Master's thesis

NTNU
Norwegian University of Science and Technology
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Initial bacterial colonization of yolk-sack fry in Atlantic Salmon (Salmo Salar) using a gnotobiotic system.

Master's thesis in MBIOT5
Supervisor: Ingrid Bakke
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Abstract

Fish larvae are germ free when hatched but gets quickly be colonized by surrounding microorganisms. The early colonization is known to be important for the health and development for the host. The gut microbiota associated with fish larvae, is highly dynamic because of a rapidly changing gut environment. The gut and skin microbiota of fish have been found to be very different from the surrounding water, but little is known about the initial colonization of the skin and gut of fish larvae. Atlantic salmon (*Salmo salar*) are relatively large fry with a long yolk-sack period, making it possible to study interactions between bacteria and the host over a long period in a germ-free environment.

The aim of this study was to investigate the early colonization of salmon fry by using germ free yolk-sack fry exposed to certain bacterial strains separately or in combination with other strains (i.e a gnotobiotic model). Salmon eggs were made germ free by surface sterilization in and then exposed to strains representing *Bacillus, Pedobacter, Arthrobacter, Psychrobacter, Janthinobacterium* and *Flavobacterium*, previously isolated from salmon fry skin and gut, by addition to the rearing water. In Experiment 1, yolk-sack fry were subject to long-term exposure from 7-35 days post hatching (dph). In Experiment 2, 10-week-old yolk-sack fry were subject to short-term exposure for 5 days. The ability of the strains to colonize the gut and skin was investigated by CFU counting. The composition of the gnotobiotic society, and possible interactions between the strains were investigated by Illumina 16S rDNA sequencing, the interactions between the *Janthinobacterium* strain and other strains was especially interesting because it is closely related to *Janthinobacterium Lividum* which has shown to have antibiological effects.

No mortalities were observed in any experiments. No significant difference in growth of the salmon fry was observed between bacterial treatments in Experiment 1. Very low levels of colonization were observed in the gut, and skin was colonized to a much larger degree. *Bacillus* were unable to colonize both the water and the fry and were believed to represent an airborn contaminant. *Janthinobacterium* was the most efficient colonizer of skin, while *Pedobacter* and *Janthinobacterium* was the most efficient colonizers of gut. All strains that were able to adhere to the fish mucus in the short-term experiment in various levels, were also the ones that were able to maintain their population in skin and gut in the long-term experiment. The *Janthinobacterium* strain seemed to benefit from the presence of other bacterial strains, indicating some sort of competition or commensal interaction, this is especially interesting because *Janthinobacterium* strains are known to have antibiological effects which could be part of the explanation for these results. Ten weeks old salmon fry were colonized by bacteria to a much larger degree then the four weeks old salmon fry.

Sammendrag

Fiskelarver er bakterie frie når de klekkes ut, men blir raskt kolonisert av omgivende mikroorganismer. Den tidlige koloniseringen er kjent for å være viktig for helsen og utviklingen for verten. Tarmmikrobiotaen assosiert med fiskelarver er svært dynamisk på grunn av et raskt skiftende miljø i tarmen. Tarm og skinn mikrobiota av fisk har vist seg å være veldig forskjellig fra det omkringliggende vannet, men lite er kjent om den innledende koloniseringen av skinn og tarm til fiskelarver. Atlanterhavslaks (*Salmo salar*) har relativt store yngel med et langt plommesekk-stadie, noe som gjør det mulig å studere interaksjoner mellom bakterier og verten over en lang periode i et bakteriefritt miljø.

Målet med denne studien var å undersøke den tidlige koloniseringen av lakseyngel ved å bruke bakteriefri plommesekkyngel som ble utsatt for visse bakteriestammer enkeltvis eller i kombinasjon med andre stammer (dvs. en gnotobiotisk modell). Laksegg ble gjort bakteriefrie ved overflatesterilisering i og deretter utsatt for stammer som representerte *Bacillus, Pedobacter, Arthrobacter, Psychrobacter, Janthinobacterium* og *Flavobacterium*, tidligere isolert fra laksyngel skinn og tarm, ved å tilsette bakterier til oppdrettsvannet. I eksperiment 1 ble plommesekkyngel utsatt for langvarig eksponering fra 7-35 dager etter klekking (dph). I eksperiment 2 ble 10 uker gammel plommesekkyngel utsatt for kortvarig eksponering i 5 dager. Stammenes evne til å kolonisere tarm og skinn ble undersøkt ved CFU-telling. Sammensetningen av det gnotobiotiske samfunnet og mulige interaksjoner mellom stammene ble undersøkt ved Illumina 16S rDNA-sekvensering, interaksjonene mellom *Janthinobacterium*-stammen og andre stammer var spesielt interessant fordi den er nært beslektet med *Janthinobacterium lividum* som har vist seg å ha antibiologiske effekter.

Ingen dødsfall ble observert i noen av eksperimentene. Det ble ikke observert noen signifikant forskjell i vekst av lakseyngel mellom bakteriebehandlinger i forsøk 1. Det ble observert meget lave nivåer av kolonisering i tarmen, mens huden ble kolonisert i mye større grad. Alle stammer som klarte å feste seg til fiskeslimet i det kortsiktige eksperimentet i forskjellige nivåer, var også de som var i stand til å opprettholde sin bestand i skinn og tarm i det langvarige eksperimentet. *Bacillus* klarte ikke å kolonisere verken vannet eller fisken, og antas å representere en luftbåren kontaminant. *Janthinobacterium* var den mest effektive kolonisatoren av skinnet, mens *Pedobacter* og *Janthinobacterium* var de mest effektive kolonisatorene i tarmen. *Janthinobacterium* stammen så ut til å dra fordel av tilstedeværelsen av andre bakteriestammer, noe som indikerer en slags konkurranse eller kommensal interaksjon, dette er spesielt interessant fordi *Janthinobacterium* stammer er kjent for å ha antibiologiske effekter som kan være en del av forklaringen på disse resultatene. Ti uker gammel lakseyngel ble kolonisert av bakterier i mye større grad enn de fire uker gamle lakseynglene.

Abbreviations

CFU	Colony forming units
CN	Copy number
CVR	Conventionally raised
CVZ	Conventionalized by adding non-sterile water
FAO	Food and agriculture organization
GF	Germfree
GI	Gastrointestinal tract
OD	Optical density
PCR	Polymerase chain reaction
qPCR	Real-time polymerase chain reaction
S1_Ba	*Bacillus*
S2_Pe	*Pedobacter*
S3_Ar	*Arthorbacter*
S4_Ps	*Psychrobacter*
S5_Ja	*Janthinobacterium*
S6_Fl	*Flavobacterium*
SGM	Salmon growth medium
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultra-violet lightning
BHI	Brain heart infusion
SD	Saboraud dextrose
dph	Days post hatching
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
rDNA	Ribosomal Deoxyribonucleic acid
rRNA	Robonucleic acid

NTC Non-template control

Innhold

1 Introduction

1.1 Atlantic Salmon

Atlantic salmon (*Salmo Salar*) is a species from the Salmonidae family that habitats the north Atlantic on both the European side and the north American side (FAO Fisheries & Aquaculture, n.d.). Atlantic salmon is an anadromous species, meaning that they live in both freshwater and marine waters. Adult salmons live in the sea but migrates to freshwater rivers to spawn when they have reached sexual maturity. During spawning they release eggs in gravel nests were the river is well oxygenated by the river flow. Upon hatching the tiny fish is called alevins and they all have a large yolk-sack which provides nutrients for the fish, meaning they don't need to feed at this stage. The spawning happens in the winter and by spring the alevins still have their yolk-sack. This period lasts a couple of months. When the yolk-sack is consumed, and they start feeding the fish is called fry. They remain as fry throughout the summer where they feed on microscopic invertebrates. During the autumn they develop into parr which feed on smaller insects and they will remain at this stage for up to 3 years before they smoltify. Smoltification involves a series of changes for the fish including adaptions to the salty water of the ocean and swimming with the current instead of against it. Smolts between one to three years migrate to the ocean to feed and grow during the spring. Salmon reach maturity in one to three years at sea and when they do, they return to the river again to spawn. Salmon has a remarkable "homing instinct" and use the earth's magnetic field to locate the river which they were hatched in(Marine Institute, n.d.). The complete life cycle of Atlantic salmon is summarized in Fig. 1.

The yolk sac stage or alevin stage is a crucial stage for the developing fry. The mean egg-to-fry survival rate is 2-35% but can vary from 0-90% between spawning nests in the same river, meaning that they are very sensitive to changes in water parameters. The most important water parameter is oxygen. High mortalities is often caused by lack of oxygen caused by low intergravel water flow, as well as the dispersion of toxic metabolic wastes (Dumas and Marty, 2006). The respiratory system of alevins is poorly developed and the oxygen uptake is cutaneous (Wells and Pinder, 1996). Temperature is another important water parameter affecting the alevins, they can tolerate temperatures between 0-16 ^{o}C and within these temperatures, the rate of yolk-sac absorption increases with increasing temperature(Peterson et al., 1977).

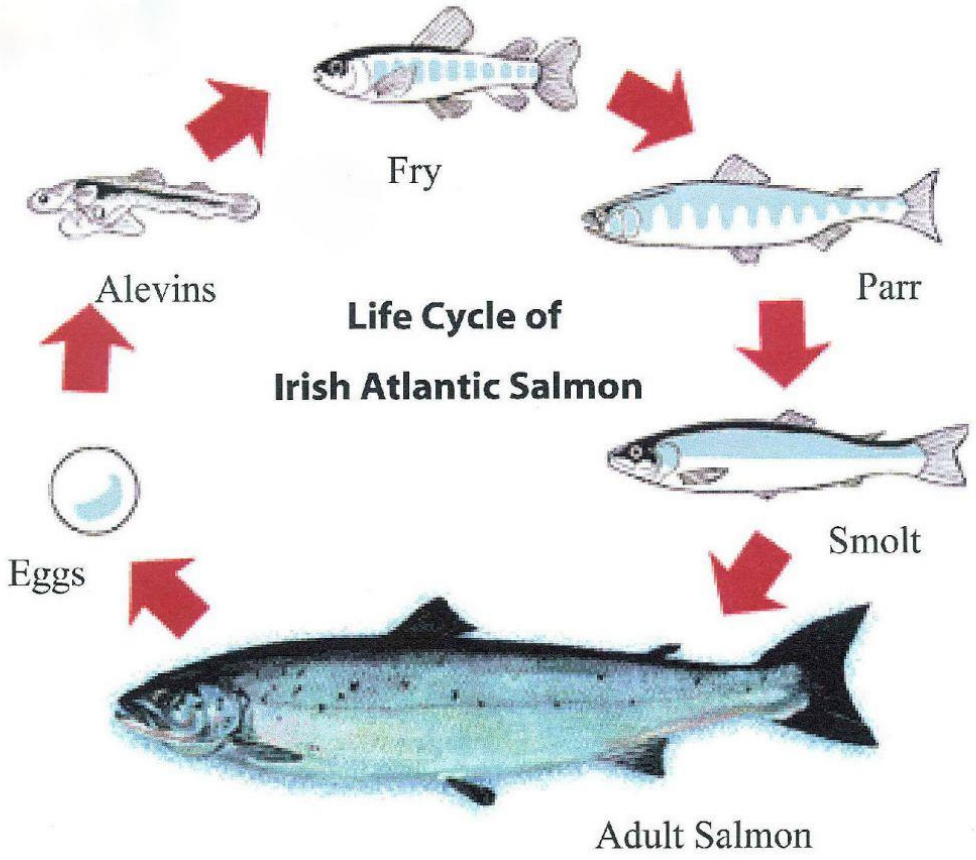


Figure 1: Life cycle of Atlantic salmon(Marine Institute, n.d.)

1.2 Aquaculture

Even though the growth of aquaculture has decreased the last few years it is still the fastest growing food-producing sector and aquaculture now accounts for almost 50 percent of the worlds food fish ("FAO Fisheries & Aquaculture - Aquaculture," n.d.). Excluding marine plants, aquaculture production has increased from 3 million tons in 1970 to 80.1 million tons by 2017 and employs approximately 26 million workers. ("Aquaculture," n.d.). In Norway the aquaculture dates to 1850 when the first brown trout hatched, but the development of commercial aquaculture began in 1970 and has now developed into a major industry in the coastal areas. Atlantic salmon is by far the most important species in Norwegian aquaculture and accounts for more than 80 percent of the total Norwegian aquaculture (FAO Fisheries & Aquaculture, n.d.).

What started as a primarily small-scaled and non-commercial activity in freshwater ponds by some Asian families, has now developed to an international aquaculture commercial industry encompassing all aquatic environments and using a range of aquatic species. The reason for this is the increasing demand of fish food and the increased understanding of aquatic biology, technical development and innovations (Subasinghe et al., 2009). In Norway the technological breakthrough came around 1970 when the first salmon cages where constructed. The cages were safer and provided much better environmental conditions than onshore tanks. Also the understanding of the biology of the salmon species is an important factor for its success in aquaculture (FAO Fisheries & Aquaculture, n.d.).

Knowing the life cycle of Atlantic salmon is important when culturing the species. Since the species is anadromous, it needs separate sections with both freshwater tanks for the early life stages until smoltification and saltwater cages for the later life stages after smoltification. Genetic favorable strains of salmon have been picked for commercial breeding. Adults from these strains are picked and transferred from the saltwater cages to freshwater tanks where they acclimatize to the freshwater for about two months before the eggs are stripped and fertilized with milt. The eggs are hatched in trays and transferred to suitable tanks according to their stages in the life cycle (FAO Fisheries & Aquaculture, n.d.). Research on the smoltification process has led to manipulation of this process using lights (Berrill et al., 2003). Selective breeding has increased growth rates of farmed salmon (Gjøen and H.B Bentsen, 1997). By combining these factors with an intensive feeding regime, the production is fastened by decreasing the age of the fish when smolting occurs. (Duston and Saunders, 1995).

Because of the high mortalities from egg to fry (Dumas and Marty, 2006), this is a challenging stage in the aquaculture industry. During this period, the fry are especially vulnerable to changes in water parameters like temperature (Gunnes, 1979; Macqueen et al., 2008), and increase in toxins like ammonia (Knoph, 1992) and cadmium(Rombough and Garside, 1982). Microbial challenges in the early stages is also important. Saprolegniose is a fungal disease that attacks dead matter so dead row is infected, and from these hyphae develops and kills nearby eggs. This disease is challenging because the spores survives disinfecting procedures like UV and ozon treatment. It has earlier been treated with Malachite, but since it was banned in 2000, it has been an increasing problem (Norwegian Veterinary Institute, n.d.). Another microbial disease which is problematic during the early stages is caused by *Yersinia ruckeri,* a gram-negative rod bacterium that causes enteric redmouth disease. This is a special host-microbe interaction where carriers of the infection transfer the disease to healthy fish only when they are being stressed (Tobback et al., 2007). Thus, host microbe interactions in the early stages of Atlantic salmon is an important field of research in order to prevent diseases in the aquaculture industry.

1.3 Host-microbe interactions

Host-microbe interaction is a well-studied field and it is known that animals provide niches for several kinds of bacteria that colonize the host (Claudia Lupp, 2007). Bacteria are able to colonize the skin, intestine, internal organs, respiratory tract and the urogenital tract (Medzhitov, 2007). The interactions between the host and its microbiota can be beneficial, commensal or pathogenic. A relationship that is beneficial for both the host and its microbes is often referred to as symbiotic, while commensal is a relationship where they co-exist without obvious benefits or harming each other. The microbiota in humans and other animals is often referred to as commensals, even though the microbiota has some obvious beneficial effects for the host. Higher animals are unable to digest much of the food they ingest on their own. This undigested food will be utilized as energy by gut microbiota and in return, they transform it by unique digestive enzymatic activities to substances that is digestible for the host. This makes the host able to take up more nutrients and is an example of a beneficial interaction for both the host and the microbiota. In contrast to this, many bacteria are known to damage their host, and this is known as a pathogenic relationship. The pathogen can be harmful by releasing toxins or it can invade and expand in the host's tissue (Steinert et al., 2000). Even though these relationships are defined, it is not always easy to determine the relationship for each type of bacteria because the relationship can vary depending on the nutrients available and the immune system of the host. For example, generally commensal opportunistic bacteria can become harmful to the host if the immune system of the host is weakened (Packey and Sartor, 2009).

Increasing production and stocking density can potentially lead to more stressful conditions for the fish. Stress is a factor that decreases the immune responses and hence enhances the chances for pathogenic infections (Lluis Tort et al., 2003). Host microbe interactions in fish are complex, and even though host-microbe interactions are well studied, most of the research has been on mammalian organisms like mice and humans. Thus the microbe-microbe interactions and host-microbe interactions in teleost fish is poorly understood (Kelly and Salinas, 2017).

1.4 Mucosal tissues

Every animal enters this world as germ free but gets quickly disposed to microbiota that colonize the mucosal tissues and the skin. Mucosal tissues are associated with the digestive, respiratory, urogenital tracts and the skin of fish and amphibians (Maynard et al., 2012; Xu et al., 2013). Mucosal tissues form the mucous membrane which generally has a layer of epithelial cells over a deep layer of connective tissue. The membrane typically contains cells especially adapted for absorption and secretion and the major substance secreted from the membrane is mucus (Encyclopedia Britannica, 2018). The predominant gel-forming macromolecules of mucus are glycoproteins called mucins. Other components are glycosaminoglycans, lysozyme, immunoglobins, carbonic anhydrase, lectins, crinotoxins, calmodulin, C-reactive protein, pheromones and proteolytic enzymes (Shephard, 1994). These components form an ideal niche for microbial adherence and growth (Gomez et al., 2013). There are several types of mucosal surfaces and the most studied is the Type 1 mucosal surfaces found in the gut alimentary, respiratory, and female upper reproductive systems in mammals. Common features of Type 1 mucosal surfaces include the presence of mucus-secreting goblet cells and the expression of polymeric immunoglobulin receptor(plgR) on the basolateral surface of epithelia. The layer acts as immune defense against pathogenic bacteria and the main protective immunoglobulin at Type 1 mucosal surfaces is Immunoglobin A(IgA) (Iwasaki, 2007). At the same time, the mucosal immune system has evolved to permit the colonization of mucosal surfaces with complex and diverse microbial communities (Gomez et al., 2013). This means that the mucosal surface is a selective barrier that prevents pathogens from entering while other molecules enters freely. The main mucosal surfaces of fish are found in the gut, and on the skin and gills where they all share many characteristics with Type 1 mucosal surface of mammals (Fig. 2). Teleost surfaces in the gut also contain mucus-producing cells arranged in a simple columnar epithelium. The mucosal surface in the gills contain one to four layers of cuboidal or squamous epithelial cells and a stratified squamous epithelium is found in the skin. Teleost fish mucosal surfaces contains B cells like IgT/IgZ and although this is different from the IgA in mammals Type 1 mucosa, they are homologues and have mainly the same functions. They also share many other immunological elements, like the presence of T cell, macrophages, mast cells, dendritic cells and the coordinated expression of cytokines. All these are illustrated in Fig. 2.

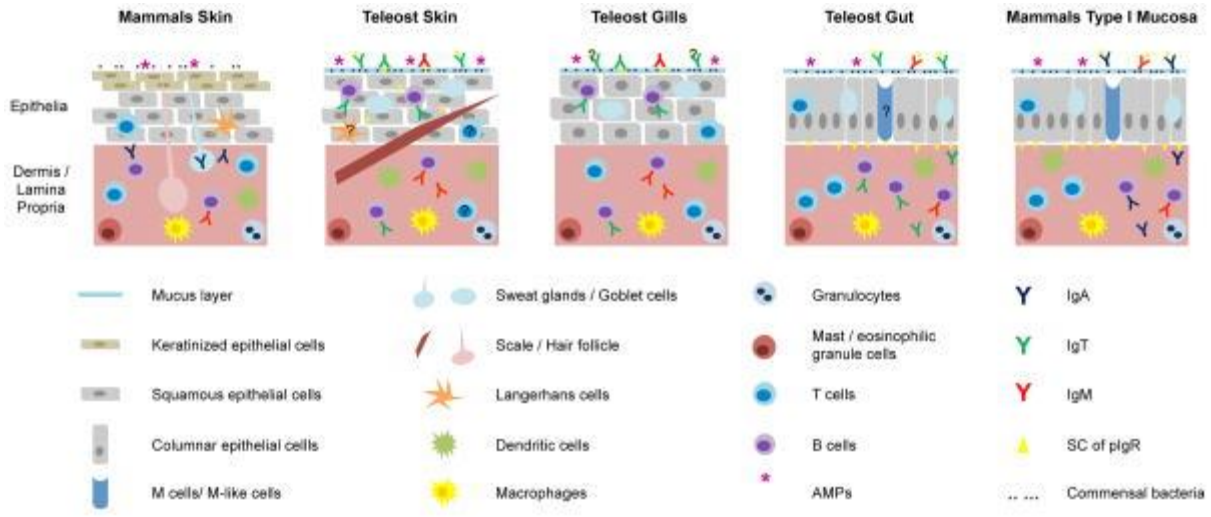


Figure 2: Comparison of mammals skin versus the mucosal surface of teleost skin and gills, and comparison of the mucosal surface in mammals gut versus the mucosal surface in teleost gut (Gomez et al., 2013)

Although there are similarities, the skin structure in fish differs from the skin in mammals. The evolutionary pressure has formed the mucosal surfaces of vertebrates, and while land living animals have adapted to terrestrial environments by evolving feathers, scales, hair, etc. The fish skin is made of a living cell layer that secretes a layer of mucus and has imbricated scales for protection (Lowrey et al., 2015).

Although we are beginning to understand the main functions of mucosal surfaces in teleost fish, the role of the residing bacteria that colonizes these surfaces is poorly understood, especially in skin and gills. While many of the responses to the gut microbiota is evolutionary conserved from fish to mammals (Rawls et al., 2004). There is little knowledge about the role of skin and gill microbiota. Whether the microbiota has a role for the host or just represent microbiota being trapped in the mucus layer is unclear.

1.5 Microbiota associated with fish

The mucosal epithelia are important mechanical and chemical barriers that prevent pathogen invasions, but permit colonization by symbiotic microorganisms, the microbiota. The microbiota is crucial for development, homeostasis and immune function of an animal's mucosal epithelia. Distinct microbial communities inhabit different anatomical sites. Thus the body site is a strong determinant factor for the composition of the microbiota in terrestrial vertebrates(Lowrey et al., 2015).

1.5.1 Gut microbiota

The gut microbiota has lived in symbiotic association with vertebrate hosts for millions of years. It is often considered as an "extra organ" because it plays a crucial role in the intestinal development and physiology, as well as overall development, growth and health (Butt and Volkoff, 2019). The gut microbiota is involved in the regulation of multiple host metabolic pathways, giving rise to interactive host-microbiota metabolic, signaling and immune-inflammatory axes that physiologically connect the gut, liver, muscle and brain (Nicholson et al., 2012). Most of the research has been on mammals and studies have shown conserved responses to the gut microbiota, including those involved in

stimulation of epithelial proliferation, promotion of nutrient metabolism, and innate immune responses (Rawls et al., 2004). Microbes in both zebrafish and mice regulate gut epithelial homeostasis (Fraune and Bosch, 2010). However, the way aquatic animals live suggest that the association between the host intestinal microbiota and the environmental microbiota is closer than for land-bred animals. Aquatic environments are better media for microorganism growth compared to air (Gomez et al., 2013). Fish embryos develop in a constant bacteria-free environment; hence they are microbe free upon hatching. After hatching they are quickly disposed to bacteria by the surrounding environment which can colonize the fish (Butt and Volkoff, 2019). The gut is believed to be colonized when the larvae opens its mouth and digestive tract. Depending on species, this usually takes a couple of days after hatching (Lescak and Milligan-Myhre, 2017). The immune system of newly hatched larva is poorly developed (Uribe et al., 2011). By stimulating mucus production, producing antimicrobial factors and contributing to the regulation of immunological responses it is believed that the microbiota residing in the mucosal tissues of the gut is a first line of defense against pathogens (Abt and Pamer, 2014). In vertebrates, a healthy gut microbiota in adult individuals is rather stable. During the larvae stage of aquatic animals, however, the gut community is highly dynamic because of the interaction between colonizing bacteria and a rapidly changing environment (De Schryver and Vadstein, 2014). Being germ free at hatching also mean that the larvae is highly vulnerable to pathogenic bacteria. Early colonization of commensals and symbionts are thought to outcompete pathogens and improve immune responses (Dimitroglou et al., 2011) (Abelli et al., 2009).

The total bacterial load varies from different species, age and environments, but based on earlier culture dependent research it is estimated to be between 10^4-10^9 CFU/g intestinal content(Shiina et al., 2006; Sugita et al., 2005; Trust and Sparrow, 1974). However, a large number of GI bacteria in fish is unculturable, so studies using culture independent research is probably closer to the reality, estimating a total bacteria load of 10^9-10^{11} CFU/g intestinal content (Shiina et al., 2006; Sugita et al., 2005). Among the bacterial colonizers, *Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes* and *Fusobacterium* are the dominating phyla (Wang et al., 2018). It was previously believed that the gut microbiota is dependent on the surrounding environment, including salinity and feeding ecology (Ringø and Olsen, 1999; Ringø and Strøm, 1994; Sullam et al., 2012). However, several studies have shown that host factors are the major deterministic filters that decide the microbial assemblage in the fish intestine (Li et al., 2014; Rawls et al., 2006; Roeselers et al., 2011; Xuemei et al., 2011; Yan et al., 2016). The water and fish-associated bacterial community in salmon larvae also differs from each other, indicating that host factors are important determinants in salmon as well. In salmon eggs, *Proteobacteria* is the most abundant, but the diversity increases after hatching, reflecting the significant abundance of *Actinobacteria, Firmicutes, Tenericutes, Spirochaetes* and *Deinococcus-Thermus* (Lokesh et al., 2019). Although the microbial gut community in salmon has been characterized by several studies, there is little knowledge on how the microbiota initially colonizes the gut in larvae upon hatching and how the bacteria interfere with each other during colonization.

1.5.2 Skin microbiota

Compared to the gut microbiota, the fish skin microbiota is less understood. The teleost skin is more similar to the Type 1 mucosal tissues of mammals than that of mammalian skin (Fig. 2) (Lowrey et al., 2015). These structural similarities could also indicate similar functionalities. Many bacteria are well adapted to evading or resisting the immunological components of fish mucus and the mucous coat may be an adhesion site for microbes. It is assumed that the skin microbiota acts as a first line of

defense by interfering with pathogen colonization by antagonistic activity and competition for nutrients or adhesion sites (Merrifield and Rodiles, 2015). The establishment of skin microbiota is also poorly studied. The skin is constantly in contact with the surrounding water, so it was earlier believed that the skin microbiota is very similar to the surrounding water. However, several studies have suggested that this is not the case. Chiarello et al. (2015) found that the fish surface from European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) was highly diverse and very different from that of surrounding bacterioplankton. Even though the skin microbiota differs from the environment, the environmental microbes is an important factor that influences the composition of the skin microbiota. Atlantic salmon is anadromous and this shift from freshwater to saltwater influences the microbiome of the skin. The transition to seawater influences the operational taxonomic unit richness and evenness (Lokesh and Kiron, 2016). Other factors like host species specificity, season and mucus composition are also believed to influence the microbial community of the skin (Larsen et al., 2013). A study from Minniti et al. (2017) showed that stress could also be an important factor for the skin microbiota in farmed Atlantic salmon. *Proteobacteria* is the dominating phyla associated with fish skin, followed by *Actinobacteria, Bacteroidetes* and *firmicutes* (Chiarello et al., 2015; Larsen et al., 2013; Lowrey et al., 2015). In farmed Atlantic salmon, *Proteobacteria* is also the most abundant, followed by *Firmicutes* and *Acidobacteria* (Minniti et al., 2017). The bacterial load on fish skin is estimated to be somewhere between 10^2-10^4 CFU/cm^2 (Austin, 2006; Effiong and Isaac, 2019). However, this is based on culture dependent methods, and these methods can underestimate the real bacterial load since many of the residing bacteria is not able to grow on culture media. Very little is known about the early colonization of fish skin, and how environmental factors and bacterial interactions affects this process.

1.6 Gnotobiotic Model Systems for Host-microbe Interactions

The study of host-microbe interactions can be challenging because of the complex composition of the host microbiota. One way of controlling the composition is by making the host germ free or axenic. The terms germ free or axenic refers to an animal that is free of microbes, including bacteria, viruses, fungi, protozoa and parasites (Al-Asmakh and Zadjali, 2015). Once the host is germ free, one or more known strains of bacteria can be added to the host in a controlled way, and this is referred to as a gnotobiotic model. The first gnotobiotic models were based on mammals, such as mice, rats, and guinea pigs. Such models have later on been developed and established successfully to several kinds of fish, including Platy *(Xiphophorus maculatus)* (Baker et al., 1942), Atlantic halibut *(Hippoglossus hippoglossus*) (Verner-Jeffreys et al., 2003), turbot *(Scopthalmus maximus)*(Munro et al., 1995), different types of salmonids (Trust, 1974), *zebrafish (Danio rerio)*(Rawls et al., 2004) *and* Atlantic cod *(Gadus Morhua)* (Forberg et al., 2011). Zebrafish is the most commonly used species for research on host – microbe interactions in teleosts. The reason for this is because of its rapid development, optical transparency and large brood size, making them excellent models for germ free studies (Lescak and Milligan-Myhre, 2017). A protocol for generating germ free Atlantic salmon *(Salmo salar)* has recently been developed at NTNU Department of Biotechnology and food science (Gomez de la Torre Canny et al., in preparation) Atlantic salmon has a relatively large size upon hatching, and a long yolk sack stage, lasting up to two months at standard rearing temperatures (6-7 ^oC) (Webb et al., 2007). Feeding introduces microorganisms and makes it harder to maintain a germ-free environment, but the long yolk-sack period means that the fry doesn't need food for a long period. This makes Atlantic salmon an excellent species for germ free or gnotobiotic model systems and for studying long-term interactions between microbes and their host.

1.6.1 Bacterial isolates from salmon fry

In order to make a gnotobiotic model in a controlled way, bacterial strains that is relevant for the host is needed. The research group ACMS at NTNU IBT has previously isolated a collection of bacterial strains from the skin and gut of yolk-sac salmon fry (Personal communication, Ingrid Bakke). This collection of bacterial strains is being used in gnotobiotic studies with salmon fry as host. The genus of the most relevant strains for this study is presented.

Janthinobacterium

Janthinobacterium is an aerobic gram-negative bacterial species, commonly isolated from the microbiota of soils and waters of rivers, lakes and springs (Ramsey et al., 2015). It is well known for colonizing the skin of some amphibians where it confers protection against fungal pathogens. The strain has capnophilic behavior, meaning that the growth is favored by high concentrations of CO_2 (5%). CO_2 is secreted by amphibian skin alveoli, and is believed to act as a signaling molecule during colonization of the skin (Valdes et al., 2015). The antifungal properties are suggested to be caused by secondary gene clusters and chitinases, including N-acetyl-D-glucosamine and a gene cluster called JQS (Haack et al., 2016). *J. lividum* is well known for its production of the purple-pigmented violacein, and even though the results from Haack et al. (2016) indicate that the antifungal properties is independent of violacein production, several studies have shown that violacein has anti-biological effects like anti-bacterial, anti-tumor and anti-protozoan activities (Aranda et al., 2011; *Masuelli* et al., 2016; Ramsey et al., 2015; Xu et al., 2019) (Pantanella et al., 2007). *Janthinobacterium* was also found to be part of the gut microbiota of Atlantic salmon (*Salmo salar*) (Gajardo et al., 2016), Gills of Turbot *(Scophthalmus maxiumus)* (Cahill, 1990). It has also been isolated from dead fish after disease outbreaks and mortalities (Austin et al., 1992; Jeremic and Radosavljevic, 2015). Thus, it can potentially act as both a pathogen or probiotic, and what determines this relationship is unknown.

Pedobacter

By 2012, the *Pedobacter* genus comprised 34 recognized species and is part of the *Shpingobacteriaceae* family. All members are obligately aerobic, non-spore forming, Gram-stain-negative rods. They lack flagella, but they are motile by gliding. They are oxidase-and catalase-positive but negative for urease activity, indole production and nitrate production. They all lack flexiruibin-type pigments but contain shpingolipids (Zhou et al., 2012). Members of this genus have been isolated from soil, fish, freshwater, a nitrifying inoculum, glacier samples and a Himalayan mountain (Gallego et al., 2006)

Arthrobacter

The *Arthrobacter* genus belongs to the family Micrococcaceae within the suborder Micrococcineae. *Arthrobacter* is unusual in that they appear as Gram-negative rods in young cultures and as Gram-positive cocci in older cultures(Jones and Keddie, 2006). They are highly aerobic and belong to the heterogeneous group of coryneform bacteria (Mages et al., 2008) *Arthrobacter* is commonly isolated from soil, sewage, food and is also abundant in the gut microbiota of fish(Comi and Cantoni, 2016; Nayak, 2010)

Psychrobacter

The *Psychrobacter* genus is part of the Moraxellaceae family and are small coccabacillus ranging from 0.9-1.3 μm in diameter and 1,5-3,8 μm in length. They are gram-negative, catalase and oxidase positive and most strains are psychrotrophic, meaning that they are able to grow below 7 ^{o}C. They are aerobic but can grow in anaerobic conditions if a suitable electron acceptor is provided. They are

found in a variety of marine and terrestrial environments, including foods, soil, sea water, sea ice and air (Betts, 2006). *Psychrobacter* has been frequently identified among fish gills and intestinal flora. It has also been isolated from moribund Atlantic salmon(McCarthy et al., 2013)

Flavobacterium

The *Flavobacterium* genus contains 30 species. They are gram-negative rods, non-spore-forming, strictly aerobic and motile by gliding. It is a diverse genus where species can be psychrofilic, psychrotolerant, mesophilic, halophilic, halotolerant or sensitive to salts (Waśkiewicz and Irzykowska, 2014). They are widely distributed in nature, occurring mostly in aquatic ecosystems ranging in salinity from freshwater to saltwater. *Flavobacterium* species are naturally occurring on healthy fish, but several species are (or potentially are) the etiological agents of fish diseases and especially in salmonids (Bernardet and Bowman, 2006). Coldwater disease is a bacterial disease that affects a broad host-species range of fishes that inhabit cold, fresh waters including salmon. Coldwater disease is caused by *F. psychrophilum* (Starliper, 2011).

1.7 Hypothesis and aims

The hypothesis of this master project is that the ability to colonize the mucosal surfaces of Atlantic salmon (*Salmo salar)* yolk-sac fry is species dependent, and that interactions between the colonizing bacteria affects this ability. Because of its antibiological effects, *J. lividum* is of main interest and it would be interesting to see how it interacts with other bacteria during colonization of salmon fry. The aims of this study are to:

1. Investigate the initial colonization of newly hatched germfree salmon fry by different bacterial strains previously isolated from salmon
2. Examine the ability of different strains to colonize the gut and skin
3. Investigate potential interactions between bacterial strains in a mock community during the colonization
4. Investigate potential commensal or competitional interactions from the *J. lividum* strain during colonization, when combined with other strains.

By investigating these aims, the goal is to improve the understanding of the initial colonization of developing fish fry.

2 Materials and Methods

During this master project, two different fish experiments was conducted. Most of the materials and methods in these experiments were the same. The differences between the experiments was essentially time of exposure to bacteria and the age of the salmon fry during the experiment. Because of this, the general procedure in both experiments will be presented and then the experimental design for each experiment will be mentioned.

2.1 Generating germ free salmon fry

The disinfection procedure was performed by researcher Sol Gomez de la Torre Canny as follows: Salmon eggs from Aquagen was acclimatized to 6-7 °C in darkness upon arrival. When acclimatized, the eggs were transferred to large petri dishes (100 eggs per dish) containing sterile salmon growth medium (SGM) (Appendix 1) and then stored dark at 6-7 °C overnight. To generate germ free eggs, the eggs were treated with a double disinfection, the first round of disinfection took place the day after arrival and the eggs were immersed in sterile SGM containing several antibiotics (Appendix 2). In the second treatment, a solution of 100 mg/l available iodine was made by adding 500µl 1:100 dilution of BUFFODINE® (Evans Vanodine International PLC) in 50 ml SGM. The eggs were kept in the disinfectant solution for 30 minutes and during this time, they were gently agitated so that the whole surface of the eggs was in contact with the solution. After 30 minutes, they were rinsed four times in sterile SGM. All handling of the eggs during disinfection was performed under a laminar flow hood, and all the equipment used were treated with UV beforehand.

The surface disinfected eggs were distributed to 500ml sterile tissue culture flasks containing 100 ml of sterile SGM. To each flask, 15 eggs were distributed, giving a stocking density of 150 eggs/l. Not all the eggs were disinfected, and these eggs were used as a control for conventionally raised (CVR) fry, meaning that they were reared in a non-sterile rearing water. The control group was reared in SGM identical to the germ free (GF) group, but without the disinfection procedures. Flasks from both groups were kept in darkness at 6-7 °C during the whole experiment. To maintain good water quality for the fish, 60% of the SGM was changed three times a week. To ensure that the disinfecting procedures had worked, a sterility-check was performed 1-week post-hatching, testing for bacteria, fungi and oomycetes. This was done by adding 100µl media from the GF flasks to Tryptic soy agar (TSA) plates and three different liquid medias: Nutrient broth, brain heart infusion (BHI) and saboraud dextrose (SD), all listed in Appendix 3. The agar plates and liquid media from the test were incubated in both room temperature and in 6-7 °C, before they were inspected for growth. The same check was also performed on one of the CVR flasks as a control.

2.2 Gnotobiotic fish experiments

2.2.1 Experiment 1

In Experiment 1, the focus was on long term mono associations; i.e. the salmon fry was exposed to one strain: S1_Ba, S2_Pe, S3_Ar, S4_Ps or S5_Ja (Table 1) or to a mix of all strains 1-week post hatching and then monitored for four weeks. Four replicate flasks with GF salmon was exposed to each treatment (Fig. 3). As control groups, three flasks were kept germ free and two flasks were kept

as CVR. The bacterial strains were prepared as described in 2.3.1. and the amount of media containing bacteria were added to give a final bacterial concentration of $1*10^5$.

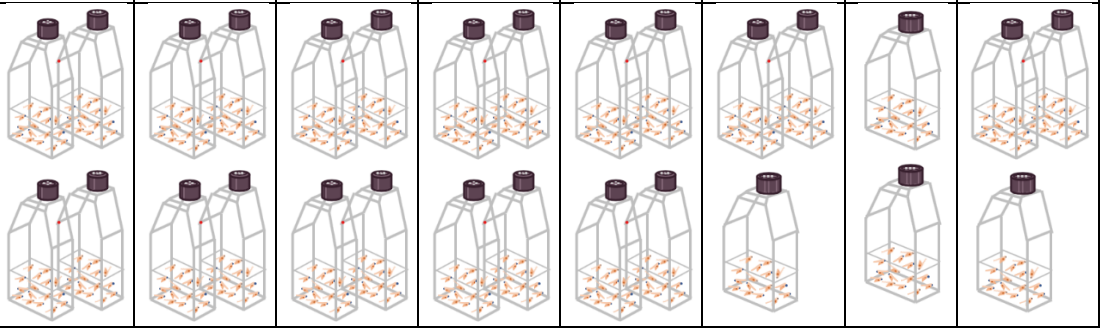
	Bacterial treatment							
	S1_Ba	S2_Pe	S3_Ar	S4_Ps	S5_Ja	Mix of all	CVR	GF
n Flasks .								

Figure 3: Distribution of bacterial strains into flasks containing GF fry.

After the salmon had been colonized, they were kept for five weeks at 6-7 °C in the dark with water changes three times a week. One fish from each replicate flask was dissected and sampled for CFU analysis and aliquoted to agar plates after one week and then weekly throughout the experiment as described in 2.4.1 and 2.5, respectively. On the last sampling day, three fish from each flask was also sampled for DNA based analyses as described in 2.4.2.

The bacterial density in the rearing water declined between the first and second sampling, possibly because of the water changes. Therefore, new batches of bacteria were added to the fish flasks after the second sampling. The second addition was done as described in 2.3.1 with a few exceptions. The washing procedure was skipped so the bacteria with TSB was directly added to the flasks. The final concentrations of the bacterial strains in the fish flasks were increased from $1*10^5$ ml^{-1} to $1*10^6$ ml^{-1}.

2.2.2 Experiment 2

In Experiment 2, the focus was on possible interactions between the bacterial strains during colonization of a short-term exposure (5 days), reflecting the initial interaction between the bacteria and the fry. The intention was to use the same bacterial strains as in Experiment 1, but due to a mix-up, S6_Fl was used instead of S4_Ps. Here, the germfree salmon fry was exposed to either one bacterial strain: S2_Pe, S3_Ar, S6_Fl or S5_Ja (Table 1), a mix of two strains, or a mix of all (four) bacterial strains, approximately 10 weeks post hatching. Individual GF salmon fry were distributed to 10ml wells containing 8 ml SGM with the respective treatment (Fig. 5). As controls, 6 wells were conventionalized after the disinfection by introducing water from a local lake (CVZ). The bacterial strains were prepared as described in 2.3.1. and the amount of media containing bacteria were added to give a final bacterial concentration of $1*10^6$ from each strain added. The wells were kept in 6-7 °C for five days without water changes before samples for CFU counting was taken as described in 2.4.1 and samples for PCR amplification as described in 2.4.3. Of the six wells, three replicate

individuals with the same treatment were used for CFU analysis, and three were used for 16S rDNA amplicon sequencing.

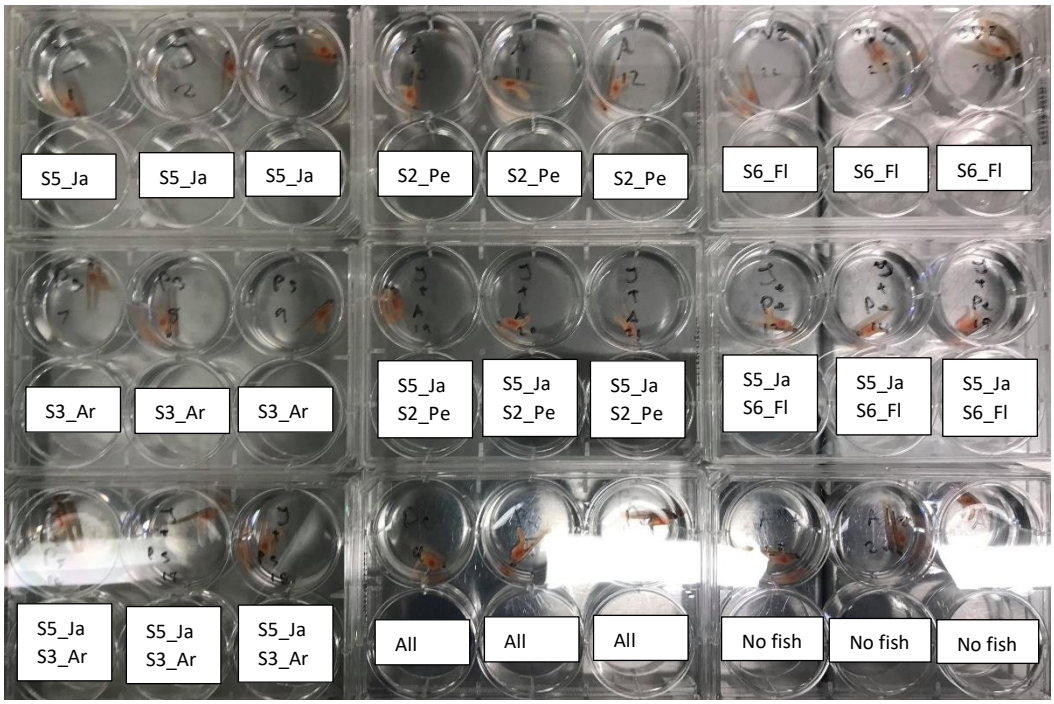


Figure 4: Distribution of fry into wells with the respective treatment.

2.3 Bacterial strains used in the gnotobiotic experiments

The intention was to use the same bacteria in both experiments, but *Flavobacterium* was mixed up with *Psychrobacter* in Experiment 2. All strains used in this project has previously been isolated from salmon by the ACMS group at NTNU (Table 1)

Table 1: Strains used in the experiments.

Phylym	Taxonomy	Isolated from salmon fry skin/gut	Gram +/-	Isolate ID
Firmicutes	Bacillus (Bacilli)	Skin and gut	+	S1_Ba
Bacteriodetes	Pedobacter (Shpingobacteria)	Skin	-	S2_Pe
Actinobacteria	Arthrobacter (Actinobacteria)	Skin and gut	+	S3_Ar
Proteobacteria	Janthinobacterium (Betaproteobacteria)	Skin	-	S5_Ja
Proteobacteria	Psychrobacter (Gammaproteobacteria)	Gut	-	S4_Ps
Bacteriodetes	Flavobacterium (Flavobacteriaceae)	Skin	-	S6_Fl

2.3.1 Exposing the salmon yolksac fry to bacteria

Before the fish were exposed to bacteria, the bacterial cultures had been prepared two days beforehand by incubating each strain in liquid tryptic soy broth (TSB) culture media (Appendix 3). They were incubated in 13ml plastic tubes with a semi-closed lid at 20 °C with shaking. In Experiment 1, the bacteria were washed by centrifuging at 2000 rpm for three minutes, so the bacteria were concentrated at the bottom of the tube and the supernatant (TSB) was replaced by SGM. The washing procedure was repeated twice. In Experiment 2 this procedure was only done if the bacterial concentration was so low that more than 150 µl media had to be added to the fish flask. To determine the bacterial density, OD_{666} was measured with a spectrophotometer (HITACHI U-5100). The OD_{666} had to be less the 0.3 to be valid. If the value were above 0,3 the solution was diluted with SGM. The density was calculated by using the McFarland standard (Equation 1)

$$Bacterial\ Density(CFU\ ml^{-1}) = OD_{666} * 1.2 * 10^{9} \quad (1)$$

Depending on the desired bacterial density, a calculated amount of bacterial suspension was added (Equation 2)

$$Addition\ of\ media = \frac{Bacterial\ Density\ of\ rearing\ water}{Bacterial\ Density\ of\ liquid\ culture} * Total\ amount\ of\ SGM \quad (2)$$

2.4 Sampling of the salmon fry

To quantify the initial bacterial colonization of the fry, two approaches was used; one culture-dependent method where CFU was counted, and one culture independent method where qPCR was used. TSA (Appendix 3) plates with series of dilutions were made to estimate the CFU in salmon fry skin, gut, and the rearing water. 100 µl rearing water and whole fish was instantly frozen in liquid nitrogen and later used to quantify the bacterial load by running the samples in qPCR. Samples were taken from all the treatments, and GF and CVR. To characterize the bacterial community, 100 µl rearing water, fish skin and gut was instantly frozen in liquid nitrogen and later purified and analyzed by illumina amplicon sequencing. All fish were euthanized by immersing them in a tricaine mesylate bath (5.2 g tricaine, 27.3 mL Tris (1 M, pH 9.0), 972.7 mL SGM) until their heart stopped beating.

2.4.1 Sampling for CFU analysis

Salmon fry was euthanized and dissected by removing the yolk sack, and the gut was separated from the fish. The fish was placed in a sterile petri dish where the dissection was performed under a magnifier, using forceps pre-steralized with 70% ethanol. The gut and the skin, represented by the rest of the fish, was placed in separate screw caps. The screw caps were prepared beforehand, by adding a small amount (approximately 200-300 µl) of 1,4mm zirconium oxide beads (Precellys), autoclaved and filled with sterile SGM. For the gut samples, 200 µl SGM was added to the screwcap and for the skin samples, 300 µl was added to the screwcap. Water samples were taken by collecting 100 µl from the rearing water of each fish flask into Eppendorf tubes.

2.4.2 Sampling for qPCR analysis

Whole fish were instantly frozen in liquid nitrogen. 3 ml rearing water was centrifuged in an Eppendorf tube twice at 14000rpm for ten minutes and the supernatant was carefully removed. The samples were kept at -80 °C.

2.4.3 Sampling for 16S rRNA gene amplification

The gut was dissected out as described in 2.4.1 before the gut and the skin (represented by the rest of the fish) was placed in separate cryotubes and instantly frozen in liquid nitrogen. Water was sampled by running all the water from each well through a Sterivex GP 0.22µl Filter unit before the filter was instantly frozen in liquid nitrogen. Samples were kept at -80 °C.

2.5 Estimation of Bacterial Numbers by CFU Analysis.

Skin and gut samples were homogenized by using the Precellys 24 tissue homogenizer. Gut samples were homogenized at 2500 rpm twice for 10 seconds and skin samples were homogenized at 4000 rpm twice for 100 seconds. To each TSA plate, 50-80µl diluted homogenate was aliquoted and spread using autoclaved glass beads. For the gut samples, undiluted homogenate and 1:10 dilutions were used. For the skin samples, undiluted homogenate, 1:10 and 1:100 dilutions were used. For the water samples, a serial dilution from undiluted and up to 1:10000 was used. The glass beads were removed, and the plates were incubated at room temperature for 48 hours before they were inspected for CFUs.

2.6 DNA Extraction

To prepare the samples for DNA extraction, the samples were thawed, and for whole fish, the yolk-sack was removed by dissecting the fish before it was transferred to the ZR BashingBead Lysis Tubes with Lysis solution. The samples were homogenized in Precellys 24 tissue homogenizer at 5000 rpm for 10 seconds. The plastic cover of the Sterivex GP 0.22µl Filter unit with water samples were removed with a knife, and then the actual filter was removed and cut into smaller pieces with a scalpel before the pieces were transferred to a lysis tube and run in the Precellys 24 tissue homogenizer twice at 5000 rpm for 10 seconds. For DNA extraction the ZymoBIOMICS DNA miniprep kit was used according to the manufacturer`s protocol (Appendix 4).

2.7 qPCR

Real-time PCR (qPCR) was evaluated as a method to estimate the bacterial numbers associated with fish and water samples. For each DNA amplification, a fluorescent signal is measured and quantified. The signal is proportional to the DNA product. The fluorescent signal is caused by the addition of a fluorescent dye, in this case SYBR®Green, which binds to the minor groove in the double bonds of DNA and acts as a fluorescent probe. The background fluorescence from SYBR®Green when in solution or when bound to single-stranded nucleic acids, is very low. When the required number of amplification cycles to cross the threshold or exceed the background level, a cycle threshold (C_t) is determined. The C_t value is inversely proportional to the amount of target nucleic acid in the sample and can be used to calculate DNA copy numbers (Dorak, 2007).

The DNA, extracted as described in 2.6, was used as template in real-time PCR (qPCR) to quantify the bacterial DNA. Previous research in the ACMS group has shown that DNA extracts from salmon fry

contains PCR inhibitors (personal communication, Ingrid Bakke). Therefore, in order to reduce the interfering from these inhibitors, all the DNA extracts containing salmon tissue were diluted 1:50 prior to being used as templates in the qPCR reaction. Triplicate reactions for each sample with a total volume of 25µl was prepared in a 96 well plate (Thermo Scientific) containing 12,.5µl of SYBR®Green master mix, 2,5µl of each primer (RT996F: 5'-GCAACGCGMRGAACCTTACCTA-3' and RT1089R: 5'-CSGGACTTAACCSAACATYTCA-3'; (Skjermo et al., 2015) 2,5µl of PCR graded water, and 5 µl diluted template (Table 2). The qPCR was run in a QuantStudio (AppliedBiosystems) qPCR instrument, with the following cycle conditions: pre-heating at 95 °C (10 min), followed by 40 cycles of denaturation at 95 °C (15 sec), and annealing/extension at 60 °C (1 min). A melt curve analysis was performed after amplification of the PCR product, with the following conditions: 95 °C(15 sec), 60 °C (1 min), 95 °C (1sec) and finally a cool down stage at 37 °C (30 sec). Along with the samples to be quantified, reactions for generating a standard curve was included, based on a DNA sample of known concentration and sequence length. This sample had been prepared previously in the ACMS group by PCR amplification of a 123 base pair region of the 16S rRNA gene for a *Vibrio sp. (RD5-30)* isolate using the primers RT996F and RT1089R, followed by purification of PCR product by using the Qiaquick kit (Qiagen).The concentration was determined using Qubit Invitrogen by Thermofischer. A series of 5-fold dilutions was made (up to 1:50), and qPCR reactions were run for template concentrations with 12ng/µl in the undiluted sample.

Table 2: Mastermix ingredients used in qPCR.

Components	Amount (µl)
Power SYBR Green PCR Mastermix	12,5
RT-966F(10 µM)	2,5
RT-1089(10 µM)	2,5
PCR grade water	2,5
Diluted DNA template	5
Total	**25**

QuantStudio Design and Analysis Software v1.5.0 (AppliedBiosystems) was used to analyze the qPCR results. By using equation 3, the concentration of DNA could be converted to copy number (CN) of the diluted templates in the standard curve dilutions. The standard curve was then obtained by plotting the C_t values from the qPCR against the calculated LogCN numbers of each *Vibrio sp (RD5-30)* sample. The standard curve was used to convert C_t values to copy numbers of the 16S rDNA PCR product for the rest of the samples.

$$CN_{stock}\,\frac{molecules}{\mu l} = \frac{DNA_{cons}\frac{g}{\mu l} * 6.22 * 10^{23}\frac{molecules}{mol}}{DNA_{length} * 660\frac{g}{mol}} \quad (3)$$

2.8 PCR amplification and gel electrophoresis.

PCR amplification and gel electrophoresis were used to prepare samples for Illumina amplicon sequencing, which was used to characterize bacterial community composition in the samples that represented fish or water exposed to more than one bacterial strain.

2.8.1 PCR for 16S rRNA gene amplification

To prepare the samples for PCR amplification of the v3 + v4 region of the bacterial 16S rRNA gene, DNA was extracted from the samples as described in 2.6 and used as template in PCR. A master mix was prepared (Table 3). The primers used for the amplification were ill338F: 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNNN CCT ACG GGW GGC AGC AG-3' and Ill805R: 5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN GAC TAC NVG GGT ATC TAA KCC-3' (Mathisen, 2019). As explained in 2.7, the salmon DNA extracts contain PCR inhibitors, so the DNA templates were diluted 1:10 for the gut and skin samples. The mastermix (24 µl) and template (1 µl) was mixed, giving a total volume of 25 µl in the PCR tubes. Along with the samples, negative (NTC) and one positive control (DNA extracted from a water sample) was also included in the PCR. The PCR amplification was run in a T100^{TM} Thermal Cycler (BioRad) with the cycle conditions specified in Table 4. The temperature cycles were repeated 31 times for water samples and 38 times for fish samples.

Table 3: Mastermix components used in PCR 16S rDNA amplification

Component	**Amount**	**Supplier**
5x Phusion buffer HF(7,5mM)	5.0 µl	Phusion Kit Illumina
dNTP (10 mM each)	0.625 µl	VWR
Primer 1 (ill338F) (10 µM)	0.375 µl	Sigma Aldrich
Primer 2 (ill805R) (10 µM)	0.375 µl	Sigma Aldrich
PCR-grade water	17.55 µl (Up to 25 µl)	
Phusion Hot Start DNA polymerase	0.1875 µl	Phusion Kit Illumina
DNA Template	1.0 µl	
Total	25 µl *amount of samples	

Table 4: Cycle conditions for PCR 16S rDNA amplification

98 ºC 60 sek.	
98 ºC 15 sek. 55 ºC 20 sek. 72 ºC 20 sek.	31-38 cycles
72 ºC 5 min.	
4 ºC 1 min.	
Hold 10 ºC ∞	

2.8.2 Gel electrophoresis

The quality and quantity of the PCR products were examined by agarose gel electrophoresis. The agarose gel (1.5%) was made by mixing 1.5g of agarose in 100ml of 1xTris-acetate-EDTA (TAE)

(Appendix 5). This mixture was boiled in a microwave oven for complete melting of the agarose. After boiling, 5 µl of GelRed (Qiagen) were added to the mixture during cooling. The gel was poured into the gel electrophoresis chamber with a comb for making wells and left to polymerize for 20-30 min. 4 µl of the PCR products was mixed together with 1 µl Loading dye (Thermo Scientific) on a piece of parafilm and added to wells, one well for each PCR product. A GeneRuler 1kb Pluss ladder (Thermo Scientific) was added to some of the wells. The gel was run in 1x TAE buffer (Thermo Fischer) solution at 115 volts for 60-80 min. The gel was visualized and photographed inside a UV-cabinet (SynGene GBOX 680X HR).

2.8.3 Illumina amplicon sequencing

Due to limited time, the amplicon library was prepared by Amalie Horn Mathisen (member of the ACMS group at NTNU) as described in her master thesis (Mathisen, 2019). In brief, PCR products were purified and normalized using the Sequal Prep Normalization plate Kit (Invitrogen). Further, each PCR product was indexed with unique sequence tags using the Nextera XT Index Kit Set D (Illumina). The indexed PCR products were again purified and normalized using the Sequal Prep Normalization plate Kit (Invitrogen). Finally, the PCR products were pooled and concentrated using Amicon Ultra 0.5 Centrifugal Filter units (Merck Millipore, Ireland). The amplicon library was sequenced on a MiSeq lane (Illumina, San Diego, CA) with V3 reagents (Illumina) at the Norwegian Sequencing Centre (NCS).

2.8.4. Processing of Illumina sequencing data

The resulting sequencing data were processed by Ingrid Bakke (ACMS, IBT NTNU) by using the Usearch pipeline (version 10; https://www.drive5.com/usearch/). In brief, the Fastq_mergepairs command was used for merging of paired reads, trimming off primer sequences, and filtering out reads shorter than 400 base pairs. The data were demultiplexed and quality trimmed using the Fastq_filter command with an expected error threshold of 1. The UPARSE-OTU algorithm was used for chimera removal and clustering at the 97% similarity level. Taxonomy assignment was performed with the Sintax script (Edgar, 2016) with a confidence value threshold of 0.8 and the RDP reference data set (version 16).

The resulting OTU table was normalized to 19 500 reads per sample (the lowest number of reads obtained for the samples) and was then manually inspected. OTUs representing *Salmo salar* sequences, chloroplast rRNA genes, or contaminants (identified as OTUs observed for the non-template PCR control and/or the negative control for the DNA extraction kit) were removed from the OTU table.

3 Results

3.1 Experiment 1

To investigate the ability to colonize fish skin and gut by different bacterial strains, germ free salmon yolk-sac fry was exposed to five different bacterial strains, previously isolated from salmon fry. The fish was exposed to bacteria 7 days post hatching (dph) and monitored for 4 weeks to investigate growth and colonization success of bacteria. Some of the fish were exposed to a mix of all strains to investigate any potential interactions between the strains.

3.1.1 Fish growth for different bacterial treatment

The strains S1_Ba, S2_Pe, S3_Ar, S5_Ja, S4_Ps and a mix of all five strains were added to four replicate flasks each while three flasks were kept as germ free, and two flasks were kept with non-disinfected fry (CVR; conventionally raised) as controls. Fish growth in length was measured at 14 dph and then every week until 35 dph. Fish growth was compared between the different bacterial treatments (Fig. 5). There is substantial growth from 14 to 35 dph, but no significant difference in growth was observed between the treatments (Anova, p=0,039), indicating that none of the strains was affecting the growth of the salmon fry.

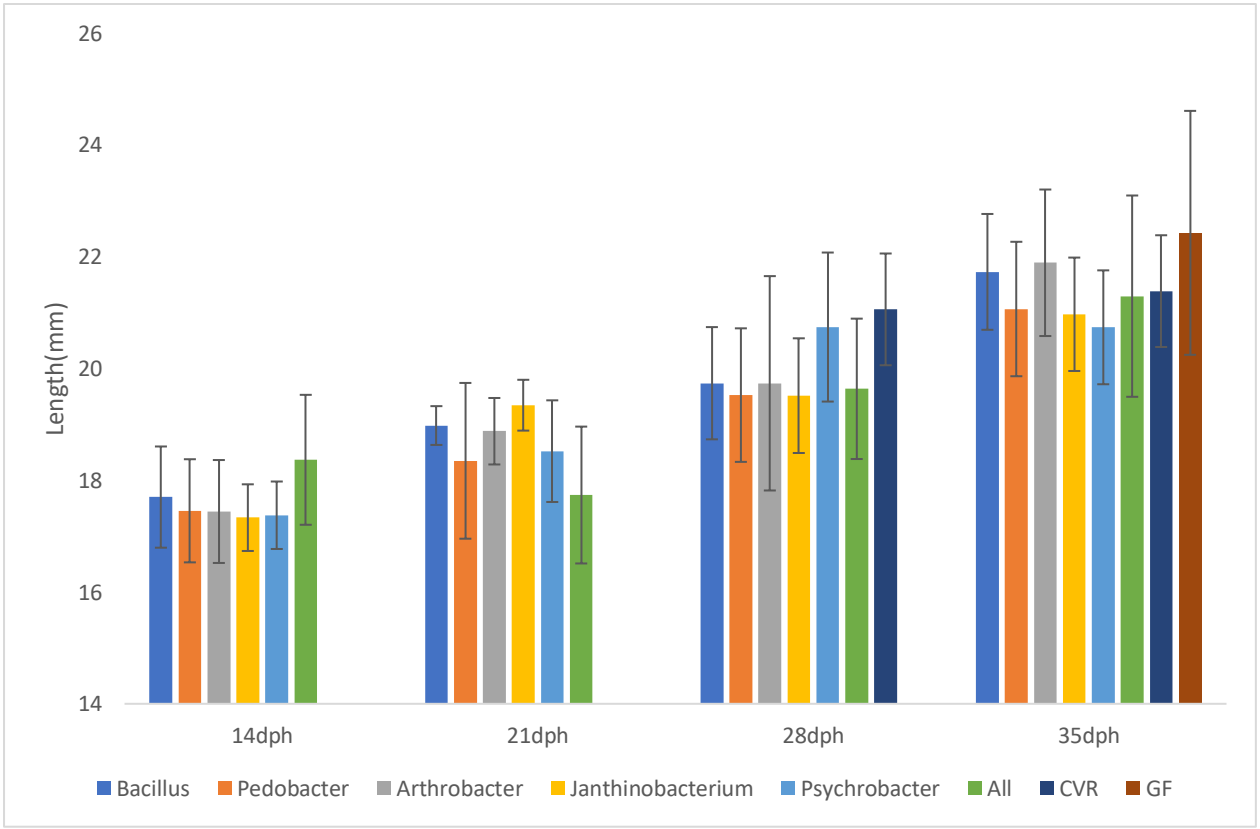


Figure 5: Average length of fry exposed to different bacterial treatments over a period from 14 dph to 35 dph. Based on the average of 1 fish from each of 4 replicate flasks at 14 and 21 dph, 2 fish from each of 4 replicate flasks at 28 dph and 5 fish from each of 4 replicate flasks at 35 dph. Error bars represent the calculated standard errors based on the average.

3.1.2 Quantification of bacterial colonization using CFU

The ability of the strains to colonize the SGM rearing water, skin and gut was investigated weekly after exposure at seven dph by counting colony forming units (CFU) on agar plates. The bacteria were added to a total density of approximately 10^5 at seven dph. The bacterial density in the rearing water on 21 dph was $1.01*10^5$ for S2_Pe, but for the other strains, the bacterial density was considerably lower than 10^5 in all flasks (Fig. 4a). The low numbers indicated that the strains were unable to maintain their population due to the heavy water exchanges with 60% dilutions three times a week. Because of this, a new and higher dose of bacteria was added to the rearing water at 24 dph. The water samples with S2_Pe had by far the highest bacterial concentration with approximately 10^6 CFU/ml throughout the experiment followed by S3_Ar and S5_Ja, both with around 10^5 CFU/ml at all sampling times (Fig. 6a). These high numbers indicate that S2_Pe is better at colonizing the rearing water than the other strains. Water samples from flasks added S4_Ps had very low CFU counts and S1_Ba disappeared completely from the water.

The number of CFUs in skin per individual varied considerably. S5_Ja, S2_Pe and S3_Ar seemed to be the only strains that were able to colonize the skin (Fig. 6b). The fish skin exposed to S5_Ja had slightly higher CFU numbers than the skin exposed to S2_Pe, followed by S3_Ar. For the fish added the mix of all strains, the CFUs mainly seemed to represent S2_Pe and S5_Ja (based on morphology of the CFUs). Because of high variations in the concentrations of the strains in the water (Fig. 6a), we determined the CFUs in skin per individual fish after normalizing to the amount of CFUs in the water (Fig. 6c). This clearly indicates that S5_Ja is the best at colonizing the skin of the fish with around a 10-fold higher numbers than for the second-best strain, which was S3_Ar.

Compared to CFUs in fish skin, the CFU counts per gut was surprisingly low (Fig. 6d). Except from S2_Pe, all gut samples exposed to the different strains had less than 10 CFUs per individual gut. The samples exposed to S2_Pe had the highest number of CFUs, followed by S3_Ar, S5_Ja and S4_Ps. Interestingly, the CFU counts for gut increased dramatically at 35 dph for the fish that was exposed to a mix of all strains. Abundance in gut after normalizing the gut samples to the amount in water was a bit variable, but S5_Ja and S3_Ar were the most abundant overall (Fig. 6e).

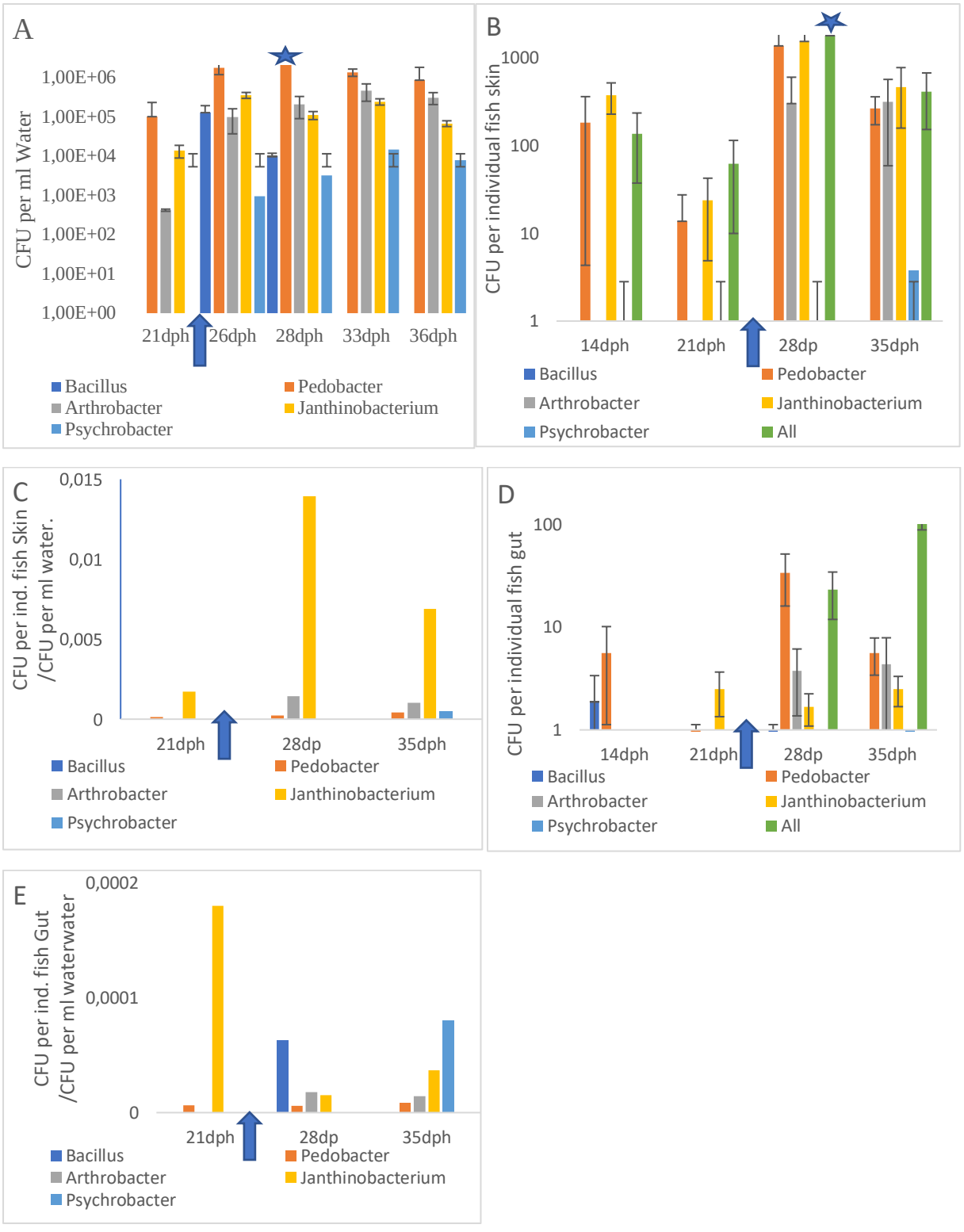


Figure 6: CFU counts for water, fish skin and fish gut samples from each sampling day during the period from 21 to 36 dph. Average bacterial colonization of water (6A). Average bacterial colonization of skin (6B). Average bacterial colonization of skin compared to the concentration of the same strain in the rearing water (6C). Average bacterial colonization of gut (6D). Average bacterial colonization of gut compared to the concentration of the same strain in the rearing water (6E). The numbers on skin represent CFU per fish after the gut and yolk-sack was removed from the fish, and the numbers in gut is represented by the gut that was removed from the fish. Blue arrows represent addition of a new batch of bacteria. Blue stars represent too high density of CFUs to count. The average values are based on one sample from each replicate flask, the error bars represent the calculated standard error based on the average.

3.1.3 Quantification of bacterial colonization using qPCR

We wanted to develop a protocol for quantifying bacteria, using qPCR. This would have made it possible to quantify all the bacteria in CVR-fish, including the unculturable strains. The samples of fish and water exposed to the S1_Ba strain were excluded from the qPCR and the later experiment because the bacterium was not able to colonize neither the water nor the fish.

First, a standard curve was made in order to correlate C_t values with amount of DNA template. A 5-fold dilution series of a 123 base pair PCR product for *Vibrio sp. RD5-30* (obtained with the qPCR primer pair RT996F and RT1089R) of known concentrations were used as template in the qPCR reaction with the same primer pair (RT996F and RT1089R). The copy number (CN) was calculated (Equation 3) and the standard curve was made by plotting the logarithmic scale of CN versus C_t values obtained from the qPCR reaction (Fig. 7). Linear regression from the standard curve was made to determine the relationship between the C_t values and the CN of the qPCR product.

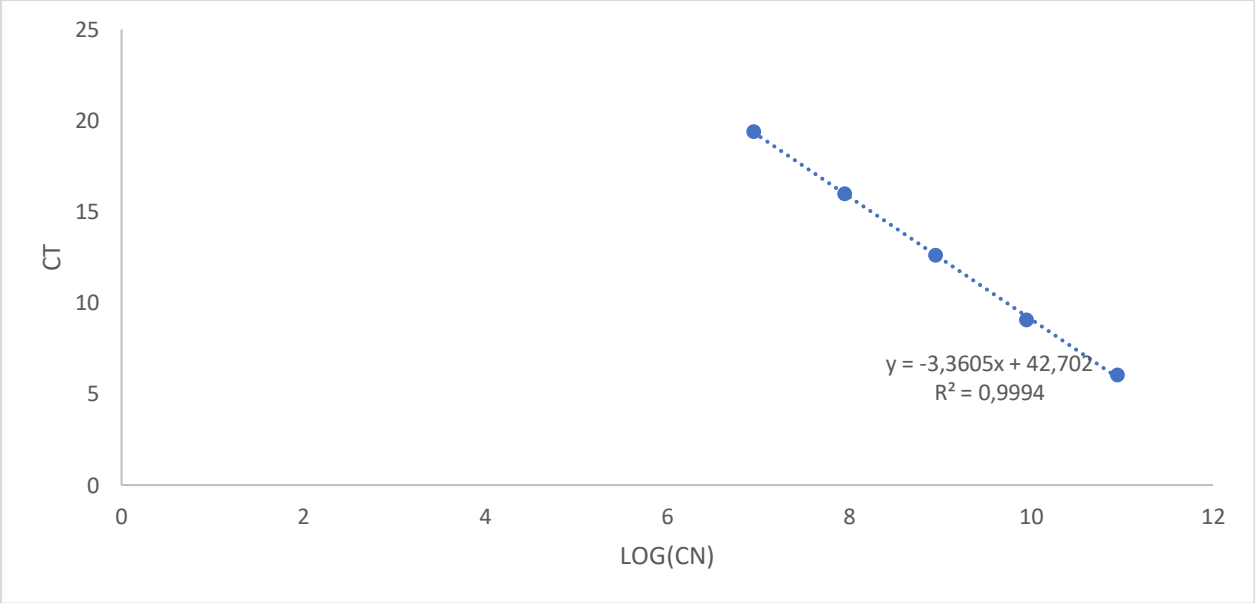


Figure 7: Showing C_t values as a function of the logarithm of the 16s rDNA copy number (LOGCN) obtained by running qPCR reactions with known concentrations of the 16s rDNA 123 bp product amplified from Vibrio sp(RD5-30) DNA as template. The concentrations of the 5-fold dilution series were measured to range from 12 ng/µl undiluted to 0,0012 ng/µl in the 1:10000 dilution.

The qPCR tests showed difference between the CVR fish samples (C_t:21.53) and the GF fish samples (C_t:29.76) (Fig. 8). But the fish samples with all the strains added had similar C_t values to GF fish samples. All the water samples had low C_t values, hence one water sample with three replicates is included as a positive control in the figure. Although there was a difference between CVR fish and GF fish, the samples from GF fish still had low C_t values. This was also the case for the non-template control (NTC) and extraction kit control, indicating that there was a lot of background noise from contaminants. All C_t values is listed in appendix 6. The melting temperature varied among the samples, indicating that the samples contained both bacterial and salmon DNA (Appendix 7).

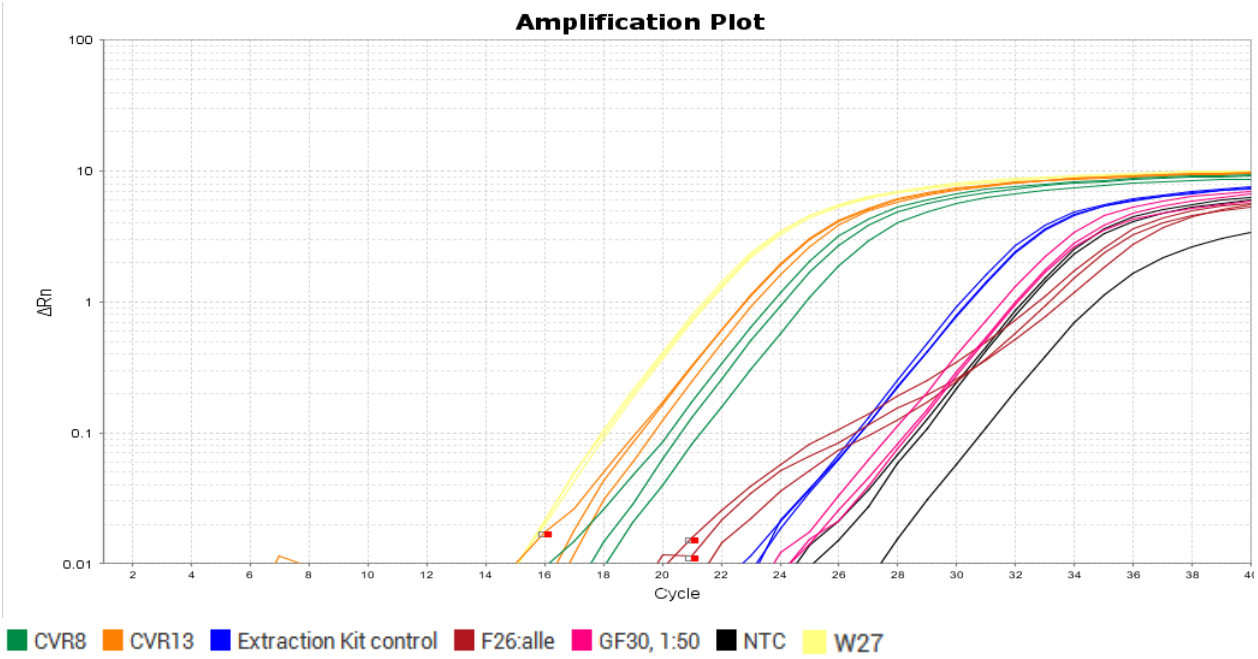
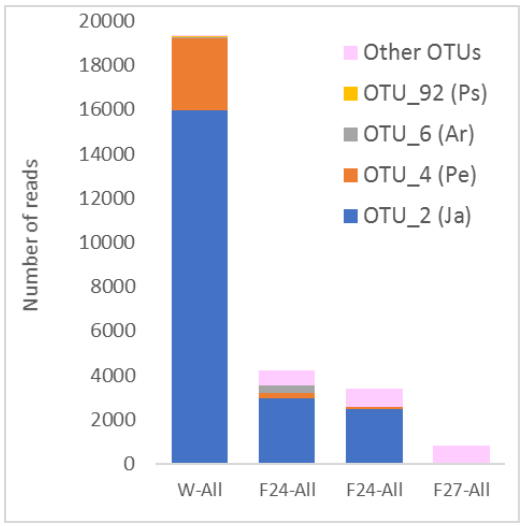


Figure 8: Amplification curves for qPCR of samples containing Conventionally raised (CVR) fish, Fish added all bacteria, germ free (GF) fish, extraction kit control, non-template control (NTC) and one water sample as positive control.

3.1.4. Composition of bacterial communities using 16S rDNA amplicon sequencing

The composition of the gnotobiotic community of water and fish samples exposed to all the five bacterial strains were assessed by 16s rDNA amplicon sequencing. The OTU table was normalized to 19500 reads per sample. After the OTUs representing salmon-DNA, chloroplast rRNA genes or other DNA from contaminants (as identified from the non-template and negative DNA extraction kit controls) was removed, the water sample had 19378 remaining reads. The three fish samples had only 4223, 3386 and 830 reads, respectively. The bacterial community in the water samples collected at the end of the experiment (35 dph), almost exclusively consisted of bacteria from the S2_Pe and S5_Ja strains. The sample had only small amounts of reads from the S3_Ar and S4_Ps strains (0.2 and 0.4% of total reads respectively) and no reads for S1_Ba (Fig. 11A). Because of few reads for the fish samples, it is uncertain how reliable the results are, but the indication is that S5_Ja is the main colonizer of fish, followed by S2_Pe. In one of the fish samples, a small amount of reads for the S3_Ar strain was observed (App. 8% of total reads), indicating that this individual was also colonized by S3_Ar.

A



B

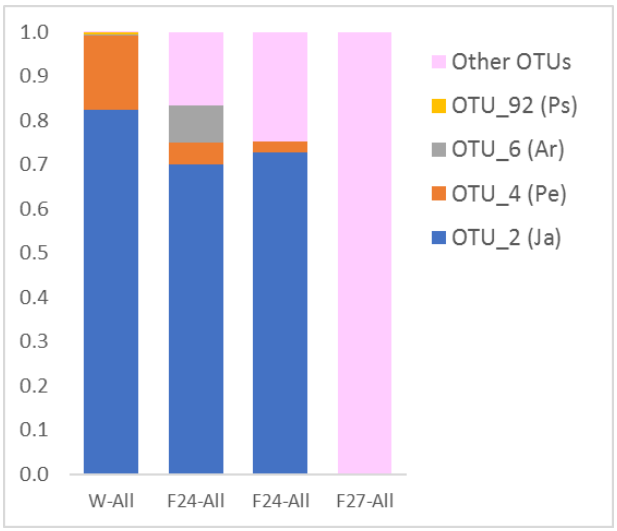


Figure 9: Composition of gnotobiotic communities in water and fish samples added S1_Ba, S2_Pe, S3_Ar, S4_Ps and S5_Ja as assesed by 16S rDNA amplicon sequencing. The OTU table was normalized to 19500 reads per samples, and subsequently OTUs representing Salmo salar, chloroplasts, and contaminating DNA as identified from non-template and negative DNA extraction kit controls. A) OTU abundances reported as number of reads. B) Relative OTU abundances. The OTUs were classified as follows: OTU_2: Janthinobacterium; OTU_4: Pedobacter; OTU_6: Arthrobacter; and OTU_92: Psychrobacter. The OTU for S1_Ba is not classified since it disappeared from the water based on CFU. "Other OTUs" refers to OTUs that were classified as other taxa, and probably represented contaminating DNA. W: water samples; F: fish samples. All: samples from fish flasks added the five bacterial strains S1_Ba, S5_Ja, S2_Pe, S3_Ar, and S4_Ps.

3.2 Experiment 2

To investigate the ability to colonize the rearing water, fish skin and gut by different bacterial strains during short time exposure, germ free salmon yolk-sac fry was exposed to combinations of 4 different bacterial strains, previously isolated from salmon fry. One of the reasons the strains was combined was to further investigate if combinations of strains would lead to an increase of CFUs in the gut, as observed in Experiment 1. The fish was transferred from flasks to wells 10 weeks post hatching and exposed to bacteria in well plates. The intention was to use the same strains as in Experiment 1, except for S1_Ba, but due to a mix-up, S6_Fl was used instead of S4_Ps. S6_Fl is another strain from the collection of strains isolated from salmon yolk-sack fry (Table 1). Strains of S5_Ja, S2_Pe, S3_Ar, S6_Fl and combinations of these were distributed to three wells in 6 wells' plates, each containing one fish. There were three replicate wells for each treatment and the strains ability to colonize the rearing water (SGM), skin and gut was investigated five days post exposure by CFU analyses. S1_Ba, was excluded from experiment 2 because it wasn't able to colonize either skin or gut in Experiment 1.

3.2.1 Quantification of bacterial colonization using CFU

Fig. 11 summarize the results for the CFUs analysis for the rearing water, skin, and gut samples. The highest CFU counts in the rearing water were found for the S2_Pe strain, followed by S5_Ja, S3_Ar and S6_Fl (Fig. 11A). This corroborates the results from experiment 1, indicating that S2_Pe is better at colonizing the rearing water than the other strains. Combining S2_Pe with S5_Ja resulted in a lower total number of CFUs in the water samples than the water samples with S2_Pe alone.

Samples of fish skin exposed to S5_Ja had the highest number of CFUs followed by S2_pe and S6_Fl (Fig. 11B). Samples of skin exposed to combinations showed higher numbers of CFU than samples exposed to single strains. Combining S5_Ja with another strain results in higher CFU counts for the skin samples. The skin samples exposed to S3_Ar were excluded from the results because of highly variable CFU numbers. Because of high variations between the strains ability to colonize the water, we decided to estimate normalized CFU counts in skin samples per individual fish after normalizing to the CFU counts in the water samples (Fig. 11D). The results indicate that S5_Ja is the best at colonizing skin and by combining S5_Ja with another strain, the growth of one or both strains are improved

The CFU counts for gut samples was low compared to the CFU counts in fish skin (Fig. 10 C), but much higher compared to the CFU counts for gut in Experiment 1, indicating that the salmon fry gut gets increasingly colonized from 4 to 10 week. As with skin, the gut samples exposed to S5_Ja has the highest CFU counts, followed by S2_Pe. Samples containing fish exposed to combinations of strains showed higher numbers of CFU counts than samples containing fish exposed to single strains. There is no dramatic increase when combining all strains like in Experiment 1, but every sample with combination shows higher number of CFUs than the samples with single strains alone, indicating that S5_Ja benefits from being in a community or it may improve growth on other bacteria. S5_Ja was the most abundant when normalizing the gut samples to the amount of CFUs in the water samples (Fig. 10E).

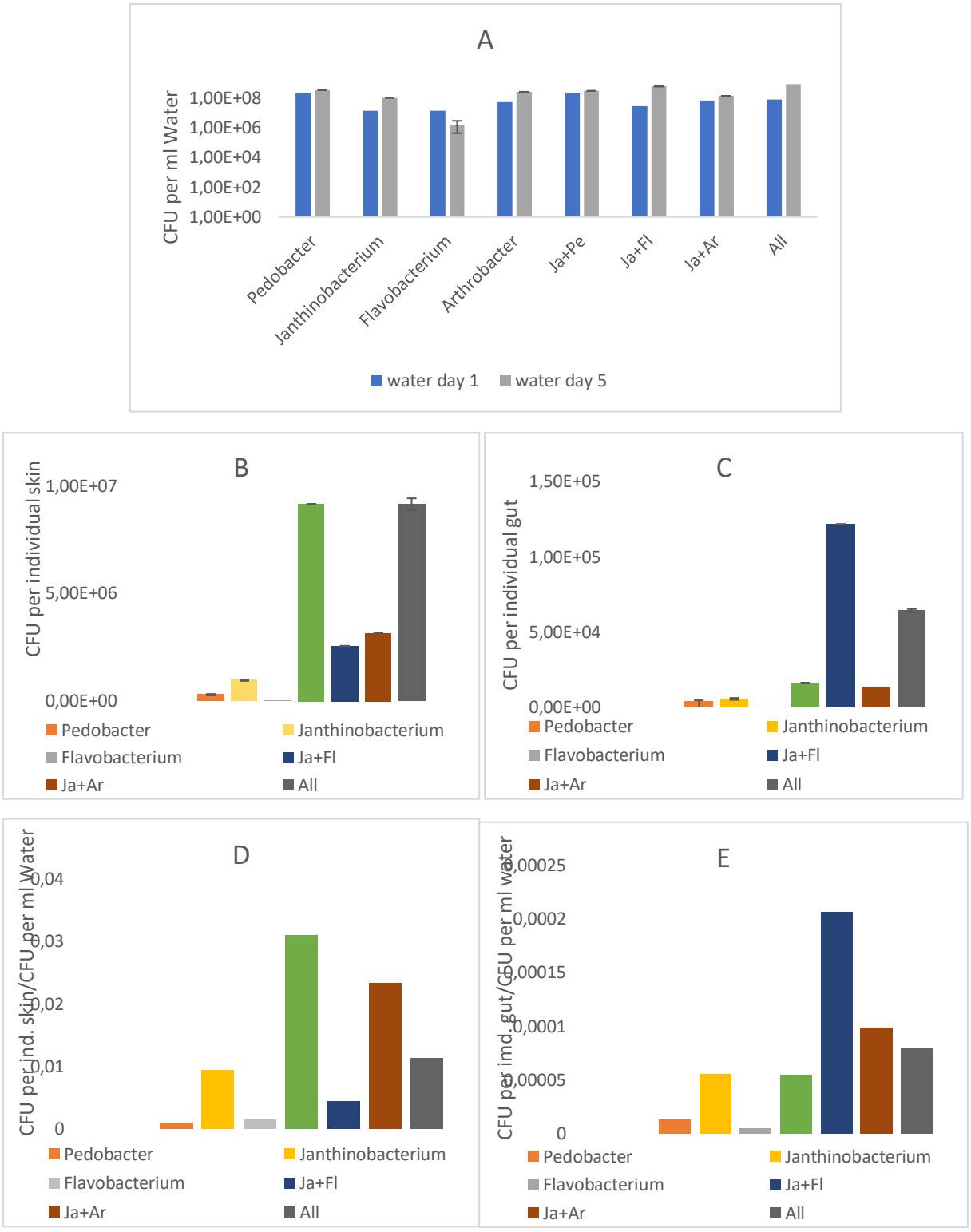


Figure 10: CFU counts for water, fish skin and fish gut samples taken five days after exposure. Average bacterial colonization of water on day one and day five of exposure (10A). Average bacterial colonization of skin (10B). Average bacterial colonization of gut (10C). Average bacterial colonization of skin compared to the concentration of the same strain in the rearing water (10D). Average bacterial colonization of gut compared to the concentration of the same strain in the rearing water (10E). The numbers on skin represent CFU per fish after the gut and yolk-sac was removed from the fish, and the numbers in gut is represented by the gut that was removed from the fish. The average values are based on one sample from each replicate flask, the error bars represent the calculated standard error based on the average.

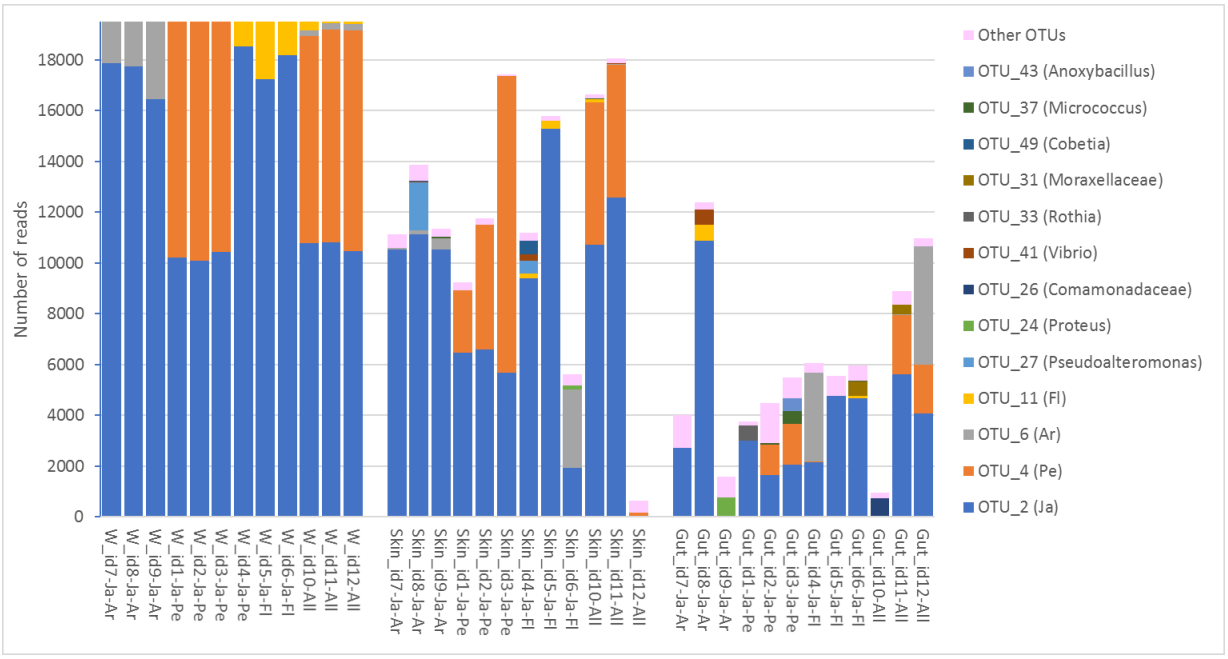
3.2.2. Composition of bacterial communities using 16S rDNA amplicon sequencing

Samples from fish flasks added more than one bacterial strain were subjected to 16S rDNA amplicon sequencing to investigate the composition of the gnotobiotic community. As described in Experiment 1, the table of OTUs was normalized to 19500 reads per sample, and the OTUs representing salmon-DNA, chloroplast rRNA genes or other DNA from contaminants (as identified from the non-template and negative DNA extraction kit controls) was removed. The mean number of reads after normalization was 19497±1.5, 11894±5088 and 5845 ±3445 for water, skin, and gut samples respectively (Table 11, Appendix 8).

The OTUs from the water samples corresponded with the added strains of bacteria (Fig. 11A), but for the samples were S5_Ja was combined with either S3_Ar or S6_Fl, OTU 2 (*Janthinobacterium*) dominated with approximately 90% of the total reads. For samples were S5_Ja was combined with S2_Pe, the composition of OTU 2 and OTU 4 (*Pedobacter*) was more evenly distributed. These OTUs was also the dominating OTUs in samples were all the four strains were added, and the abundance of OTU 3(*Arthrobacter*) and OTU 11 (*Flavobacterium*) never exceded 1.7% of the total reads (Fig. 14 A).

The fish samples, and especially the gut samples had very low number of reads, and for this reason, there is some uncertainty regarding the reliability of the fish sample results. But they do seem to confirm the impression from Experiment 1, that S2_Pe and S5_Ja were able to colonize the skin and gut of salmon yolk-sack fry. The abundance of S3_Ar was substantial in some of the fish samples, but it didn't seem to be any compliance between skin and gut samples from the same individual (Fig. 14 B).

A



B

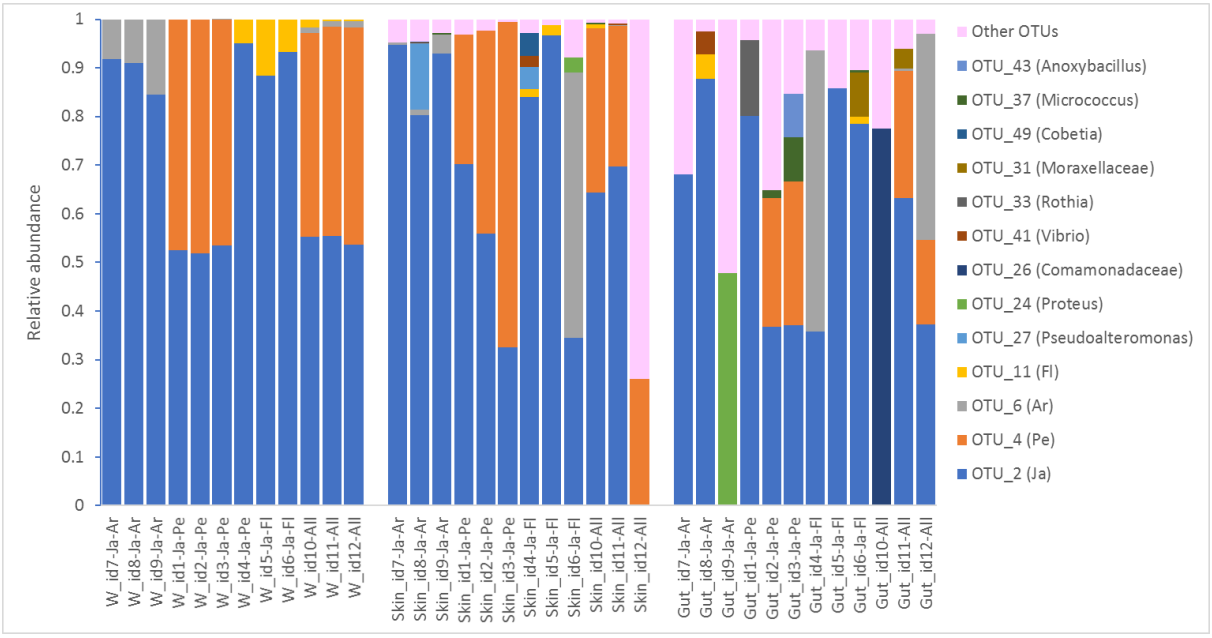


Figure 11: Composition of gnotobiotic communities in water and fish samples (Experiment 2) as assed by 16S rDNA amplicon sequencing. The OTU table was normalized to 19500 reads per sample, and subsequently OTUs representing Salmo salar, chloroplasts, and contaminating DNA as identified from non-template and negative DNA extraction kit controls were removed. A) OTU abundances reported as number of reads. B) Relative OTU abundances. OTU_2, OTU_4, OTU_6, and OTU_11 represents the added bacterial strains; S5_Ja, S2_Pe, S3_Ar, and S6_Fl, respectively. The remaining OTUs probably represented contaminating DNA. Taxonomy is given for the most abundant of these OTUs. W: water samples; Ja: S5_Ja; Pe: S2_Pe; Ar: S3_Ar; All: samples added all the four strains used in Experiment 2. Skin and gut samples annotated with the same id were taken from the same individual.

4 Discussion

The aim of this study was to study the early bacterial colonization of yolk-sack salmon fry. The ability to colonize the gut and skin of salmon yolk-sack fry by six bacterial strains was investigated and how these affect their host and each other during colonization. This was done by conducting two similar experiments; one experiment with long term exposure to bacteria with the main focus being how the strains are able to colonize the skin and gut of salmon yolk-sack fry, and how they affect the host. The second experiment was an experiment with short term exposure, where the ability to adhere to fish mucus could be compared with the ability to survive over time by comparing the results in Experiment 1 and Experiment 2. In Experiment 2, there was also more focus on interactions between the strains colonizing skin and gut. The bacterial community was analysed with CFU counting and Illumina 16S rDNA amplicon sequencing, qPCR was evaluated as a method to quantify the bacteria in samples containing salmon DNA.

4.1 The strains effect on yolk-sack fry growth and survival

To investigate how the bacterial strains, affect the salmon fry during long term exposure, the growth of the fry was monitored throughout Experiment 1 (Fig. 5). There was no significant difference in growth between the bacterial treatments, CVR and GF. This suggest that the initial colonization and microbial community by the strains used in this project had little effect on the growth of salmon at the yolk-sac stage. Fish health and survival is associated with a high growth rate (Graeb et al., 2004) and a recent study from Chapagain et al (2019) showed that the diversity of the fecal microbiota did not differ between fast and slow-growing Rainbowtrout (*Oncohynchus mykiss*), but they did have different indicator taxa. Thus, observing and comparing the growth rate of the fry during the experiment, could identify a strain that affects the growth for salmon fry. Before the yolk-sack is consumed, the salmon fry gets all its nutrients from the yolk-sack, so it is possible that they don't need bacterial help for digestion. This could be the reason for no significant difference in growth between the treatments. The commensal bacteria residing in the mucus is suggested to help with immune responses and by outcompeting pathogens (Abt and Pamer, 2014; Merrifield and Rodiles, 2015). Mammals and fish show conserved responses to the gut microbiota, including innate immune responses (Rawls et al., 2004). In Experiment 1, no significant difference in growth was observed between the CVR fish and GF fish, indicating that there were no pathogens in the CVR bottles. Previous experiments in the ACMS group have shown that there are increased mortalities in CVR fish compared to GF fish (Personal communication, Ingrid Bakke). In Sea bass (*Dicentrarchus labrax* L.), it has been observed a significant improvement in growth of newly hatched larvae when grown under germ free conditions (Rekecki et al., 2009). Having no obvious negative effect of being in a germ free environment, it could be that the main function of the commensal bacteria residing in the mucosal surfaces are to outcompete pathogens or to improve the immune system rather than to improve nutritional uptake in yolk-sack fry.

4.2 Strains colonizing the rearing water

To investigate the colonizing ability and bacterial community of the rearing water, the total bacterial counts were quantified by CFU counting and the relative abundance of the strains were investigated by using Illumina 16s rDNA amplicon sequencing. The results from both Experiment 1 and Experiment 2 show that S2_Pe had the highest amount of CFUs among the water samples exposed to a single strain of bacteria, followed by S3_Ar and S5_Ja (Fig. 6A and 10A). The 16s rDNA amplicon sequencing however, showed that S5_Ja and S2_Pe was more evenly distributed and the most abundant strains when water samples were exposed to multiple strains (Fig. 9 and 11). The OTUs from these two strains accounted for more than 98% of the total OTUs when combining all strains in Experiment 2. The results show that all strains except S1_Ba were able to maintain their population in the water when added separately to the water (Fig. 6A and 10A), but when combining multiple strains, it seems that S3_Ar, S4_Ps and S6_Fl was outcompeted by S2_Pe and S5_Ja (Fig. 9 and 11). Both the *Pedobacter* and the *Janthinobacterium* genera are aerobic, Gram-stain-negative rods and are usually isolated from soils and water from lakes, rivers or springs (Ramsey et al., 2015; Zhou et al., 2012). *Pedobacter aquatilis sp.* has also been isolated from tap water in Spain (Gallego et al., 2006), thus it is not surprising that these strains were abundant in the rearing water.

4.2.1 Colonization ability of each strain

The CFU analysis from both Experiment 1 and 2 indicate that S5_Ja is best at colonizing skin, but also gut, followed by S2_Pe and S3_Ar (Fig. 6B, C, D, E and 10B, C, D E). The 16s rDNA amplicon sequencing corroborate with the CFU analysis. Both short term exposure and long term exposure show similar results, indicating that the strains that most easily adhere to the mucosal surfaces of skin and gut, also are the ones that are able to thrive and grow over time.

The S1_Ba strain was unable to colonize either skin or gut and it also disappeared completely from the rearing water by two weeks (Fig. 6A). The strain added has been found to be closely related to *Bacillus altitudins, B. stratosphericus* and *B. Aerophilus,* revealed by 16S rDNA sanger sequencing (Mallasvik, 2019). These have not been previously associated with fish or aquatic environments, indicating that this strain was an airborne contaminant from the previously isolated strains of salmon fry, so this strain was not added to any wells during Experiment 2.

The S2_Pe strain was the most abundant in the water and was also able to colonize both skin and gut relatively well. *Pedobacter* is common in soil and water but not usually associated with fish (Gallego et al., 2006; Ramsey et al., 2015; Zhou et al., 2012). A more common host is the Nematode *Acriobeloides Maximus*, where *Pedobacter* is one of the most abundant genera in the gut (Baquiran et al., 2013). It is hard to say if the ability to colonize fish is because of the high abundance in the water or if it actually is an effective colonizer. If it is an effective colonizer, the high abundance of S2_Pe in skin and especially gut could be explained by the fact that no feed runs through the gut, so the nutritional benefit from being in the gut is lowered.

The S3_Ar strain was able to maintain their population in the rearing water on their own (Fig. 6A) but were outcompeted when combined with other strains (Fig. 9 and 11). The colonization on skin and gut was highly variable and the abundance was high in some of the samples. The results indicate that S3_Ar can potentially colonize both skin and gut well, but the results are to unreliable to be conclusive. They were also excluded from the results in Experiment 2 because of this. *Arthrobacter* is

commonly isolated from soil, sewage, food and is also abundant in the gut microbiota of fish (Comi and Cantoni, 2016; Nayak, 2010).

The S4_Ps strain was able to maintain their population in the rearing water on their own (Fig. 6A) but were outcompeted when combined with other strains (Fig. 9). The S4_Ps strain was almost unable to colonize either skin or gut. *Psychrobacter* is commonly isolated from the GI tract Atlantic cod (*Gadus morhua* L.), and the alimentary tract of Atlantic salmon (*Salmo salar*)(Askarian et al., 2012; Ringø et al., 2006). *Psycrobacter glacincola* was also one of the most dominating strains in the hindgut of Arctic charr (*Salvelinus alpinus* L.)(Ringø et al., 2006). Hence, the low CFU numbers from samples with fish exposed to S4_Ps was a bit surprising. Atlantic cod and Arctic charr are both saltwater species, and Atlantic salmon is anadromous. The shift from saltwater to freshwater influences the microbiome of the salmon skin (Lokesh and Kiron, 2016). So, it is possible that the S4_Ps strain prefer a host living in saltwater.

The S5_Ja was abundant in water, gut and was by far the best colonizer of skin. *Janthinobacterium lividum* is commonly found in skin of humans and it especially well known for colonizing the skin of amphibians (Valdes et al., 2015). The salmon skin share similarities with the amphibian skin by containing mucosal tissues (Maynard et al., 2012) and it is possible that these similarities makes salmon skin a good environment for the S5_Ja strain. *Janthinobacterium* is capnophilic, meaning that it is attracted to high concentrations of CO_2 (Valdes et al., 2015). Salmon yolk-sack fry have poorly developed gills but do have high skin area to mass ratio and a large well-vascularized yolk-sack. Cutaneous surfaces accounts for more than 95% of the total area available for respiration (Wells and Pinder, 1996). By secreting CO_2 through the skin, it could be attracting S5_Ja in the same way as it is believed to attract *Janthinobacterim lividum* in amphibians (Valdes et al., 2015).

The S6_Fl strain was able to maintain their population in the rearing water on their own (Fig. 10A) but were outcompeted when combined with other strains (Fig. 11). It was able to colonize both skin and gut, but to a less degree than S5_Ja and S2_Pe. *Flavobacterium* is a diverse genus occurring mostly in aquatic ecosystems (Waśkiewicz and Irzykowska, 2014). The genus is commonly found on healthy fish, but several species can be the etiological agents of fish disease on salmonids among others (Bernardet and Bowman, 2006). No pathogenic relationship was discovered between S6_Fl and the fry, indicating that this strain is not pathogenic.

4.2.2 Colonization of gut.

In general, there was very little colonization of the gut compared to skin, revealed by both CFU analysis and amplicon sequencing (Fig. 6, 9, 10 and 11) The PCR products were dominated by salmon DNA and contaminating DNA, and this is probably because of the low amount of bacterial DNA in the gut samples. But the low numbers are interesting results by its own. Salmon fry opens its mouth at 7 dph and by this time it has a digestive system that is morphologically distinct with an open anus (Sahlmann et al., 2015). Based on this it is reasonable to believe that the gut is colonized at the same time, however the results from both experiments indicate that there is little colonization of gut and that the skin is much more colonized than gut during the yolk-sack period of salmon fry. The low colonization of gut could be because of the low drinking rate of juvenile Atlantic salmon (Fuentes et al., 1996), meaning that few bacteria pass through the gut. The most abundant strains in the gut samples were also abundant in the water, indicating that the bacterial composition reflected the composition in the water to some degree. Several studies have shown that the composition of mucosal surfaces in fish may differ a lot from the surrounding water ((Chiarello et al., 2015; Li et al., 2014; Rawls et al., 2006; Roeselers et al., 2011; Xuemei et al., 2011; Yan et al., 2016), but this was not

the case in these experiments. This can indicate that the bacterial composition in the rearing water may still be an important factor for the composition of gut in salmon yolk-sack fry, but this was a very small community with only four strains. The colonization pressure increases when more strains are competing, so a more complex community would be needed to investigate if this was the case. One reason for the similarities between the gut and the rearing water could be that the fry is not feeding. When no food is going through the gut, the nutritional advantage for colonizing gut is lowered, hence the gut mucosal tissue may not provide the same niches as when the fry is starting to feed.

4.2.3 Colonizing ability is dependant of the hosts age.

The total number of CFUs were significantly higher in Experiment 2 than in Experiment 1. This can to some degree be explained by the larger mucosal surface area in a larger fish than a smaller one, and by the fact that more bacteria were added to the rearing water in Experiment 2. But numbers were so much higher in Experiment 2 that this still indicates that the bacterial colonization of mucosal surfaces increases when the fry ages.

4.2.4 Interactions between strains during colonization

The total CFU numbers increased dramatically for the samples with fish exposed to all strains, compared to samples with fish exposed to a single strain in Experiment 1 (Fig. 6D). This dramatic increase was not observed in Experiment 2, but the CFU numbers were significantly higher for skin and gut samples exposed to S5_Ja combined with one or more strains than any of the samples exposed to single strains (Fig. 10B, C, D and E), indicating that the strains might benefit from each other. However, the 16s rDNA amplicon sequencing revealed that S5_Ja was by far the main colonizer when strains were added together, indicating that the growth of S5_Ja improves when combined with one or more other strains. This indicates that S5_Ja has a commensal or competitional interaction with other bacteria. *Janthinobacterium* is well known for its production of the purple-pigmented violacein which has antibiological effects, including antibacterial (Aranda et al., 2011). It is possible that S5_Ja kills surrounding bacteria that will release nutrients that S5_Ja may be benefitting from.

4.3 Errors in CFU and 16S rDNA amplicon sequencing

The CFU results from the rearing water were single strains were added indicated that S2_Pe was best at colonizing the water, while Illumina amplicon sequencing indicated that S2_Pe and S5_Ja were more evenly distributed. Thus, the results appeared to be a bit conflicting. The 16s rRNA amplicon sequencing are performed by analysing the prokaryotic 16s ribosomal RNA. The copy number of the rRNA gene varies among bacterial species, meaning that a species with a high number of rRNA gene copies will yield a higher number of reads than a species with lower copy numbers (Fadrosh et al., 2014). Among species in the *Janthinobacterium* genus, the typical copy number of the 16s rRNA gene is eight, while in the *Pedobacter* genus this number is typically four (rrnDB, https://rrndb.umms.med.umich.edu/) indicating that a sample with S5_Ja could potentially give twice amount of reads compared to a sample with the same amount of S2_Pe cells. Primer bias could also affect amplification efficiency and contribute to non-quantitative results. Primer bias can overestimate or underestimate the PCR product, leading to wrong amplification of the bacterial strain. On the other hand, the morphology of the S5_Ja colonies were quite different from the S2_Pe colonies, and some of the colonies seemed to be flowing together, potentially underestimating the

number of CFUs for the S5_Ja strain. Since the S5_Ja strain may be underestimated based on CFU, while it may be overestimated in the 16S rDNA amplicon sequencing, it is important to keep in mind that the results are calculated estimates. Calculated estimates can give a strong indication of how things work but it is not 100% correct. The reliability of the results increases with more data showing the same results. There was very little colonization of the gut, so it is a question of how reliable the results are, especially for the amplicon sequencing, where most of the PCR product appeared to represent either salmon DNA sequences or contaminanting bacterial DNA. Some of the OTUs representing contaminating DNA was not found in the negative controls. These OTUs may represent contaminating DNA from the Illumina sequencing itself. In addition to this, all samples with fish tissue is dominated by salmon DNA that is also known to contain PCR inhibitors of the bacterial 16S rDNA (Personal communication, Ingrid Bakke). This make the PCR product vulnerable to contaminating DNA and non-specific PCR product obtained from salmon DNA.

4.4 Evaluation of qPCR

qPCR based on broad-coverage primers that targeted the 16S RNA-gene was evaluated as a method for quantifying bacterial load of samples containing whole fish or water in Experiment 1. This method has been tested before in the ACMS group with not so promising results (Mallasvik, 2019), but we was hoping that the bacterial load would be higher in this experiment because the fry were older and they were exposed to bacteria for a longer period, and hence potentially easier to quantify. All water samples were quantified by qPCR, but the qPCR results were not in agreement with the CFU analysis. They indicated that S4_Ps was the most abundant and that S2_Pe was the least abundant. The results were also hard to interpret so they were excluded from results. Primer bias is a potential problem, and this can lead to better amplification of one strain than another, giving a false quantification results (Gaby and Buckley, 2017). Another factor to consider, is the fact that different strains of bacteria contain different copy numbers of the 16S rRNA-gene (rrnDB, https://rrndb.umms.med.umich.edu/), meaning that a strain with more copy numbers compared to another strain will be overestimated.

A test with samples containing fish tissue was run and it clearly indicates that the CVR fish contains more bacterial DNA than the GF fish by having a lower C_t-value. But there is little difference between the GF fish and the samples were all strains of bacteria were added. In addition to this, all samples had low C_t-values, including NTC, GF fish and the negative extraction kit control (app. 28-30), indicating that there was a problem with contaminating DNA. qPCR is an excellent method for quantifying bacteria. However, this method has proven to be difficult when studying host-microbe interactions where there is little target DNA. Reagents used during handling of the samples can contain contaminating DNA, including the PCR grade water, PCR reagents and DNA extraction kits. This problem increases with samples containing low amounts of bacterial DNA (Salter et al., 2014). In this experiment, the samples were dominated by salmon DNA which can be problematic because salmon DNA are known to contain PCR inhibitors for the bacterial 16S rRNA gene (Personal communication, Ingrid Bakke) and large amount of salmon DNA can lead to non-specific amplification of salmon DNA. The melting curves show a variable melting temperature between the samples, indicating that some of the samples could contain non-specific salmon DNA. However, the GF and NTC had approximately the same C_t-values, indicating that non-specific amplification is not the main problem.

4.5 Future work.

There was no observable difference in growth between the bacterial treatments, indicating that the microbiome is not crucial for survival and growth of salmon yolk-sack fry. It is more likely that the commensal bacteria are of more importance when the fry is exposed to potential pathogens, but more research on the commensal bacteria living in mucosal surfaces of fish is needed. There is still little knowledge on how commensal bacteria can strengthen the mucus barrier or how opportunistic bacteria can become pathogenic to the fish in certain circumstances. Future research related to this experiment could be to investigate if the strains used in these experiments have any beneficial effects on strengthening the mucus barrier of the fish. This experiment indicated that the S5_Ja strain outcompeted other commensals and that S5_Ja improved its own growth when combined with other strains. The closely related *Janthinobacterium lividum,* is known for its antibiological effects. So It would be interesting to monitor health and survival of salmon yolk sack fry during a pathogenic challenge experiment where S5_Ja is combined with known pathogens, or if pathogens are added after S5_Ja has successfully colonized the fish. In this experiment, there was no significant difference in growth when the fish was exposed to different bacteria, but a recent study from Chapagain et al (Chapagain et al., 2019) showed that there was difference in the indicator taxa between fast and slow-growing Rainbowtrout (*Oncohynchus mykiss*). It would be interesting to see how some of the strains identified as indicator taxa for fast and slow-growing Rainbowtrout would affect the growth and survival of yolk-sack fry in a similar gnotobiotic experiment. 10-week-old salmon fry were colonized by bacteria more effectively than 4-week-old salmon fry. It would be interesting to conduct an experiment to investigate how the age of the fish affects the colonization. For example, newly hatched CVR fish can be sampled once a week up to 10 weeks for quantifying total bacterial load. An experiment like this can reveal if the colonization of mucosal surfaces in salmon yolk-sack fry increases gradually or if it increases at a certain age.

5 Conclusion

qPCR was evaluated as a method for quantifying bacteria in skin and gut of salmon yolk-sac fry. Promising differences in the C_t values between GF and CVR fish samples were observed, but low C_t-values were obtained for GF fish samples, non-template PCR controls and negative extraction kit controls, indicating problems with contaminating DNA.

Growth and survival were investigated between fish exposed to different strains of bacteria over a period of 4 weeks. No mortalities and no significant difference in growth between the fish that were subject to different bacterial treatments was observed, indicating that none of the strains have any significant effect on growth and survival of the fish.

In total 6 strains were added to the rearing water and in investigated by CFU counting and Illumina 16S rDNA amplicon sequencing. All strains of bacteria were able to maintain their population in the water when added separately, but when several strains were combined, S2_Pe and S5_Ja outcompeted the other strains.

Samples of gut and skin (represented by the rest of the fish after removal of gut) was investigated by CFU analysis and Illumina 16S rDNA amplicon sequencing. The main findings from both experiments was that the skin is colonized to a much larger degree than gut during the yolk-sac period, regardless of the age of the fish. S5_Ja was the best colonizer of fish skin, followed by S2_Pe and the best colonizers of gut was S5_Ja and S2_Pe. The best colonizers were similar in both short-term and long-term exposure, indicating that the strains that were able to adhere to the mucosal surfaces of fish, was also the ones that were able to grow on these surfaces. Considerably higher CFU numbers were observed in both gut and fish during Experiment 2 when the fish was approximately 10 weeks old, compared to Experiment 1 when the fish was 0-4 weeks old. This indicates that the colonization of salmon mucosal surfaces increases with age. The S5_Ja strain seemed to benefit from the presence of other bacterial strains, indicating some sort of competition or commensal interaction.

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7 Appendixes

7.1 Appendix 1. Salmon gnotobiotic media (SGM)

Sol Gómez de la Torre Canny, modified from on US EPA/600/4-90/027F artificial water recipe

7.1.1 Salt Stocks

MgSO4•7H2O 100X
Dissolve 12.3 g in 1 l. Autoclave.
KCl 100X
Dissolve 0.4 g in 1 l. Autoclave.
NaHCO3 100X
Dissolve 9.6 g in 1 l. Autoclave.
CaSO4•2H2O 5X
Dissolve 0.3 g in 1 L. Filter sterilize.

7.1.2 SGM prep

MgSO4•7H2O 100X 10 ml
KCl 100X 10 ml
NaHCO3 100X 10 ml
CaSO4•2H2O 5X 200 ml
Miiq H2O 700 ml

1000 ml
Prepare in pre-autoclaved 1 L glass bottles.
Autoclave and store in fish room.

7.2 Appendix 2. Growth medias for microorganisms

7.2.1 Tryptic soy broth (TSB):

Table 5: Components of TSB per litre of deionized water.

Pancreatic digest of Casein (Tryptone)	17g
Papaic digest of soybean meal	3g
Dipotassium Phosphate (K_2HPO_4)	2.5g
Glucose	2.5g
Sodium chloride	5g

7.2.2 Tryptic soy agar (TSA):

TSB with 15g Agar

Nutrient broth:

Table 6: Components of Nutrient broth per litre of deionized water.

"Lab lemco" powder (meat extract)	1g
Yeast extract	2g
Peptone	5g
Sodium Chloride	5g

7.2.3 Brain heart infusion (BHI):

Table 7: Components of BHI per litre of deionized water.

Infusion from calf brain	200g
Infusion from beef heart	250g
Proteose peptone	10g
Dextrose	2g
Sodium chloride	5g
Disodium phosfate	2.5g

7.2.4 Saboraud dextrose broth (SD):

Table 8: Components of SD per litre of deionized water.

Mycological Peptone	10g
Dextrose	20g

7.3 Appendix 3. Antibiotic Cocktail

AB-GSM
Sol Gómez de la Torre Canny

7.3.1 Antibiotic Cocktail Preparation

7.3.1.1 Rifampicin (Rif)

(557303-1, VWR)
Stock: 50 mg/ml in DMSO
Dissolve 1000mg of powder in 20 ml of DMSO.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.
NOTE—To facilitate dissolving, I recommend splitting powder from original vial, shake at room temperature, and make sure to diffuse any clumps of powder at the bottom of the vial before adding DMSO. Shaking at RT for about an hour helped getting powder into solution.

7.3.1.2 Kanamycin (Kan)

(420311-5, VWR)
Stock: 50 mg/ml in H2O
Dissolve 1000mg of powder in 20 ml of filtered/autoclaved mqH2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

7.3.1.3 PenicillinG (PenG)

(A1837.0025, VWR)
Stock: 100 mg/ml in H2O
Dissolve 5000mg of powder in 50 ml of filtered/autoclaved mqH2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

7.3.1.4 Ampicillin (Amp)

(171254-5, VWR)
Stock: 100 mg/ml in H2O
Dissolve 5000mg of powder in 50 ml of filtered/autoclaved mqH2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

7.3.1.5 Oxolinic acid (Ox)

(J66637.06, VWR)
Stock: 12,5 mg/ml in 0,05N NaOH
Dissolve 1000mg of powder in 80ml of 0,05 N NaOH.
NOTE—0,05 N NaOH was prepared by diluting filter-sterilized 1N NaOH with filtered/autoclaved mqH2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

7.3.1.6 Amphotericin B (Fun)

Stock: 250 µg/ml pre-made solution
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

7.3.1.7 Erythromycin (Ery)

(329815-5, VWR)
Stock: 50 mg/ml in 90% EtOH
Dissolve 1000mg of 20ml of 96% OH.
NOTE—96% EtOH was prepared by diluting absolute EtOH in filtered/autoclaved mqH2O (19,2 ml of EtOH + qs 20 ml mqH2O=
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

7.3.1.8 Antibiotic working concentrations

Rifampicin 10 mg/l
Erythromicin 10 mg/l
Kanamycin 10 mg/l
Ampicillin 100 mg/l
Amphotericin B 250 ug/l
Penicillin 150 mg/l
Oxolinic acid 75 mg/l
Rifampicin 0,2 ml
Kanamycin 0,2 ml
Ampicillin 1 ml
Amphotericin B 1 ml
Penicillin 1,5 ml
Oxolinic acid 6 ml
qs 1 L GSM

7.3.1.9 Preparation

1. Thaw the Abx stocks in advance.
2. Prepare solution in a pre-autoclaved GSM bottle, by the addition of the Abx stocks as described above inside of the laminar flow cabinet.
NOTE: Do not irradiate Abx with UV light.
3. Filter sterilize the solution Abx cocktail and aliquot 100 ml in the polycarbonate bottles (qs for a large petri Dish of ~150 salmon embryos.
4. Frozen aliquots or freshly made Abx work well for derivations.
NOTE: Upon thawing, there will be a white precipitate in the Abx

7.4 Appendix 4. ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research)

Protocol

1. Add sample to a ZR **BashingBead™ Lysis Tubes (0.1 & 0.5 mm)**. Add 750 µl **ZymoBIOMICS™ Lysis Solution** to the tube and cap tightly.

   ***Note:** For samples stored and lysed in DNA/RNA Shield™ Lysis Tubes, do not add ZymoBIOMICS™ Lysis Solution and proceed to Step 2.*

Sample Type	Maximum Input
Feces	200 mg
Soil	250 mg
Liquid Samples[1] and Swab Collections[2]	250 µl
Cells (isotonic buffer, e.g. PBS)	50-100 mg (wet weight) (10^9 bacterial and 10^8 yeast cells)
Samples in DNA/RNA Shield™[3]	≤ 1 ml

2. Secure in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for ≥ 5 minutes.

   ***Note:** Processing time will vary based on sample input and bead beater. Times may be as little as 5 minutes when using high-speed cell disrupters (FastPrep® -24) or as long as 20 minutes when using lower speeds (e.g., Disruptor Genie®).[4]*

3. Centrifuge the ZR **BashingBead™ Lysis Tubes (0.1 & 0.5 mm)** in a microcentrifuge at ≥ 10,000 x *g* for 1 minute.

4. Transfer up to 400 µl supernatant to the **Zymo-Spin™ III-F Filter** in a **Collection Tube** and centrifuge at 8,000 x *g* for 1 minute. Discard the Zymo-Spin™ III-F Filter.

5. Binding preparation:

Feces and All Non-Soil Samples	OR	Soil Samples
Add 1,200 µl of **ZymoBIOMICS™ DNA Binding Buffer** to the filtrate in the Collection Tube from Step 4. Mix well.		Add 800 µl of **ZymoBIOMICS™ DNA Binding Buffer** and 400 µl of 95% ethanol to the filtrate in the Collection Tube from Step 4. Mix well.

6. Transfer 800 µl of the mixture from Step 5 to a **Zymo-Spin™ IIC-Z Column** in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute.

7. Discard the flow through from the Collection Tube and repeat Step 6.

8. Add 400 µl **ZymoBIOMICS™ DNA Wash Buffer 1** to the Zymo-Spin™ IIC-Z Column in a new Collection Tube and centrifuge at 10,000 x *g* for 1 minute. Discard the flow-through.

9. Add 700 µl **ZymoBIOMICS™ DNA Wash Buffer 2** to the Zymo-Spin™ IIC-Z Column in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute. Discard the flow-through.

For Technical Assistance:
1-888-882-9682 or E-mail
tech@zymoresearch.com

[1]For water samples, filter using desired filter (not provided). Cut the filter into small pieces and place into ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm).

[2]Swabs can also be cut or broken, then placed directly in bead beating tube. For more information on processing swab samples, see Appendix B.

[3]Up to 1 ml of sample in DNA/RNA Shield can be processed directly in ZR BashingBead™ Lysis Tube. Adjust final volume to 1 ml with ZymoBIOMICS™ Lysis Solution or DNA/RNA Shield, if necessary.

[4]For optimal lysis efficiency and unbiased profiling, all bead beater devices beyond those validated by Zymo Research should be calibrated using the ZymoBIOMICS™ Microbial Community Standard (see Appendix C).

For **Technical Assistance**: 1-888-882-9682 or E-mail tech@zymoresearch.com

[5] In some cases a brown-colored pellet may form at the bottom of the tube after centrifugation. Avoid this pellet when collecting the eluted DNA.

[6] If fungi or bacterial cultures were processed; the DNA is now suitable for all downstream applications.

10. Add 200 µl **ZymoBIOMICS™ DNA Wash Buffer 2** to the Zymo-Spin™ IIC-Z Column in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute.

11. Transfer the Zymo-Spin™ IIC-Z Column to a clean 1.5 ml microcentrifuge tube and add 100 µl (50 µl minimum) **ZymoBIOMICS™ DNase/RNase Free Water** directly to the column matrix and incubate for 1 minute. Centrifuge at 10,000 x *g* for 1 minute to elute the DNA[5, 6].

12. Place a **Zymo-Spin™ III-HRC Filter** in a new Collection Tube and add 600 µl **ZymoBIOMICS™ HRC Prep Solution.** Centrifuge at 8,000 x *g* for 3 minutes.

13. Transfer the eluted DNA (Step 11) to a prepared Zymo-Spin™ III-HRC Filter in a clean 1.5 ml microcentrifuge tube and centrifuge at exactly 16,000 x *g* for 3 minutes.

    The filtered DNA is now suitable for PCR and other downstream applications.

7.5 Appendix 5. TAE Buffer

Recipe for 50x TAE-buffer are presented in Table x. 1x TAE-buffer was prepared by diluting40 mL 50x TAE-buffer in 1960 mL MQ-water.

Table 9: Recipe for 50x TAE-buffer.

Components	**Amount**
Tris base	242g
Glacial acetic acid	57.1 ml
0.5M EDTA pH 8.0	100 ml
dH_2O	Up to 1 L

7.6 Appendix 6. C_t -values for each sample run in qPCR

Table 10: C_t -values for each sample run in qPCR (Experiment 1). Vibrio sp(RD5-30 was used for the standard curve. V=Vibrio, CVR=Conventionally raised, F=Fish, W=water, GF= Germ free, NTC= Non-template control.

Type of bacteria	Sample Name	C_t
Vibrio	V1	5,913
Vibrio	V1	6,406
Vibrio	V1	5,837
Vibrio	V2	8,997
Vibrio	V2	9,079
Vibrio	V2	9,117
Vibrio	V3	12,773
Vibrio	V3	12,643
Vibrio	V3	12,490
Vibrio	V4	16,087
Vibrio	V4	15,980
Vibrio	V4	15,935
Vibrio	V5	19,405
Vibrio	V5	19,438
Vibrio	V5	19,315
CVR	F:CVR8	22,081
CVR	F:CVR8	21,682
CVR	F:CVR8	22,810
CVR	F:CVR13	21,160
CVR	F:CVR13	20,755
CVR	F:CVR13	20,708
Pedobacter	W6	27,767
Pedobacter	W6	27,726
Pedobacter	W6	27,581
Pedobacter	W7	27,279
Pedobacter	W7	27,112
Pedobacter	W7	26,911
Pedobacter	W8	27,402
Pedobacter	W8	27,412
Pedobacter	W8	27,397
Arthrobacter	W10	23,612
Arthrobacter	W10	23,675
Arthrobacter	W10	23,591
Arthrobacter	W13	23,505
Arthrobacter	W13	23,598
Arthrobacter	W13	23,491
Arthrobacter	W14	34,796
Arthrobacter	W14	32,294
Arthrobacter	W14	35,633
Janthinobacterium	W15	21,881
Janthinobacterium	W15	22,063

Janthinobacterium	W15	21,514
Janthinobacterium	W16	21,170
Janthinobacterium	W16	21,135
Janthinobacterium	W17	22,276
Janthinobacterium	W17	21,488
Janthinobacterium	W17	21,647
Psychrobacter	W19	20,116
Psychrobacter	W19	20,255
Psychrobacter	W19	20,355
Psychrobacter	W22	19,683
Psychrobacter	W22	19,542
Psychrobacter	W22	19,500
Psychrobacter	W23	19,963
Psychrobacter	W23	20,588
Psychrobacter	W23	20,119
All	W25	23,576
All	W25	23,553
All	W25	23,385
All	W26	19,932
All	W26	20,144
All	W26	20,193
All	W27	19,455
All	W27	19,540
All	W27	19,383
GF	F:GF30	29,955
GF	F:GF30	29,893
GF	F:GF30	29,443
All	F26:all	30,083
All	F26:all	29,200
All	F26:all	30,251
	Extraction kit control	28,340
	Extraction kit control	28,331
	Extraction kit control	28,134
	NTC	32,396
	NTC	30,201
	NTC	30,348

7.7 Appendix 7. Melting curve analysis for qPCR

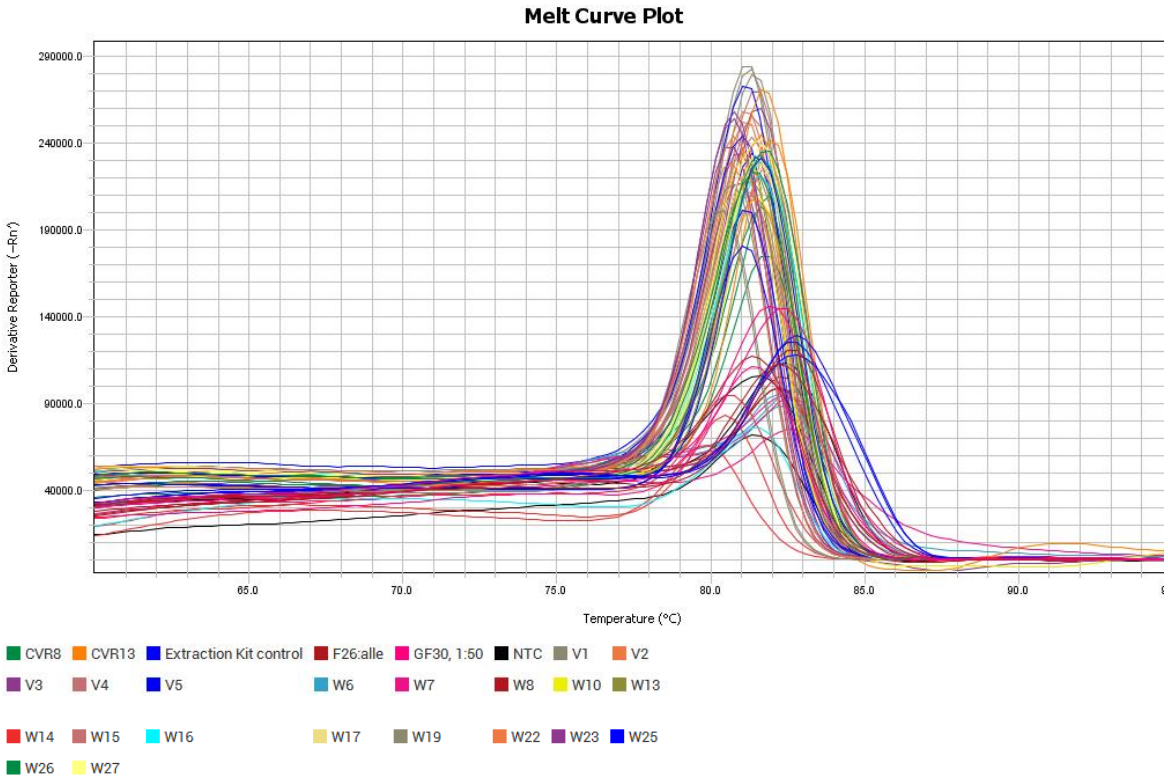
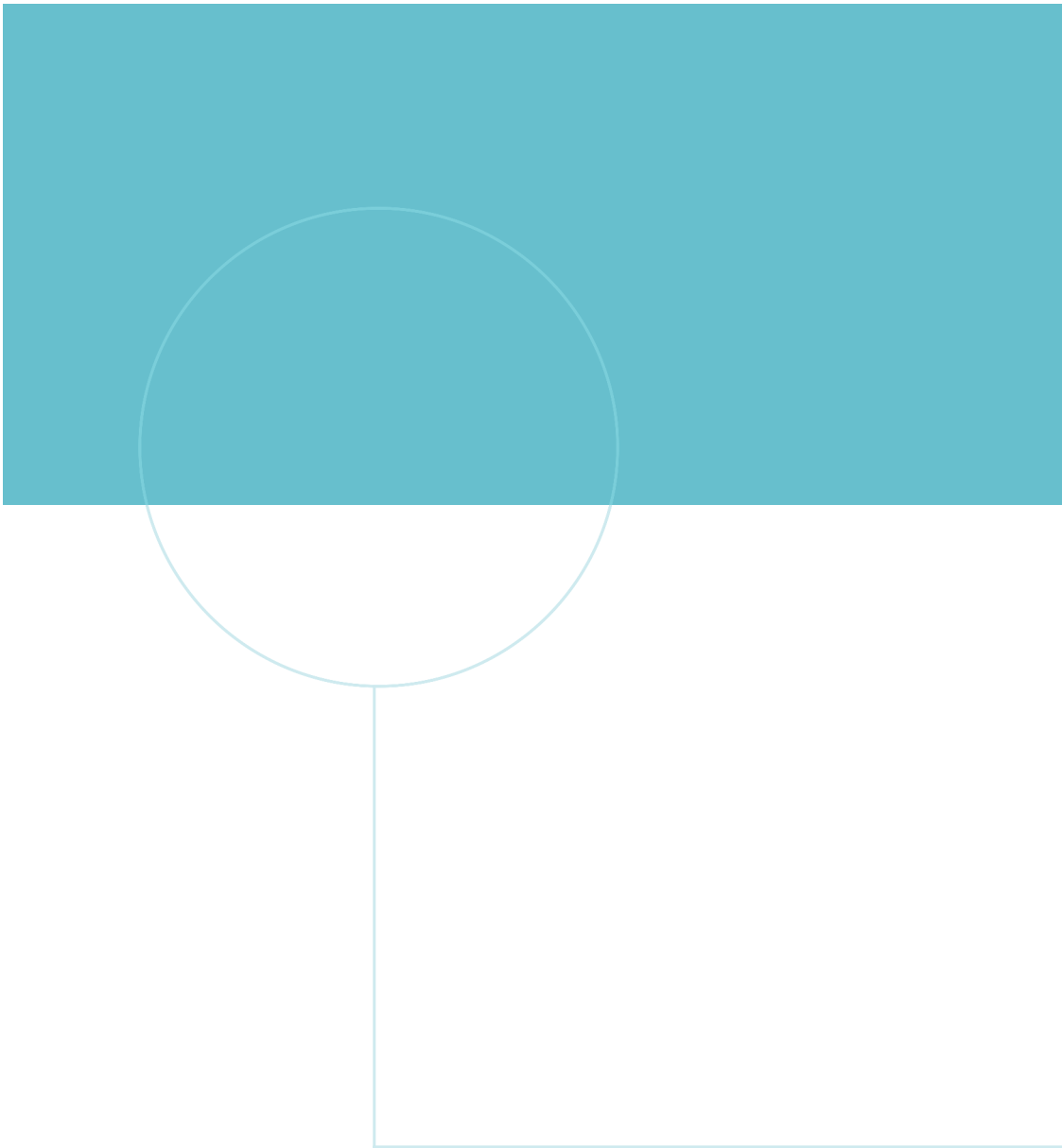


Figure 5: Melting curve analysis for all samples run in qPCR (Experiment 1). Vibrio sp(RD5-30 was used for the standard curve. V=Vibrio, CVR=Conventionally raised, F=Fish, W=water, GF= Germ free, NTC= Non-template control, Alle= all strains.

7.8 Appendix 8. Reads per sample after normalization (Experiment 2)

Table 11: No of reads per sample for Experiment 2 after normalization to 19500 reads per and subsequent removal of OTUs representing Salmo salar DNA, chloroplast rRNA genes, and OTUs identified as contaminants due to presence in non-template PCR and negative DNA extraction kit controls.

Sample	No of reads
W_id7-Ja-Ar	19496
W_id8-Ja-Ar	19496
W_id9-Ja-Ar	19496
W_id1-Ja-Pe	19496
W_id2-Ja-Pe	19498
W_id3-Ja-Pe	19497
W_id4-Ja-Pe	19497
W_id5-Ja-Fl	19498
W_id6-Ja-Fl	19498
W_id10-All	19499
W_id11-All	19500
W_id12-All	19497
Skin_id7-Ja-Ar	11121
Skin_id8-Ja-Ar	13868
Skin_id9-Ja-Ar	11344
Skin_id1-Ja-Pe	9231
Skin_id2-Ja-Pe	11775
Skin_id3-Ja-Pe	17444
Skin_id4-Ja-Fl	11191
Skin_id5-Ja-Fl	15796
Skin_id6-Ja-Fl	5621
Skin_id10-All	16627
Skin_id11-All	18056
Skin_id12-All	643
Gut_id7-Ja-Ar	4015
Gut_id8-Ja-Ar	12405
Gut_id9-Ja-Ar	1590
Gut_id1-Ja-Pe	3761
Gut_id2-Ja-Pe	4475
Gut_id3-Ja-Pe	5508
Gut_id4-Ja-Fl	6063
Gut_id5-Ja-Fl	5541
Gut_id6-Ja-Fl	5976
Gut_id10-All	949
Gut_id11-All	8894
Gut_id12-All	10975



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