.01
OTU-33	*Pseudoalteromonas*	Gammaproteobacteria	4.66	5.92	0.42	6.24
OTU-17	*Chryseobacterium*	Flavobacteriia	3.88	8.41	1.56E-03	0
OTU-35	*Tumebacillus*	Bacilli	3.52	7.21	0.58	0

3.5.2 *Effect of sampling time on the skin microbiota*

The temporal dynamics of the skin microbiota in salmon fry was examined by a PCoA plot (Fig 3.20a-d). Fries in production batch 1R and 1A showed same trend where the skin microbiota of each production batch was more similar at t1 and t2 and distinct from t3 (Fig 3.20b-c). Skin microbiota of fries in production batch 5S clustered distinctly at all sampling time (Fig 3.20d). There was no significant difference in skin microbiota of each production batch at all sampling times (t1, t2, t3) (PERMANOVA, $p > 0.05$).

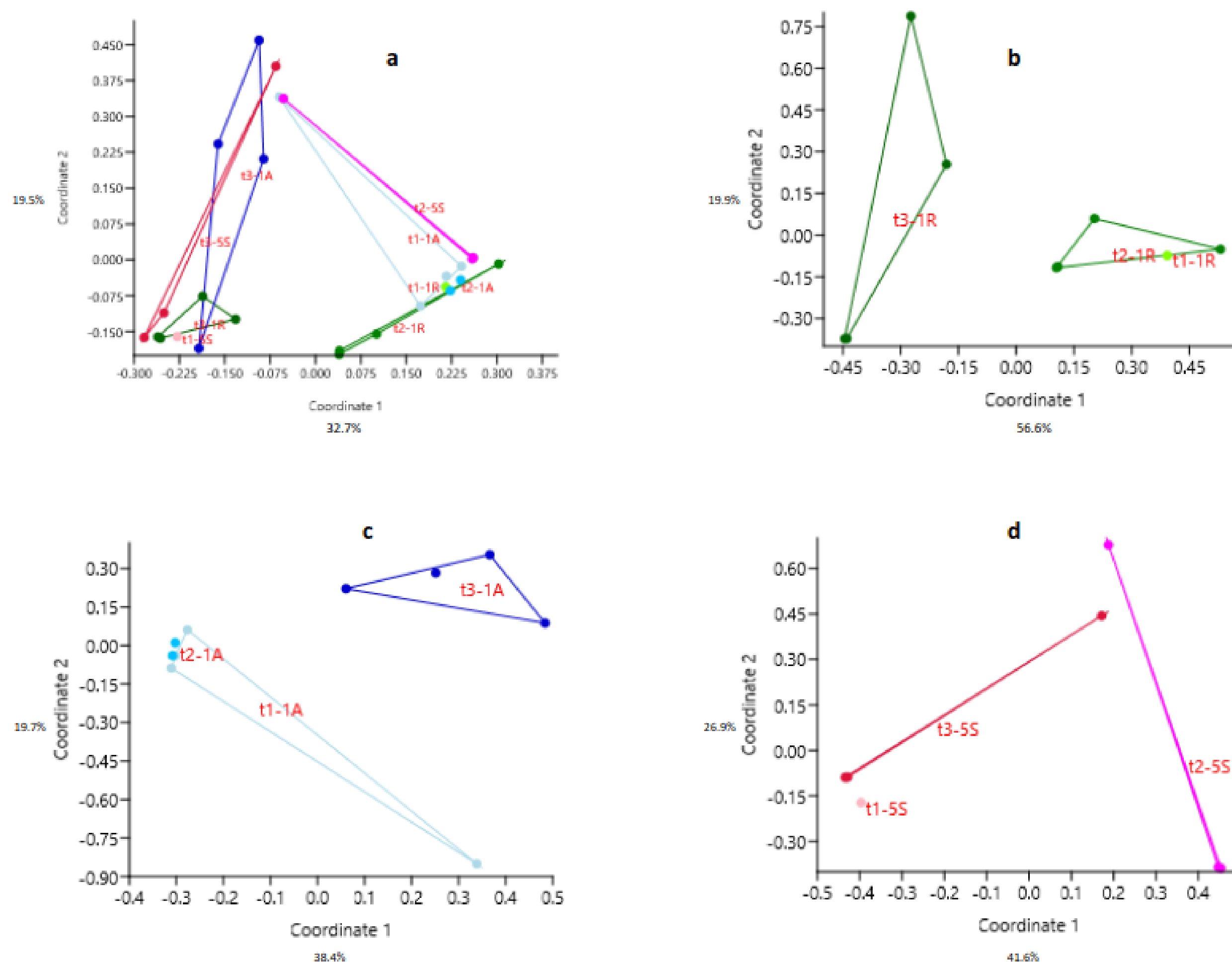

Figure 3.20: PCoA plots showing the variation in individual's skin microbiota between three sampling time (t1, t2, t3) for each production batch (1R, 1A, 5S). Green circles: Fries in production batch 1R. Blue circles: Fries in production batch 1A. Red circles: Fries in production batch 5S. Darker versions of the above-mentioned colours represent fries in same production batch but at a new sampling time. **a:** For all production batches (1R, 1A, 5S), variation in individual's skin microbiota between three sampling times (t1, t2, t3) **b:** Variation in individual's skin microbiota between three

sampling time (t1, t2, t3) for fries in production batch 1R (t1). **c:** Variation in individual's skin microbiota between three sampling time (t1, t2, t3) for fries in production batch 1A. **d:** Variation in individual's skin microbiota between three sampling time (t1, t2, t3) for fries in production batch 5S. The ordination plot was based on Bray-Curtis distances. Percentages on axis indicate how much of the total variance that were explained by the two coordinates.

In production batch 1R and 1A, the between sampling time similarity was highest between t1 and t2 (1R; t1 vs t2: Br-Cs= 0.60 ± 0.03, 1A; t1 vs t2: Br-Cs= 0.48 ± 0.28) but lower for fries in production batch 5S (5S; t1 vs t2: Br-Cs= 0.08 ± 0.06). The fries in production batch 5S showed a different trend where highest similarity in the between sampling time comparison was between t1 and t3 (5S; t1 vs t3: Br-Cs= 0.38 ± 0.29).

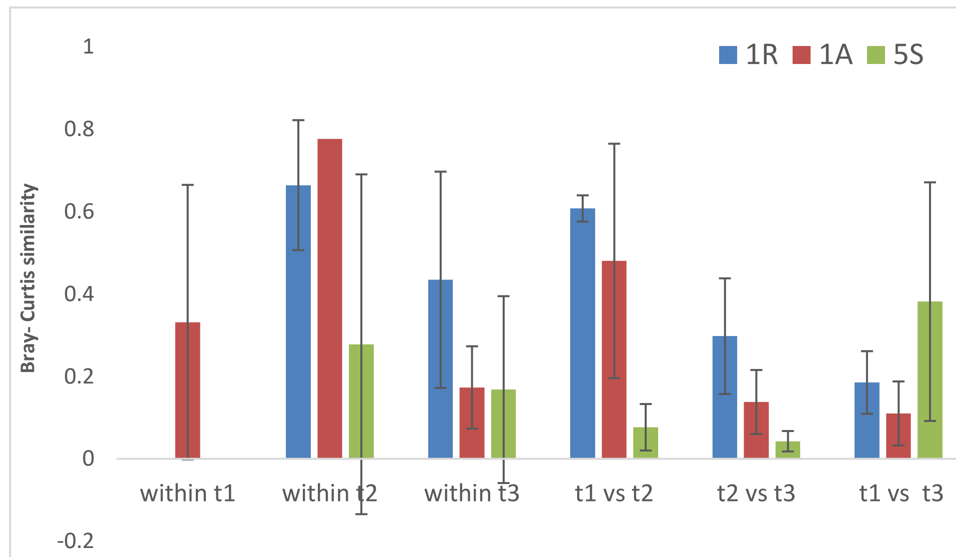

Figure 3.21: Average Bray-Curtis similarity for comparisons of skin microbiota within and between three-sampling time (t1, t2, t3) for fries in each production batch (1R, 1A, 5S). Error bars represent standard deviation of mean. Labels with no bar represent production batches with only one sample. Bars without an error bar represent production batches with two samples.

SIMPER analysis of the skin microbiota of fries in production batch 1R, implicated OTU-1 (*Propionibacterium,* Actinobacteria) as contributor of most difference (17.11%) between all sampling time. OTU-1 was more abundant at t3 (43.80%) than t2 (27.0%) and t1 (10.30%). OTU-9 accounted for second most difference between all sampling time in fries in production batch 1R. Fries in production batch 1A had OTU-6 (*Bacillus,* Bacilli) responsible for the most dissimilarity (11.51%) at all sampling time. This OTU was more abundant at t1 (23.90%) than t2 (0%) and t3 (0.52%). For fries in production batch 5S OTU-10 (*Streptomyces,* Actinobacteria) contributed

second most to the dissimilarity (13.56%) after OTU-1. OTU-10 was more abundant at t2 (29.69%) than t1 (0%) and t3 (0.56%). Table 3.6 reports the 5 OTUs contributing most to the dissimilarity between three sampling times in each production batch.

Table 3.6: OTUs that contributed to most to the dissimilarity in gut microbiota between three sampling times (t1, t2, t3) in each production batch. Analysis was made by SIMPER which also estimated the mean abundance of these OTUs at each sampling time.

OTU ID	Taxa (Genera)	Taxa (Class)	Amount of dissimilarity (%)	Mean abundance in sampling time t1 (%)	Mean abundance in sampling time t2 (%)	Mean abundance in sampling time t3 (%)
Production batch 1R						
OTU-1	*Propionibacterium*	Actinobacteria	17.11	10.30	27.0	43.80
OTU-9	*Zoogloea*	Betaproteobacteria	12.09	17.30	18.40	0
OTU-8	*Delftia*	Betaproteobacteria	8.44	25.40	7.64	5.54
OTU-17	*Chryseobacterium*	Flavobacteriia	5.23	0	0	8.41
OTU-35	*Tumebacillus*	Bacilli	4.49	0	0	7.21
Production batch 1A						
OTU-6	*Bacillus*	Bacilli	11.51	23.90	0	0.52
OTU-1	*Propionibacterium*	Actinobacteria	10.9	7.48	10.40	24.90
OTU-9	*Zoogloea*	Betaproteobacteria	10.07	9.86	27.70	0.56
OTU-42	*Bacillus*	Bacilli	5.045	5.84E-04	0.09	10.60
OTU-8	*Delftia*	Betaproteobacteria	5.003	10.40	10.80	2.57
Production batch 5S						
OTU-1	*Propionibacterium*	Actinobacteria	17.81	44.30	2.26	32.70
OTU-10	*Streptomyces*	Actinobacteria	13.56	0	29.60	0.56
OTU-9	*Zoogloea*	Betaproteobacteria	12.42	0	27.40	4.68E-03
OTU-5	*Staphylococcus*	Bacilli	6.69	0.25	0.46	5.13
OTU-8	*Delftia*	Betaproteobacteria	5.51	6.25	11.60	0.49

4. Discussion

This thesis consists of two independent parts; The first was an attempt to establish a PCR protocol for the salmon fry gut samples from the Frøya experiment. The second part was processing and analysis of the Illumina sequencing data obtained from skin and gut samples collected from Atlantic salmon fry and parr at a commercial farm at Follafoss (SalMar Settefisk AS).

4.1 Optimization of PCR protocol for the Frøya experiment

The attempt to optimize the PCR protocol for the Frøya experiment samples was hampered by many challenges. These challenges included small amount of PCR products, presence of co-amplified eukaryotic rRNA gene regions, PCR products for the non-template negative control and formation of primer dimers.

The presence of PCR products in the non-template negative control was probably due to contamination of the PCR reagents. This problem was resolved by improving routines during the pre-PCR stage and distribution of newly acquired PCR reagents in aliquots. This helped prevent cross-contamination between users. DNA extracted from the gut of salmon fries was low in concentration because of the small size of the gut samples. This lead to a low yield of PCR products after amplification. This issue was resolved by precipitation of the extracted DNA before amplification. Precipitation helped concentrate and purify the extracted DNA. This improved PCR yield for the target 16Sr RNA gene but co-amplified a region of the salmon 18S rRNA and mitochondrial 12S rRNA gene. This result was observed when the primers ILL515F and ILL805R was used to amplify the V4 region. Co-amplification of eukaryotic genes during amplification of the bacterial 16S rRNA from cod larvae was also reported by Bakke et al., (2011). This co-amplification was due to the extensive sequence homology between the primers and these salmon rRNA genes.

The most promising PCR protocol was obtained when ILL338F and 532R primer were used at 55°C annealing temperature and 36 cycles to amplify the V3 region of the bacterial 16S rRNA gene from DNA templates concentrated by precipitation. The target V3-16S rRNA gene was explicitly amplified. However, to enable the Illumina sequencing of the amplified V3 region, use of the

primer pair ILL338F and ILL532R at same PCR conditions, resulted in primer dimer formation. This observation might suggest that the Illumina adapter sequence from the reverse primer may have a good sequence homology with a region of the Illumina-adapted forward primer. The annealing of these primers outcompeted the annealing of the primers to the specific target region. The use of other primer sets also encountered problems with primer dimer formation or co-amplification of undesired genes. Peng et al., (2015) observed that use of barcoded primers experienced PCR bias associated with non-uniform amplification and primer dimer formation. A potential strategy to circumvent this primer dimer formation problem, might be to include an extra round of PCR: First with primers consisting of only the target sequences, and then do a second PCR with primers with the same target sequences, but with the Illumina adapters included. However, there was not enough time to continue optimizing the PCR protocol.

The testing of PCR protocols also showed the importance of including a eukaryotic positive control in addition to the bacterial positive control. In our study, DNA extracted from salmon muscle was assumed to be bacteria free, and helped to determine if eukaryotic rRNA gene regions were amplified. This was a quick and easy way to discriminate between bacterial and eukaryotic amplicons of similar size during testing the specificity of different PCR protocols.

4.2 Diversity in gut and skin microbial communities

The gut and skin microbiota during early development of teleost is vital in organ development, nutrition and immunity (Acheson and Luccioli, 2004; Rawls et al., 2004; Perez et al., 2010; Ye et al., 2014; Yan et al., 2016). Perturbation of gut and skin microbiota have been implicated in diseases susceptibility (Gómez and Balcázar, 2008; Reid et al., 2107) and high mortality rate (Pilar et al., 2000). Therefore, a better understanding of these symbiotic relationships is essential in exploiting its benefits and thereby maximize salmon fries' health and productivity.

The sequencing depth for the skin samples was lower than for the gut samples. This was shown as the sequencing depth covered 90% and 85% of the estimated OTU richness for gut and skin samples respectively. There were also challenges in explicitly amplifying the 16S rRNA gene from the skin samples. Sequences reads from the skin samples contained a high amount of salmon 12S

rDNA gene sequences that were filtered out before processing of sequence reads. The observed OTU richness, Chao-1 index and Shannon's diversity showed that the gut microbiota had a higher alpha diversity than the skin samples. The same trend was observed in a study carried out by Øygarden (2017) were the Shannon's diversity of the gut microbiota of wild and aquaculture salmon fries was significantly higher (t-test, p = 0.01) than their skin microbiota. In another study, Ray (2016) revealed that in three wild freshwater teleost species, the gut microbiota possessed higher number of OTUs than the skin microbiota. Similarly, Webster et al., (2018) also reported a higher number of OTUs in gut microbiota of wild and aquaculture Atlantic salmon fries than the skin microbiota. These observations might be attributed to the nutrient rich environment of the gut, where the feed and gut mucosal surface provides favourable habitat for adhesion and proliferation of various microbial taxa. Evidence of the effect of feed on gut microbiota during early development was found as a rise in microbial diversity in the gut of rainbow trout after the first feeding (Ingerslev et al., 2014b). Diversity in the bacterial substrates available to the bacteria in the gut environment might have favoured a more diverse community. Contrary views support the notion that the skin may house a more diverse microbiota than the gut (Lowrey et al., 2015), possibly due to the skin microbial exchange rate with rearing water (Carlson et al., 2017) and proximity to the water bacterial community. More so, the skin may foster a high bacterial diversity due to its nutrient-rich mucosal surface that could serve as a site for microbial adhesion and proliferation.

The alpha diversity of the skin and gut samples increased with age. The production batches containing older fries (1A and 5S) had a higher richness than the production batch in the youngest fries (1R). A similar pattern in alpha diversity was observed in gut microbiota of Atlantic cod larvae (at 17 and 61dph) and discus fish over time (Bakke et al., 2015; Sylvain and Derome, 2017). Another study by Zhang et al., (2018) also showed that the diversity of the gut microbiota in Southern catfish also increased with age. These distinct shifts in the gut and skin microbiota could signify an active assemblage and retention of acquired microbes as time progressed. Gnotobiotic Zebra fish have been shown to retain transplanted microbiota from Zebra fish or mice (Rawls et al., 2006), although the relative abundance of different taxa changed to mimic the usual gut microbiota of the recipient host. There is also a possibility that the developing alimentary canal provided new niches for more microbes to thrive. Nevertheless, diversity can gradually decrease with age as seen in Zebra fish (Zac et al., 2016).

The measure of the beta diversity by the Bray-Curtis similarity showed that the microbial community composition among the skin samples irrespective of production batch and sampling time was less similar than among the gut samples, i.e. the skin samples had a higher beta diversity than the gut samples. This signifies that the investigated factors (production batch, sampling time) might have influenced the skin microbiota of the salmon fries more than the gut microbiota. Evidence of a more resilient gut microbiota was shown in a study conducted by Webster et al., (2018) on wild and aquaculture Atlantic salmon fries where various factors (location, population, fork length) had a significant stronger influence on the skin microbiota than the gut microbiota (location and population). Ray (2016) also reported similar trend where seasonal changes had a more significant impact on the skin microbiota of three freshwater fishes than the gut microbiota. In another study, freshwater Tambaqui faecal microbiota was more resistant to an acidic rearing water than the skin microbiota (Sylvain et al., 2016). The observations from our study indicates that the skin microbiota is more exposed to changes in the environmental factors and microbes.

Proteobacteria followed by Actinobacteria and Firmicutes were the most abundant phyla in gut and skin samples. This is similar to the findings in another study (Øygarden, 2017) where the gut and skin samples of wild and aquaculture strains of salmon fries were found to be dominated by Actinobacteria followed by Proteobacteria and Firmicutes .In another study, the skin microbiota of Atlantic salmon transferred from fresh water to sea water also showed that, the skin was dominated by the phyla Proteobacteria, followed by Bacteroidetes, Actinobacteria and Firmicutes in both skin microbiota of fries bred in fresh and sea water (Lokesh and Kiron, 2016). Furthermore, the gut of Salmon parr reared in an aquaculture system was dominated by Firmicutes, Proteobacteria, and Tenericutes (Dehler et al., 2017a).

In our study, the classes Actinobacteria, Betaproteobacteria, Bacilli and Alphaproteobacteria were more abundant in the skin microbiota than gut microbiota, although only Actinobacteria was significantly more abundant in the skin microbiota than their gut counterpart (t-test, $p = 0.02$). Gammaproteobacteria was more abundant in the gut than skin microbiota. Webster et al., (2018) reported that the skin microbiota of aquaculture Atlantic salmon fries were dominated by class Gammaproteobacteria and Betaproteobacteria and their gut microbiota dominated by Bacilli and Gammaproteobacteria. Actinobacteria and Bacilli were also abundant in the digesta of

post smolt Atlantic salmons while Alphaproteobacteria and Betaproteobacteria were both found in the digesta and gut mucosa microbiota (Gajardo et al., 2016). In another study, Alphaproteobacteria, Actinobacteria and Bacilli dominated the gut microbiota of salmon parr and post smolt Atlantic salmons (Rudi et al., 2018).

Although *Propionibacterium* (Class: Actinobacteria) was the most abundant genus in the gut and skin microbiota, it was speculated that this abundant *Propionibacterium*-OTU might have represented a contaminating DNA from the DNA extraction kit. This observation was discussed more in depth in 4.3. Nevertheless, this genus also contributed most to the dissimilarity between the gut and skin microbiota because it was more abundant in the skin samples. *Propionibacterium* belongs to a group of gram-positive facultative anaerobic bacteria that synthesizes propionic acid. A Similar observation was made in the study of salmon fry microbiota by Øygarden (2017), where this genus contributed most to dissimilarity between gut and skin samples in farmed and wild genetic group. This genus has also been found to be abundant in the gut microbiota of newly hatched larvae (younger than 49dph) of Atlantic salmon (Lokesh et al., 2017) and water samples used in breeding pre-smolt Atlantic salmons (Dehler et al., 2017b). Furthermore, *Propionibacterium* was present in low abundance in the microbiota of gut mucosa and digesta of post smolt Atlantic salmon (Gajardo et al., 2016) and in the gut microbiota of adult salmon (Godoy et al., 2015). This might suggest that this microbial genus is prevalent at an early lifestage, and could be retained until the adult salmon stage where their relative abundance is reduced. A dominance of this genus was also observed in the gut microbiota of salmons fed soya bean concentrate (Green et al., 2013) and fishmeal free feed (Schmidt et al., 2016). Moreover, *Propionibacterium* was detected in the microbiota of salmon faecal samples (Zarkasi et al., 2014) and on the gills of salmons (Schmidt et al., 2016). This genus is also part of the core microbiota of other teleost species such as Atlantic killfish, Pinfish, Black sea bass and Spanish mackerel (Givens, 2012) and is scarcely reported on the skin microbiota of teleost. Whether *Propionibacterium* in these observations also represent contaminating DNA, or is actually an abundant member of fish microbiota is difficult to tell.

In our study, *Zoogloea* was the second most abundant genus in the gut and skin microbiota. The genus was more abundant in the skin than gut microbiota of salmon fries. *Zoogloea* are gram-negative rod-shaped aerobic bacteria that are found in freshwater and systems with high organic load, like biological wastewater treatment system such as the suspended growth system (Kutz,

2013). It has been shown to reduce turbidity and Chemical oxygen demand (COD) in activated sludge system. (Ahn et al.,1997). There is limited information on the presence and role of this genus on skin microbiota of fish. A species of *Zoogloea* was implicated in production of poly-β-hydroxybutyrate (PHB) (Kalia and Avérous, 2011), a form of polyhydroxyalkanoate (PHA). PHAs can be produced by microbes through lipid or sugar fermentation during low nitrogen levels. PHAs aids in the removal of nitrate in aquaculture and waste water treatment systems by acting as a carbon source for denitrifying bacteria (Gutierrez-Wing, 2006). Although nitrate is the least harmful form of inorganic nitrogen compound in aquaculture systems (Timmons and Ebeling, 2007), high level of nitrate in aquaculture system can be harmful especially for freshwater fishes (Camargo et al., 2005). These observations suggest the genus *Zoogloea* found in the skin and gut microbiota may have originated from the rearing water, where it contributes in maintaining good water quality. Furthermore, presence of PHB accumulating bacteria helped protect brine shrimp from pathogenic *Vibrio* species (Defoirdt et al., 2007). Although the abundance of this genus has scarcely been reported, a study conducted by Federici et al (2015) reported the abundance of *Zoogloea* on the skin of a stream frog infected with a cutaneous disease.

OTU-8 (*Delftia*, Betaproteobacteria) was responsible for the third most difference between the gut and skin microbiota of salmon fries. *Delftia* are gram-negative rod shaped aerobic bacteria that has been found in the gut microbiota of teleost like orange-spotted grouper (Sun et al., 2009), rainbow trout (Navarrete et al., 2012; Ingerslev et al., 2014b). This genus was also found in the digesta and gut mucosa microbiota of post smolt Atlantic salmon (Gajardo et al., 2016). *Delftia* were also present in the gut microbiota of early (84dph) and late (140dph) fresh water stage Atlantic salmon (Lokesh et al., 2017). *Delftia* has been reported in the skin microbiota of three teleost species (Larsen et al., 2013).

Vibrio are gram-negative rod shaped facultative anaerobes that are found in seafoods and marine environments. In our study, OTU-12 (*Vibrio*, Gammaproteobacteria) was found to be three times more abundant in the gut microbiota than skin microbiota of salmon fries. *Vibrio* can be found in both fresh water and marine fishes (Nayak, 2010) and has been detected in the gut microbiota of salmonids through both culture dependent and independent techniques (Hovda et al., 2007; Ciric et al., 2018). Ringø and Birkbeck, 1999 also reported the presence of this genus in gut microbiota of various teleost's larvae. In another study the genus *Vibrio* was found in the fore and hind gut

microbiota of adult Atlantic salmon (Hovda et al., 2007) fed with a diet mix containing fish meal, fish oil and soya bean meal. Furthermore, some species of this genera are pathogenic (Austin and Austin, 2007) while other species have served as probiotics in gut microbiota of salmonids like Atlantic salmon where they prevent the proliferation of other pathogenic species (Austin et al., 1995). The genus *Vibro* has also be found in the skin microbiota of fish. In another study this genus was detected in low abundance or absent in the skin microbiota of six seawater fish (Larsen et al., 2013). Llewellyn et al., (2017) reported a high abundance of *Vibrio* on the skin mucosa of post smolt Atlantic salmon exposed to sea lice.

OTU-4 (*Brevinema*, Class: Spirochaetia) was 740 times more abundant in the gut microbiota than skin microbiota of salmon fries. *Brevinema* are gram-negative helical shaped microaerophilic bacteria that can be found in the gut microbiota of freshwater fishes (Larsen et al., 2014). In teleost like Atlantic cod, the genus was the 8th most abundant OTU in the gut microbiota (Riiser et al., 2017). In our study, *Brevinema* was the 4th most abundant OTU in the gut microbiota of salmon fries. Belkova et al., (2017) also reported the abundance of this genus in the gut microbiota of juvenile coregonid fish where they were the 4th most abundant OTU. *Brevinema* was more abundant in the gut microbiota of farmed rainbow trout fed with micro algae supplemented feed than the control group (Lyons et al., 2017a). This genus was the third most abundant genera in the gut microbiota of a species of tonguefish after exposure to algae infection (Han et al., 2018).

4.3 Effect of age/developmental stage on the gut microbiota

In our study, an age effect on fry gut and skin microbiota was reflected both in the production batches and sampling times. Salmon fries in the production batches represent distinct age groups and rearing systems while the sampling times reflected an increase in age of fries in each production batch. Besides the age difference of fries in production batch 1R and 1A, the age difference was larger between fries in different production batches (at t1; fry in 1R were 50dph, in 1A 97dph, and in 5S 287dph) than between sampling times in each production batch (t1:22-02-2017, t2: 30-03-2017 (36 days after t1), t3: 26-04-2017 (63 days after t1)). Fries in the different production batches not only differed in age, but also in the rearing systems used. Two different RAS were used for fries in 1R and 5S, and FTS for fries in 1A.

It was expected that age and different rearing system might affect the gut and skin microbiota of salmon fries. Differences between salmon fries gut or skin microbiota should be higher between production batches than between sampling time because of the larger age difference between distinct age groups and different aquaculture system used in each production batch. In other words, differences in fries' gut or skin microbiota between sampling times of each production batch should be smaller than between production batches. Surprisingly, the pcoA analysis (Fig. 3.14a and 3.18a) showed that the samples clustered more according to sampling time than to production batch.

The unexpected result at the third sampling time was evident in the SIMPER analysis, which showed that OTU-1 (*Propionibacterium,* Actinobacteria) was responsible for the most difference (11.77%) between the three sampling times irrespective of production batch. This OTU was more abundant in the third sampling time (28.40%) than the second (10.6%) and first sampling time (6.32%). Further analysis showed that this OTU varied much more according to sampling time than production batch. For each production batch, there was a large increase in the abundance of OTU-1 in the fries' gut and skin microbiota at t3 (Table 3.4, Table 3.6). *Propionibacterium* has been identified as one of the bacterial strains that contaminate DNA extraction kits (Glassing et al., 2016; Salter et al., 2014). Gut and skin samples from t3 had been extracted with a different batch of the Mobio Powersoil DNA extraction kit than the samples (gut and skin) from t1 and t2, which were extracted with the same batch of the kit. From our observations, the unexpected result at t3 might be associated with the use of a different production batch of the DNA extraction kit. Different production batch of the same DNA extraction kit might introduce bias in the actual microbial composition of the samples. This is due to the presence of contaminating DNA from different bacterial taxa for each production batch of the DNA extraction kit (Kim et al., 2017; Salter et al., 2014). Due to the above-mentioned reasons, the results from skin and gut microbiota of each production batch at t3 will be excluded from further discussions of the results. These observations show the importance of amplifying and sequencing of negative controls for the DNA extraction kit to be able to identify bacterial taxa in the data set that may be contaminant in the samples.

Aquaculture system design can affect the water chemistry (Bye, 2017; Marcin et al., 2004) and microbial composition of the rearing water (Åm et al., 2015). The water microbial community in

turn can affect the gut microbiota of the fish, as seen for cod larvae (Truong et al., 2012) and tilapia larvae (Giatsis et al., 2015). Although no significant difference was observed in the gut microbiota of fries between production batches, the PCoA plot suggest that the production batch might have influenced the gut microbiota of salmon fries (Fig 3.14ab-d). The gut microbiota seemed to be more influenced by age component of each production batch than by the type of aquaculture system. At the sampling times (t1 and t2), gut microbiota of younger fries in production batch 1A and 1R (50dph and 97dph at t1) were more similar than to the oldest fries in production batch 5S (Fig 3.15), although the younger fries (1R and 1A) were reared in the RAS and FTS respectively. Other studies have reported that differences in rearing systems influenced the gut microbiota of tilapia larvae reared in RAS (Recirculating aquaculture system) and AS (Active suspension system) (Giatsis et al., 2015). Furthermore, another study done with salmon parr of same age reared in recirculating system and fresh water cage system showed that they harboured different gut microbial composition (Dehler et al., 2107a). The observation of a higher similarity between younger fries in production batch 1R and 1A than older fries in production batch (5S) (287dph at t1) was corroborated by the results of Zhang et al., (2018), who showed that the gut microbiota of younger catfishes at 8dpf and 18dpf (day post fertilization) were more similar to each other but distinct from their much older pairs (65dpf and 125dpf). Here we found that both sampling times (t1 and t2), the fries in production batch 5S had lower interindividual similarity than younger fries in production batch 1A and 1R (Fig 3.15). Similar observation was made by Zhang et al., (2018), who found that the gut microbiota of Southern catfish varied more between individuals in the oldest age group than the between individuals in the younger age groups. In another study with Atlantic cod larvae, Bakke et al., (2015) showed that there was a higher variability between individual's microbiota at 61dph (days post hatch) than at younger stages. These observations could be attributed to the physiological changes in a developing alimentary channel which could affect the composition of the gut microbiota (Li et al., 2103) there by leading to a higher diversity among individuals. On the contrary Bledsoe et al., (2016) found out that the similarity of the gut microbiota among individual channel catfish increased with age.

At t2, OTU-4 (*Brevinema*), OTU-12 (*Vibrio*), OTU-20 (*Moritella*) and OTU-1312 (*Eubacterium*) were more abundant in fries in production batch 5S than in the younger fries in production batch 1R and 1A (Table 3.3; OTU-20 and OTU-1312 not shown). These observations indicate the above mentioned OTUs may be markers of an older gut physiology in the gut microbiota of fries. Also at

first and second sampling time, OTU-8 (*Delftia*), OTU-9 (*Zoogloea*), OTU-13 (*Acinetobacter*) and OTU-23 (*Comamonas*) were more abundant in the younger fires in production batch 1A and 1R than older fires in production batch 5S (Table 3.3; OTU-13 and OTU-23 not shown). However, OTU-12 representing *Vibrio* were sporadically abundant in some members. This might be the reason for the high interindividual variation for the oldest fries in production batch 5S. This observation further suggests that some of the individuals were heavily colonized by this strain. In our study, gut microbiota between production batches, at the second sampling time (t2) had a higher abundance of OTU-1 (*Propionibacterium*), OTU-9 (*Zoogloea*) and OTU-8 (*Delftia*) in younger fries in production batch 1R and 1A than in older fries in production batch 5S (Table 3.3, data not present OTU-8 at t2). Stephens et al., (2016) reported the presence of *Zoogloea* in gut microbiota of Zebra fish larvae where its relative abundance was highest and later dropped in the juvenile and adult stage. Also, gut microbiota between production batches at t2 had a higher abundance of OTU-4 (*Brevinema)* and OTU-12 (*Vibrio*) in the older fries in production batch 5S than younger fries in production batch 1A and 1R (Table 3.3, data not present for OTU-4 at t2).

The PCoA plot indicated that the gut microbiota in each production batch clustered according to sampling time (t1-t2) (t2 36 days after t1). This indicates that the gut microbiota might be less influenced by the sampling time than production batch. The sampling times reflected an increase in age of fries in each production batch (t1-t2) (t2 36 days after t1). It is also possible that the environmental microbial communities changed between this timepoints in each production batch. Temporal changes were reported in Atlantic salmon smolt faecal microbiota (Zarkasi et al., 2014; Zarkasi et al., 2016) and gut microbiota of other teleost's species like Asian silver carp (Lin et al., 2016). Conversely, the gut microbiota of juvenile rainbow trout (Heikkinen et al., 2006) and gizzard shad (Lin et al., 2016) wasn't influenced by temporal changes but by feed type and location respectively. Our study showed that gut microbiota among individual fries in production batch 1A and 5S became more similar at t2 than t1 (Fig 3.17). Furthermore, fries across all production batches shared 29 and 59 microbial genera at sampling time t1 and t2 respectively. This may be the reason for a more similar gut microbiota in production batch 1A and 5S at t2. Furthermore, the more developed gut physiology at t2 provided more niches for gut microbial colonization of fries in all production batches. Between sampling times (t1-t2) in each production batch the gut microbiota in production batch 1R increase in abundance of OTU-9 (*Zoogloea*) while fries in production batch 1A and 5S had a decrease in abundance of this genus (Table 3.4: OTU-9 not

shown for 5S). Also, for younger fries in production batch 1R and 1A the average abundance of OTU-8 (*Delftia*), OTU-12 (*Vibrio*) decreased with increasing sampling time (t1-t2) (Table 3.4). Lastly, the average abundance of OTU-12 (*Vibrio*) and OTU-4 (*Brevinema*) increased and decreased respectively in production batch 5S as sampling time increased (t1-t2) (Table 3.4).

4.4 Effect of production batch and sampling time on the skin microbiota of salmon fries.

The type of water environment has been shown to affect the skin microbiota of Atlantic salmon when bred in freshwater and seawater (Lokesh and Kiron, 2016). However, in another study, the skin microbiota of post smolt salmon was highly dissimilar from the water microbiota (Minniti et al., 2017). Information on the effect of rearing systems and age on the skin microbiota of developing fish larvae is limited. Our study indicated that the production batch might have been a higher determinant in the architecture of the skin microbiota than the sampling time. Skin microbiota seemed to be more influenced by the distinct age groups of the production batches rather than the type of aquaculture system. This was because, the skin microbiota of younger fries in production batch 1A and 1R were more similar to each other and distinct from the older fries in production batch 5S. The younger fries in production batch 1A and 1R had a more similar skin microbiota, although they were reared in the FTS and RAS, respectively. A similar trend was observed when the effect of production batch was investigated in the gut microbiota. In our study, Skin microbiota between production batches at t1 and t2 had variations in the abundance of *Propionibacterium, Zoogloea* and *Delftia* (Table 3.5). Also, at t1 and t2, the abundance of *Vibrio* and *Brevinema* was low or absent and varied (data not shown: 0.0-0.5%) between skin microbiota of the production batches.

The sampling time seemed to be a less important determinant of the skin microbial composition than the production batches probably because the sampling time reflect a smaller change in age. In another study the skin microbiota of some saltwater fish was influenced by temporal changes, although location and species where stronger determinants (Larsen et al., 2013). Skin microbiota in each production batch had the younger fries in production batch 1R and 1A cluster together at both t1 and t2 unlike the oldest fries in production batch 5S which had a distinct skin microbiota

at both t1 and t2 (Fig 3.20b-d) suggesting the skin microbiota of the oldest fries in production batch 5S had larger differences in skin microbiota between sampling times (t1 and t2) than fries in production batch 1R and 1A (Fig 3.21). Interindividual variation in skin microbiota have been associated with host related selective pressure, with a correlation between host genotype and prevalence of specific microbial taxa (Boutin et al., 2014). Apart from showing variation in skin microbiota between body parts of same fish, Chairello et al. (2015) also reported a high interindividual variation in the skin microbiota of sea bream and sea bass. Skin microbiota between sampling times (t1 and t2) of each production batch had the younger fries in production batch 1A and 1R increase in the abundance of *Propionibacterium* while the older fries in production batch 5S had a decrease in abundance of this genus in its skin microbiota (Table 3.6). Also, the skin microbiota of each production batch had the abundance of *Zoogloea* and *Vibrio* increase and, vary respectively between sampling times (t1 and t2) (Table 3.6, data not shown for *Vibrio*).

5. Conclusion

Due to our inability to improve the PCR protocol for amplification of the bacterial 16S rRNA gene from the samples of the first feeding experiment at Frøya, we tried to optimize PCR protocols by adjusting the annealing temperature, PCR cycles, PCR primers and DNA concentrations. We did not succeed in establishing a PCR protocol for the Frøya samples. The gut and skin microbiota for fry from a commercial smolt production system (SalMar Settefisk) was therefore characterised, and the effect of age and production systems on gut and skin microbiota of salmon fries was analysed. The gut and skin microbiota were different, and were dominated by phyla *Proteobacteria, Actinobacteria* and *Firmicutes*. OTUs that contributed most dissimilarity between gut and skin microbiota were OTU-1 (*Propionibacterium*), OTU-9 (*Zoogloea*), OTU-8 (*Delftia*), OTU-12 (*Vibrio*) and OTU-4 (*Brevinema*). *Propionibacterium*, *Zoogloea* and *Delftia* were more abundant in the skin microbiota while *Vibrio* and *Brevinema* were more abundant in the gut microbiota. This is first study to show a high abundance of *Zoogloea* in the skin microbiota of a teleost. The gut microbiota had a higher alpha diversity then the skin microbiota while the skin microbiota had a higher beta diversity than its gut counterpart. Despite the absence of a statistically significant differences in the gut and skin microbiota between production batches and sampling times, production batches seemed to have more effect on the gut and skin microbiota. This was probably because of the higher age difference that exist between the production batches than the sampling times. The age component of the production batch seemed to be a larger determinant than the aquaculture system, because the gut and skin microbiota for the younger fries in production batch 1A and 1R that had been reared in distinct systems (FTS and RAS, respectively) was more similar to each other than to the older fries in production batch 5S. Furthermore, the gut microbiota of fries in production batch 1A and 5S became more similar to each other as sampling time increased, indicating a development of the gut microbiota with age. The skin microbiota of oldest fries in production batch 5S had the most difference among individuals at second sampling time (t2) indicating that the skin microbiota became more variable among individuals with increasing age. Nevertheless, The results obtained here indicate that using different batches of the DNA extraction kit may introduce differences in the community profiles of the samples due to contaminating DNA associated with the kit. We therefore suggest that if possible, the same production batch of DNA extraction kit should be used for all samples, or apply DNA extraction protocols that are less hampered by contaminating DNA. It is also important to include a DNA extraction blank sample every time DNA

extraction is performed and also sequence all the DNA extraction blank. This would help identify OTUs that are potential contaminants.

6. Future Studies

It would be interesting to see how the effect of genetics and feed affect the gut microbiota of salmon fries, if a PCR protocol that optimize the PCR amplification is achieved. Moreover, a study that shows how age and rearing system affect the gut and skin microbiota of Atlantic salmons at older lifestages by use of same sampling design from the Follafoss commercial production system should be considered. For an in-depth study of the effect of age and rearing system, an experimental design that enables the separation of these effects will be helpful. In such study, a good experimental design that investigates temporal changes over a longer time would help reveal the changes with age within the same group of fishes. More so,, if we compare different groups at only one time, we would be unable to clearly state if what we see is a general trend or a trend characteristic to only that time point. Lastly, a higher number of replicates should be included during the sampling to enable proper statistical testing during measurement of the beta diversity.

7. References

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Appendices

Appendix 1: DNA extraction protocol for gut and skin samples

1. Add sample in a 1.5 ml microcentrifuge tube. If the samples are frozen do not thaw them before you add the ATL buffer
2. Vortex after Adding 180µL Buffer ATL 20µL Proteinase K
3. Incubate at 56 °C for 3 hours and vortex for every 30 minutes
4. Briefly centrifuge the 1.5 ml tube to remove drops from inside the lip and add 200µL Buffer AL before Vortexing for 15s
5. Incubate at 70 °C for 10 min and briefly centrifuge the 1.5 ml tube to remove drops from inside the tip
6. Add 200µL ethanol (96 – 100%) and Vortex for 15s
7. Briefly centrifuge the 1.5 ml tube to remove drops from inside the tip and transfer the mixture from previous step (including the precipitate) to the **QIAamp mini spin column** (in a 2ml collection tube) without wetting the rim
8. Close the cap and centrifuge at 6000xg (8000 rpm) for 1 min
9. Place the **QIAamp mini spin column** (in a 2ml collection tube provided) and add 500µL Buffer AW1 to the spin column
10. Close the cap and centrifuge at 6000xg (8000 rpm) for 1 min
11. Place the **QIAamp mini spin column** (in a 2ml collection tube provided) and add 500µL Buffer AW2 to the spin column
12. Close the cap and centrifuge at full speed (20.000xg) for 3 min
13. Place the **QIAamp mini spin column** in the same collection tube and centrifuge at full speed (20.000xg) for 1 min
14. Place the **QIAamp mini spin column** in a 1.5 ml collection tube (not provided) and add 100µL Buffer AE
15. Incubate at room temperature for 5 min and centrifuge at 6000xg for 1 min

Appendix 2: Protocol for DNA precipitation

1. Add 1/10 volume of Sodium Acetate (3 M, pH 5.2) to the DNA prep in an Eppendorf tube.
2. Add 2.5 X volume (calculated after addition of sodium acetate) of at least 95% ethanol.
3. Incubate at room temperature or on ice for at least 15 minutes. In case of small DNA fragments or high dilutions, overnight incubation gives better results.
4. Centrifuge at > 14,000 × g for 30 minutes at room temperature (or 4°C).
5. Discard supernatant carefully, making sure the DNA pellet (which may not be visible) is not discarded.
6. Rinse with 200-300 µl 70% ethanol (70% ethanol-30% water mixture).

7 Centrifuge again at > 14,000 x g for 15 minutes.
8 Discard supernatant.
9 Let pellet air dry by opening the lid of the Eppendorf tube and leave on the bench for 10-30 minutes.
10 Dissolve pellet in water. Make sure that the water comes in contact with the whole surface of the tube since a significant portion of DNA may be deposited on the walls instead of in the pellet.

Example of volumes for precipitation in Eppendorf tubes and 10 times concentration:

200 µl DNA-prep + 20 µl NaAc + 550 µl EtOH.

Dissolve the final pellet in 20µl H_2O (after washing with 79% EtOH).

NB! Take care to not introduce DNA contaminations; i.e. work in the UV cabinet with clean pipettes and solutions.

Appendix 3: preparation of TEA buffer

1 Preparation of 50 × TAE-buffer per liter

a Add 242g of Tris base to 57.1ml glacial acetic acid

b Add 100ml of 0.5M EDTA (PH 8.0) to the initial mix and fill up to 1L with distilled water

c Autoclave the resultant mix

2 Preparation of 1 × TAE buffer

a Dilute 40ml 50 ×TAE buffer in 1960ml Mili-Q water

Appendix 4: Concentration of extracted DNA from the gut and skin of salmon fry

t3 (26/04/17)

Number	Batch	Organ	Individual	DNA conc (ng/µL)
1	1R	Gut	1	14.85
2	1R	Gut	2	9.51
3	1R	Gut	3	4.68
4	1R	Gut	4	5.87
5	1R	Gut	5	4.5
6	1R	Skin/mucus	1	6.15
7	1R	Skin/mucus	2	7.21
8	1R	Skin/mucus	3	5.67
9	1R	Skin/mucus	4	5.83
10	1R	Skin/mucus	5	5.91
11	1A	Gut	1	3.62
12	1A	Gut	2	5.88
13	1A	Gut	3	3.72
14	1A	Gut	4	4.53
15	1A	Gut	5	5.17
16	1A	Skin/mucus	1	13.81
17	1A	Skin/mucus	2	8.9
18	1A	Skin/mucus	3	11.18
19	1A	Skin/mucus	4	17.59
20	1A	Skin/mucus	5	8.35
21	5S	Gut	1	3.84
22	5S	Gut	2	4.06
23	5S	Gut	3	4.92
24	5S	Gut	4	6
25	5S	Gut	5	9.4
26	5S	Skin/mucus	1	21.24
27	5S	Skin/mucus	2	16.4
28	5S	Skin/mucus	3	16.4
29	5S	Skin/mucus	4	10.11
30	5S	Skin/mucus	5	7.75

t2 (30/03/17)

Number	Batch	Organ	Individual	DNA concentration (ng/µL)
31	1R	Gut	1	100.99
32	1R	Gut	2	70.68
33	1R	Gut	3	20.35
34	1R	Gut	4	52.51
35	1R	Gut	5	68.89
36	1R	Skin/mucus	1	109.24
37	1R	Skin/mucus	2	121.62
38	1R	Skin/mucus	3	118.87
39	1R	Skin/mucus	4	108.8
40	1R	Skin/mucus	5	93.17
41	1A	Gut	1	5.29
42	1A	Gut	2	1.84
43	1A	Gut	3	2.84
44	1A	Gut	4	1.74
45	1A	Gut	5	2.49
46	1A	Skin/mucus	1	49.99
47	1A	Skin/mucus	2	43.3
48	1A	Skin/mucus	3	61.26
49	1A	Skin/mucus	4	39.11
50	1A	Skin/mucus	5	58.36
51	5S	Gut	1	4.7
52	5S	Gut	2	6.66
53	5S	Gut	3	3.13
54	5S	Gut	4	7.17
55	5S	Gut	5	4.9
56	5S	Skin/mucus	1	12.72
57	5S	Skin/mucus	2	26.17
58	5S	Skin/mucus	3	25.11
59	5S	Skin/mucus	4	12.63
60	5S	Skin/mucus	5	15.26

t1(22/02/17)

Number	Batch	Organ	Individual	DNA concentration (ng/µL)
61	1R	Gut	1-5	20
62	1R	Skin/mucus	1-5	108.12
63	1A	Gut	1	53.89
64	1A	Gut	2	14.64
65	1A	Gut	3	31.28
66	1A	Gut	4	30.08
67	1A	Gut	5	3.4
68	1A	Skin/mucus	1	17.82
69	1A	Skin/mucus	2	44.58
70	1A	Skin/mucus	3	2.36
71	1A	Skin/mucus	4	42.18
72	1A	Skin/mucus	5	10.22
73	5S	Gut	1	1.86
74	5S	Gut	2	1.53
75	5S	Gut	3	1.02
76	5S	Gut	4	1.95
77	5S	Gut	5	5.46
78	5S	Skin/mucus	1	32.28
79	5S	Skin/mucus	2	32.73
80	5S	Skin/mucus	3	41.77
81	5S	Skin/mucus	4	30.75
82	5S	Skin/mucus	5	44.51

Appendix 5: Composition of fish oil and vegetable oil enriched feed

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

Appendix 6: Amount of reads for all samples after filtering of non-bacterial OTUs. Samples with $\leq 1000\ reads$ are marked with the colour red.

Gut samples	Number of reads	Skin samples	Number of reads
t1-1R-Gut	4819	t1-1R-Skin	1495
t1-1A-Gut-id1	2523	t1-1A-Skin-id1	17641
t1-1A-Gut-id2	2752	t1-1A-Skin-id2	42822
t1-1A-Gut-id3	23621	t1-1A-Skin-id3	54705
t1-1A-Gut-id4	22973	t1-1A-Skin-id4	89480
t1-5S-Gut-id1	28056	t1-5S-Skin-id1	124
t1-5S-Gut-id2	20503	t1-5S-Skin-id2	149
t1-5S-Gut-id3	32661	t1-5S-Skin-id3	174
t1-5S-Gut-id4	57593	t1-5S-Skin-id4	436
		t1-5S-Skin-id5	69251
t2-1R-Gut-id1	818	t2-1R-Skin-id1	2216
t2-1R-Gut-id2	2570	t2-1R-Skin-id2	2689
t2-1R-Gut-id3	1333	t2-1R-Skin-id3	4162
t2-1R-Gut-id4	6833	t2-1R-Skin-id4	17093
t2-1A-Gut-id1	31040	t2-1A-Skin-id1	494
t2-1A-Gut-id2	37910	t2-1A-Skin-id2	532
t2-1A-Gut-id3	46337	t2-1A-Skin-id3	5157
t2-1A-Gut-id4	43320	t2-1A-Skin-id4	8996
t2-5S-Gut-id1	34667	t2-5S-Skin-id1	855
t2-5S-Gut-id2	41690	t2-5S-Skin-id2	1852
t2-5S-Gut-id3	37063	t2-5S-Skin-id3	5066
t2-5S-Gut-id4	70419	t2-5S-Skin-id4	34335
t3-1R-Gut-id1	28946	t3-1R-Skin-id1	3624
t3-1R-Gut-id2	31786	t3-1R-Skin-id2	5462
t3-1R-Gut-id3	46147	t3-1R-Skin-id3	18224
t3-1R-Gut-id4	58229	t3-1R-Skin-id4	59590
t3-1A-Gut-id1	31951	t3-1A-Skin-id1	9837
t3-1A-Gut-id2	39998	t3-1A-Skin-id2	8809
t3-1A-Gut-id3	42641	t3-1A-Skin-id3	16052
t3-1A-Gut-id4	52772	t3-1A-Skin-id4	20581
t3-5S-Gut-id1	40717	t3-5S-Skin-id1	13
t3-5S-Gut-id2	34615	t3-5S-Skin-id2	13098
t3-5S-Gut-id3	61304	t3-5S-Skin-id3	14253
t3-5S-Gut-id4	63182	t3-5S-Skin-id4	38743
NKC	70474		

Appendix 7: Rearing condition and feeding information of fries reared in the RAS and FTS

Dato	Innleggsnavı	Kar	System	Temperatur	pH	Utfôring kg	SFR %
22.02.2017	1A-17-01-11	208	FT	14		2,1	4,40
22.02.2017	2R-17-07-11	R11	RAS	13,4	6,9	0,7	3,20
22.02.2017	5S-16-29-11	614	FT	3,7		15,4	0,52
30.03.2017	1A-17-01-11	605	FT	11,3		43,5	2,45
30.03.2017	2R-17-07-11	R11	RAS	14,4	7,1	7,7	3,96
30.03.2017	5S-16-29-11	U12	FT	2		22,5	0,33
25.04.2017	1A-17-01-11	605	FT	12		50,2	1,51
25.04.2017	2R-17-07-11	R11	RAS	14,3	7,1	23,8	3,62
25.04.2017	5S-16-29-11	U24	FT	3,2		37,7	0,30

Appendix 8: Volume of the master mix composition used in a single PCR

PCR component	Volume (ul)
Mili Q water	15.31
5 × Phusion buffer HF	5.0
dNTP (10Mm)	0.5
DNA polymerase (5U/µL)	0.18
$MgCl_2g$ (50Mm)	0.25
ILL 805R (10µM)	0.75
ILL 515F (10µM)	0.75
Spermidine (10µM)	1.25

Appendix 9: Normalization Plate kit protocol by Invitrogen

SequalPrep™ Normalization Plate (96) Kit

Catalog no: A10510-01 **Store at room temperature (15–30°C)**

Materials Needed

- PCR reactions containing amplicons of the desired length (see **Generating PCR Amplicon**, above)
- DNase-free, aerosol barrier pipette tips
- *Optional:* automated liquid handling workstation capable of handling and manipulating 96-well plates
- *Optional:* PureLink™ Foil Tape (see previous page)

Binding Step

1. Transfer the desired volume of PCR product (5–25 µl PCR reaction mix, at least 250 ng amplicon/well) from the PCR plate into the wells of the SequalPrep™ Normalization plate.
2. Add an equivalent volume of SequalPrep™ Normalization Binding Buffer.
   For example: To purify 10 µl of PCR product, add 10 µl SequalPrep™ Normalization Binding Buffer.
3. Mix completely by pipetting up and down, or seal the plate with PureLink™ Foil Tape (page 1), vortex to mix, and briefly centrifuge the plate.
4. Incubate the plate for 1 hour at room temperature to allow binding of DNA to the plate surface. Mixing is not necessary at this stage.
   Note: Incubations longer than 60 minutes do not improve results. However, depending on your workflow you may perform overnight incubation at room temperature for the binding step.
5. **Optional:** If >25 ng DNA/well yield is desired, transfer the amplicon/Binding Buffer mixture from Step 4 to another, fresh well/plate to sequentially bind more DNA. Perform DNA binding at room temperature for 1 hour.
   Note: After binding is complete, you can remove the amplicon/Binding Buffer mixture from the well and store at -20°C for up to 30 days to perform additional purifications at a later time.
6. Proceed to **Washing Step**, next page.

Washing Step

1. Aspirate the liquid from wells. Be sure not to scrape the well sides during aspiration.
   Note: If you wish to store the amplicon/Binding Buffer mixture for additional purifications at a later time, aspirate the liquid from wells into another plate and store at -20°C for up to 30 days.
2. Add 50 µl SequalPrep™ Normalization Wash Buffer to the wells. Mix by pipetting up and down twice to improve removal of contaminants.
3. Completely aspirate the buffer from wells and discard.
   To ensure complete removal of wash buffer and maximize elution efficiency, you may need to invert and tap the plate on paper towels depending on the pipetting technique or instrument used. A small amount of residual Wash Buffer (1–3 µl) is typical and does not affect the subsequent elution or downstream applications.
4. Proceed to **Elution Step**, below.

Elution Step

Review **Elution Options** (previous page).

1. Add 20 µl SequalPrep™ Normalization Elution Buffer to each well of the plate.
   Note: Do not use water for elution. If you need to elute in any other buffer, be sure to use a buffer of pH 8.5–9.0. If the pH of the buffer is <8.5, the DNA will not elute efficiently.
2. Mix by pipetting up and down 5 times or seal the plate with PureLink™ Foil Tape (page 1), vortex to mix, and briefly centrifuge the plate. Ensure that the buffer contacts the entire plate coating (up to 20 µl level).
3. Incubate at room temperature for 5 minutes.
4. Transfer and pool the purified DNA as desired or store the eluted DNA at 4°C (short-term storage) or -20°C (long-term storage) until further use.

Expected Yield and Concentration

The expected DNA concentration is 1–2 ng/µl when using 20 µl elution volume. The expected DNA yield is ~25 ng/well normalized.

Appendix 10: How to Use Amicon® Ultra-0.5 Centrifugal Filter Devices

1. Insert the Amicon® Ultra-0.5 device into one of the provided microcentrifuge tubes.

2. Add up to 500 µL of sample to the Amicon® Ultra filter device and cap it.

3. Place capped filter device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.

4. Spin the device at 14,000 × g for approximately 10–30 minutes depending on the NMWL of the device used

5. Remove the assembled device from the centrifuge and separate the Amicon® Ultra filter device from the microcentrifuge tube.

6. To recover the concentrated solute, place the Amicon® Ultra filter device upside down in a clean microcentrifuge tube. Place in centrifuge, aligning open cap towards the center of the rotor; counterbalance with a similar device. Spin for 2 minutes at 1,000 × g to transfer the concentrated sample from the device to the tube. The ultrafiltrate can be stored in the centrifuge tube.

NOTE: For optimal recovery, perform the reverse spin immediately.

Appendix 11: Procedure for performing Indexing PCR

1 Add the following volumes in the order listed to each well of a new Hard-Shell skirted PCR plate. Pipette to mix. u TD (10 µl) u Normalized gDNA (5 µl)

2 Add 5 µl ATM to each well. Pipette to mix.

3 Centrifuge at 280 × g at 20°C for 1 minute.

4 Place on the preprogramed thermal cycler and run the tagmentation program. When the sample reaches 10°C, immediately proceed to step 5 because the transposome is still active.

5 Add 5 µl NT to each well. Pipette to mix.

6 Centrifuge at 280 × g at 20°C for 1 minute.

7 Incubate at room temperature for 5 minutes. The PCR plate contains 25 µl tagmented and neutralized gDNA, all of which is used in the next step.

8 Amplify libraries with temperature cycling program in Table 2.4