Master's thesis

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Initial bacterial colonization of Atlantic salmon (*Salmo salar*) yolk-sac fry using a gnotobiotic model system

Master's thesis in Biotechnology
Supervisor: Ingrid Bakke
May 2019

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Kunnskap for en bedre verden

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Trondheim, May 2019 **Hanne Mallasvik**

Abstract

Mucosal tissue, covering the body cavities of animals and the skin of fishes, constitutes a major barrier against microbes and other agents in the external environment. The gut and the resident mucosal microbiota are one of the most studied mucosal surfaces and the gut bacterial community has shown to contribute to the mucosal barrier through a number of mechanisms. The fish skin mucus is also colonized by an indigenous microbiota, but its function and composition are poorly understood. This makes the skin an interesting structure to investigate, as we know little about the colonization by bacteria, and which function they plays in the skin mucosal barrier.

The main goal for this study was to investigate the initial colonization by single bacterial isolates of developing salmon fry by conducting a short-term gnotobiotic experiment. Germ-free salmon fry (10 days-post hatching) was inoculated with single bacterial strains that had previously been isolated from salmon fry skin and gut. To investigate whether the strains were pathogenic to the fish, survival was examined every day during the experiment. Quantification of the bacterial load in the skin and gut were determined by conducting colony forming units (CFU) counts on agar plates and qPCR. Prior to the gnotobiotic experiment, the strains were classified based on almost the complete 16S rRNA gene and characterized based on their growth on general and mucin medium.

The strains were classified at the genus or species level as *Bacillus sp.*, *Pedobacter sp.*, *Arthrobacter sp.*, *Janthinobacterium lividum* and *Psychrobacter cibarius*. All the strains were able to grow on mucin, and none of them were observed to be pathogenic to the salmon fry. The quantification using CFU counts revealed that the gut of the salmon fry was not colonized with any of the strains after three days of exposure, while the skin had the highest bacterial loads for the *Janthinobacterium lividum* strain. The *Bacillus sp*. strains did not colonize, neither the water nor the fish and were believed to represent a contamination, not originated from the salmon fry. The quantification of the bacterial load using qPCR showed to be unsuccessful, probably because the fish had too low bacterial numbers and that the samples were dominated by host DNA. The overall results showed that different strains have various abilities to colonize the mucosal surfaces of salmon yolk-sac fry, and that *J. lividum* was clearly the “best” colonizer of the skin. This is very interesting since *J. lividum* have also been found to be a member of the skin of amphibians and even humans, and have shown to exhibit ani-bacterial, anti-fungal and anti-cancer properties.

Sammendrag

Slimhinnevev kan bli funnet tilstede rundt alle dyrs kroppshulrom, i tillegg til fiskens skinn. Dette vevet utgjør en barriere mot mikrober og andre komponenter i det eksterne miljøet. Tarmen, og den tilhørende mikrobiotaen, er en av de mest studerte slimhinne overflatene, hvor det bakterielle samfunnet har vist seg til å bidra til denne barrieren gjennom en mengde mekanismer. Slimhinnen på fiskeskinnet er også kolonisert av en slik mikrobiota, men dens funksjon og komposisjon er lite forstått. Dette gjør at fiskeskinnet er en interessant struktur å undersøke, ettersom vi vet lite om koloniseringssuksessen av bakterier, og hvilken funksjon disse har ved slimhinne barrieren.

Hovedmålet for denne oppgaven var å undersøke den første koloniseringen av enkle bakterielle isolater på ny-klekte lakseyngler, ved å utføre et gnotobiotisk eksperiment. Bakteriefrie lakseyngler ble inokulert med enkle bakterie stammer, som tidligere hadde blitt isolert fra lakseyngel tarm og skinn. For å undersøke om noen av stammene var patogene, ble overlevelse av lakseyngler registrert hver dag under eksperimentet. Det ble også utført kvantifisering av den bakterielle mengden tilstede på skinnet og tarmen ved bruk av telling av kolonier på agar plater (CFU) og qPCR. I forkant av det gnotobiotiske forsøket, ble bakteriestammene klassifisert basert på nesten hele 16S rRNA genet, og karakterisert basert på deres vekst på generelt og mucin medium.

Bakterie stammene ble klassifisert på slekt- eller artsnivå som *Bacillus sp*., *Pedobacter sp*., *Arthrobacter sp*., *Janthinobacterium lividum* and *Psychrobacter cibarius*. Alle stammene kunne vokse på mucin, og ingen av dem ble observert til å være patogene for lakseynglene. Kvantifiseringen ved hjelp av CFU avslørte at ingen av bakteriene koloniserte tarmen til fisken, mens den høyeste bakterielle mengde på skinn ble observert for stammen *J. lividum*. Stammen *Bacillus sp*. koloniserte verken vannet eller fisken, og ble mistenkt til å representere en kontaminering som ikke originalt kom fra lakseyngel. Kvantifiseringen av den bakterielle mengden ved bruk av qPCR viste seg å være mislykket, ettersom fisken hadde for lave mengder bakterier på seg og prøvene var antageligvis dominert av verts DNA. De samlede resultatene viser at ulike bakterie stammer has ulik evne til å kolonisere slimhinne vevene til lakseyngler, hvor *J. lividum* var klart best på å kolonisere skinnet. Dette er veldig interessant siden *J. lividum* har også blitt vist å være medlem av skinn mikrobiotaen i noen amfibier og huden til mennesker, samtidig som den har vist å ha anti-bakterielle, anti-sopp og anti-kreft egenskaper.

Abbreviations

CFU	Colony forming units
CN	Copy number
CVR	Conventionally raised
EPS	Extracellular polymeric substance
FAO	Food and agriculture organization
GF	Germ-free
GI	Gastrointestinal tract
OD	Optical density
PCR	Polymerase chain reaction
qPCR	Real-time polymerase chain reaction
RDP	Ribosomal database project
S_ab	Seqmatch score
S1	*Bacillus*
S2	*Pedobacter*
S3	*Arthrobacter*
S4	*Janthinobacterium*
S5	*Psychrobacter*
SGM	Salmon growth medium
TSA	Tryptic soy agar
TSB	Tryptic soy broth
VIO	Violacein

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1 Introduction

1.1 Aquaculture

Aquaculture is probably the fastest growing food industries in the world, with an increase from 3 million tonnes in 1970, to almost 78 million tonnes today (FAO 2019a). One of the most important aquatic fish species produced is the Atlantic salmon (*Salmo salar*), and Food and agriculture organization (FAO) has reported that the world production of salmon has increased from 12 000 tonnes in 1980 to over 2,4 million tonnes (Figure 1.1) (FAO 2019c). Norway is one of the largest contributors to salmon production which accounts for more than 80 % of the total Norwegian aquaculture production (FAO 2019b).

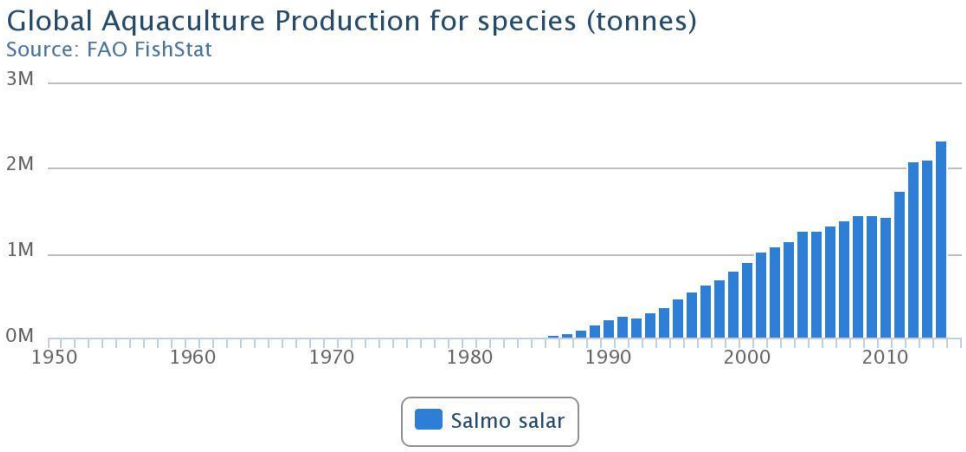

Figure 1.1: The trend of global aquaculture production of the Atlantic salmon (*Salmo salar*) over a time period of 60 years. (FAO 2019c)

Aquaculture is very important as a source of money, employment, food security and social development in a lot of countries. One of the reasons why aquaculture has been so successful may probably be based on the increased knowledge of the biology of fish species and how to control their reproduction, together with the development of new and improved technology (Brugère et al. 2010). However, with increasing pressure on the aquaculture industry, a lot of challenges arise, regarding development, feasibility, and sustainability of the production. One of the most pressing challenges is observed in large scale production, where the fish is reared in high densities, resulting in stressful conditions. This will subsequently result in higher susceptibility to pathogens which leads to problems with diseases (Esteban 2012). This linkage between stress such as different types of handling, shift in environmental conditions and high densities, with susceptibility to diseases has been reported in several different fish species (Tort,

Balasch, and Mackenzie 2003; Minniti et al. 2017). It has also been demonstrated that the ability of fish to maintain a balance between commensal, mutualistic and pathogenic bacteria at their skin mucosal surfaces is a key factor of preserving fish health (D Gómez and Balcazar 2008; Minniti et al. 2017). A result of an unbalanced microbiota can lead to diseases like skin disorders, which are often reported as a problem and cause of mortality in several aquaculture production sites. Ulceration is one example, which is often observed in Norwegian aquaculture of Atlantic salmon (Karlsen et al. 2012; Karlsen et al. 2017). The etiology is anticipated to be related to environmental factors together with the status of the skin health (Karlsen et al. 2017). That is why the understanding of the composition and relationship between the skin-mucus and microbiota of farmed fish may represent a step towards improving the welfare of important aquaculture fish species. With the pressing demand for increased fish production, the need for a better understanding of how fish interact with their surrounding microbes, both commensal and pathogenic is extremely important, which will promote good fish health in aquaculture settings.

1.2 Atlantic salmon life cycle and aquaculture

The Atlantic salmon belongs to the family of Salmonidae and is located in the Northern Atlantics (FAO 2019c). The salmon is an anadromous species, characterized by their large scale migration between fresh- and marine waters (Webb et al. 2007). The reproduction phase takes place in fresh water, and they return to their rivers of origin to spawn between October and January (FAO 2019c). A schematic overview of the life cycle of the salmon is presented in Figure 1.2. The eggs are released and placed in gravels until they hatch at approximately 500 day-degrees (Webb et al. 2007). The newly hatched fish, called alevins, stay located in the gravels until they have consumed their yolk-sac at approximately 300 day-degrees post-hatching. They emerge from the gravels, and enter a juvenile phase, feeding on insect and larvae. They spend approximately 2-5 years as fry and later parr, in the freshwater stage, until they undergo smoltification and becomes “smolt”. Smoltification is characterized by physical and chemical changes to adapt to life in marine environments. The smolts migrate to the ocean, where they head to deep feeding grounds to grow and mature (FAO 2019c).

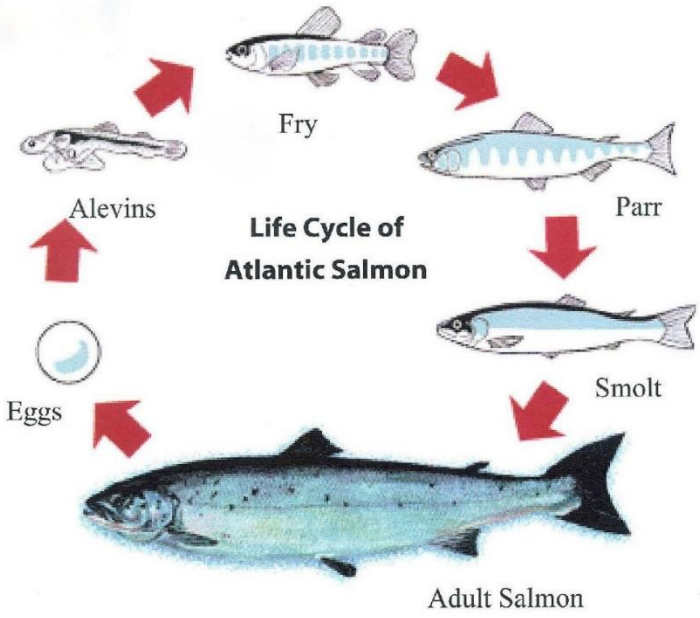

Figure 1.2: The life cycle of the Atlantic salmon (*Salmo salar*). Eggs are released in gravels and stay there until they hatch and become yolk sac fry, or alevins and later parr. Here they feed on insects and larvae until they become smolt and undergo the process of smoltification, making them ready for the migration to the sea. The phases from egg to parr occurs in freshwater, meanwhile, smolt and adult salmon is found in the sea (MarineInstitute 2019).

Because of the anadromous trait, the production of salmon in aquaculture needs to contain both freshwater and seawater stages. Broodstock fish are selected, and usually transferred to freshwater tanks, where favorable eggs and milk are collected. The eggs are stripped and fertilized, followed by incubation, hatching, and feeding with formulated feed. By using artificial light manipulation, the first smoltification process can be induced. All of these processes, hatching, nursery, and smoltification takes place in land-based, freshwater systems. The next stage is ongrowing at sea, which usually takes place in offshore marine cages. After ongrowing, which lasts between 14 to 30 months, the fish get harvested, handled, and processed to further use (FAO 2019c).

Many Salmonids, like the Atlantic salmon, have features and biological characteristics that make them highly suitable for intensive farming. The eggs are easy to obtain and incubate due to their large size. Also, the eggs' survival rate is high and hatches to create large-sized, robust offspring. The Atlantic salmon is characterized as a tolerant species, as it can easily adapt to new farm conditions and tolerate moderate degrees of population densities. The fish can reach a relatively large body size, up to 4-5 kg, which is an advantage in processing and harvesting (Jobling et al. 2010).

1.1 Host-microbe interactions

Extensive research has been made regarding interactions between microbes and the animal they colonize (Lupp 2007). The animal host provides numerous niches that can be colonized by microbes, which include skin, intestine, respiratory tract, urogenital tract and internal organs (Medzhitov 2007). Interactions with bacteria and their host can be viewed as a collection of symbiotic, commensal and pathogenic relationships (Hooper and Gordon 2001). Symbiosis refers to a relationship between two different species, where at least one partner benefits without hurting the other (Perret, Staehelin, and Broughton 2000). A commensal relationship is referred to as a co-existent between two partners, without any harmful effect, but with no obvious benefit (Hooper and Gordon 2001). Both symbiotic and commensal bacteria are viewed as mutualistic. One example of this is that some gut bacteria contribute to unique digestive enzymatic activities, which makes the intestinal content available for the host, resulting in increased nutritional uptake (Milligan-Myhre et al. 2011). As an opposite to this, a pathogenic relationship exerts damage to the host. This occurs sometimes by actively releasing toxins or by invading and expanding inside the host's tissue (Steinert, Hentschel, and Hacker 2000). However, defining a host-associated microbe as completely mutualistic or pathogenic can be difficult, because the outcome of any host-microbe interaction may be affected by circumstances like microbial ecology, or the status of the host's immune system (Milligan-Myhre et al. 2011).

Most of the research on host-microbe interactions have mainly been focused on terrestrial mammalian organisms, like mice and humans (Kelly and Salinas 2017). However, aquatic environments make an ideal medium for bacterial growth compared to air, which generates an intimate relationship between the animals living there and the surrounding microbes (Gomez, Sunyer, and Salinas 2013). This has made host-microbe interactions in fish an interesting field of research, which is increasingly being studied today (Lescak and Milligan-Myhre 2017).

The mucosal tissues and the residing bacterial community

The collection of mutualistic, commensal and pathogenic microorganism, also known as the microbiota, can be found at surfaces called mucosal tissues (Butt and Volkoff 2019). The mucosal tissues form a membrane which lines different cavities in the animal body and covers the surfaces of inner organs (Rogers 2015). The structure and function of these tissues can vary a lot depending on the animal and the location of the tissue (Salinas 2015). The main mucosal surfaces in fish are the gut, skin, and gills. These surfaces, all share characteristics with the well-studied type I mucosal surfaces of mammals, which is represented by the mammalian gut, respiratory tract, and uterus (Figure 1.3). Generally, the mucosal surfaces consist of one or multiple layers with epithelial cells, overlaying a deep connective tissue (dermis/ lamina propria). The epithelium in mammalian and fish gut is arranged as a simple columnar layer, while the fish skin and gills can have multiple squamous or cuboidal epithelium cells at their mucosal surfaces. Despite the differences in epithelium organization, all the surfaces share similar components like immunological elements like T cells, macrophages, mast cells and dendritic cells (Gomez, Sunyer, and Salinas 2013). Another key element that is present in all mucosal surfaces, is the mucus-producing goblet cells. The mucus is viscous and dynamic and is predominantly made up of highly glycosylated proteins named mucins. Mucins have strong adhesive properties and play a major role in the mucosal defense system. Alongside with mucins, the mucus contains proteins, lipids and ions, which creates a perfect niche for microbial attachment and growth. This together with the fact that mucus is also continuously produced and shed, creates a protective layer that removes entrapped microbes (Esteban 2012).

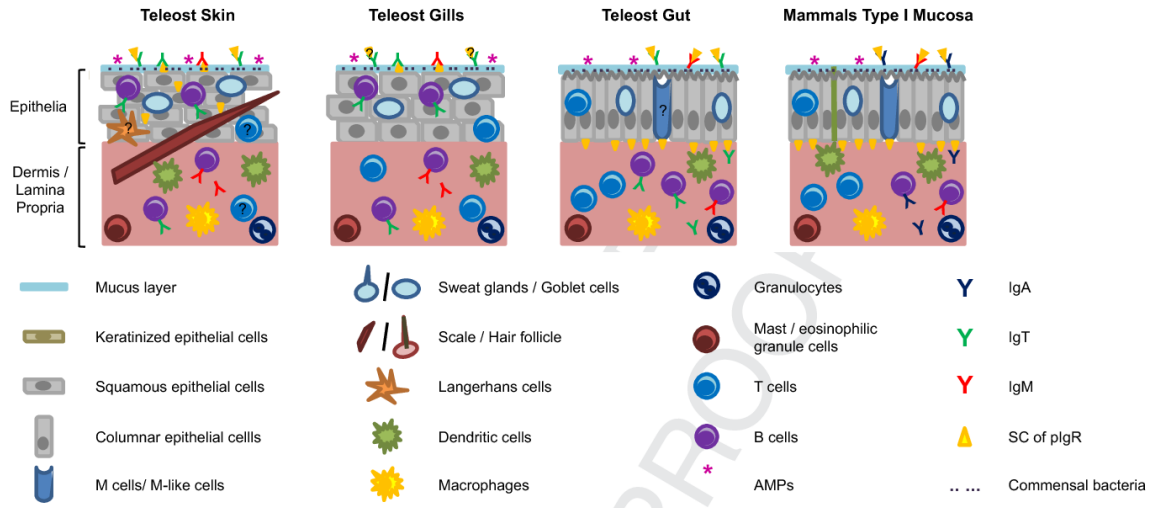

Figure: 1.3: Structural similarities and differences between mucosal surfaces present at fish skin, gut and gill, and mammals' type I mucosa. There are structural differences in type and number of epithelium cells. The bottom layer is connective tissue, called dermis for the skin, and lamina propria for the gut and gills. Similarities in cellular compounds can be found such as Dendritic cells, Langerhans cells, macrophages, granulocytes, mast cells, etc. There are differences in the location of B and T cells, isotype of immunoglobulins and the present of secretory components (SC) of the polymeric immunoglobulin receptor (pIgR). And finally, the presence of commensal bacteria and antimicrobial peptides in the outer layers.

The mucosal surfaces of vertebrates have undergone drastic changes during evolution, due to the transition from water to land. One surface that has been especially affected by these evolutionary pressures, is the skin. While birds and mammals possess hair, feathers, and scales as an adaption to the terrestrial environment, fish skin constitutes a living cell layer that produces and secrete mucus (Lowrey et al. 2015). The fish skin represents an ancient vertebrate mucosal structure, that covers the whole body, with a morphology successfully adapted to many of its functions (Elliott 2011). The epidermal layer of the fish skin is covered by a mucus layer primarily composed of water and glycoproteins. This layer has a wide range of functions which includes defense against pathogenic infections, as well as reproduction, osmoregulation, respiration, excretion, communication and feeding (Subramanian, MacKinnon, and Ross 2007). However, there exists limited research on fish skin mucosal surfaces and their residing microbiota. The fish gut mucosal surface however, is more studied and shares a lot of similarities to the mammalian gut mucosa, where it is described as a protective semi-permeable barrier, which prevents invasion of pathogen and allows exchange of nutrient, water and electrolytes (Ernst 2015).

1.1.1 Microbiota associated with fish gut

Establishment and functionality of the gut microbiota in fish

The microbiota in the fish gut is well-studied compared to the communities present on fish skin and gills. The intestine of small fish larvae starts as a straight sterile line upon hatching, which is quickly colonized by the surrounding bacteria after opening of the mouth (Ringø and Birkbeck 1999). The mouth typically opens 2-5 days post-hatching, depending on the fish species (Lescak and Milligan-Myhre 2017; Dimitroglou et al. 2011). This indicates that the gut microbiota in fish is early established after hatching. The established microbial community in the fish larvae may originate from different sources. It is generally believed that the process of early colonization depends on the microbiota on the egg surface, rearing water and live feed (Nayak 2010). Nevertheless, studies on cod have shown that the communities present in the rearing water and feed can differ from that of the larvae microbiota (Bakke et al. 2015). After opening of the mouth, the gastrointestinal tract (GI) is considered as one of the most important and intimate locations where interactions occur with the external environment (Dimitroglou et al. 2011). This also means that the fish gut is highly exposed to pathogenic bacteria, early in larval development. The immune system of hatched larvae is poorly developed and is mainly dependent on the innate immune system (Uribe et al. 2011). This is why the commensal bacteria residing on the intestinal mucosal surface in the gut, is believed to play a major role as the first line of defense against pathogens (Dimitroglou et al. 2011)

It is generally demonstrated that the gastrointestinal (GI) tract microbiota of mammals has multiple functions, like digestion, mucosal system development and immunity against pathogens (Wang, Yao, et al. 2017). Compared to mammalian research, microbial functionality in the fish gut is less understood. Commensal bacteria in the mammalian gut has shown to outcompete the pathogens for nutrients, as well as producing signal molecules and other products, inhibiting pathogens (Abt and Pamer 2014). The commensal bacteria in the fish gut has also shown similar roles in the immune system of fish, by stimulating mucus production, producing antimicrobial factors and contributing in the regulation of immunological responses (Abt and Pamer 2014; Hill, Cowley, and Andremont 1990). It has also been suggested that gut microbiota in fish plays a part in epithelial renewal, nutrition, and immunity. This was demonstrated by Rawls, Samuel, and I Gordon (2004), which showed that the gut microbiota

could regulate 212 genes, where some were related to stimulating of epithelial proliferation, promotion of nutrient metabolism and innate immunity response.

Microbial composition in fish gut and the factors affecting their structure

Based on studies using culture-dependent and culture-independent methods, it is generally assumed that the fish gut contains between 10^7 to 10^{11} bacteria per gram intestinal content (for review see: Nayak (2010)). By using next-generation sequencing, studies have shown that the bacterial community in fish gut is dominated by members of the phyla Proteobacteria, Bacteriodetes, Actinobacteria, Fusobacteria and Firmicutes (Wang et al. 2018). However, the microbial community consists of diverse bacteria, which has shown to vary significantly between fish species. For example, the GI tract of freshwater species has shown to be dominated by members, such as *Aeromonas, Pseudomonas, Lactococcus*, *Fusobacterium and some Bacteroidetes,* while the intestinal microbiota of marine fish species tends to be dominated by *Aeromonas, Alcaligenes, Alteromonas, Carnobacterium, Flavobacterium, Moraxella, Pseudomonas* and *Vibrio* (Pérez et al. 2010; Wang et al. 2018; Romero, Ringø, and L. Merrifield 2014). Multiple studies have also suggested that the microbial communities in the fish gut can evolve over time. This means that the community can vary between different fish sizes, life stages, interindividual differences, specific location in the gut and between different seasons (Merrifield and Rodiles 2015). Lokesh et al. (2019) investigated the difference between embryonic and intestinal bacterial communities in different life stages of Atlantic salmon. They discovered a significant transition of intestinal communities during development. Proteobacteria was observed to be dominant in the early developing stage (both embryonic and intestinal) and in the early seawater stage. Firmicutes was however observed to be dominant in the late freshwater stage. This shows that the microbiota of the fish gut can be very dynamic.

Several factors have shown to affect the level, stability, composition and the diversity of these communities. The environment is one of these factors, which includes water quality, salinity, season, and temperature (Merrifield and Rodiles 2015). This was observed for Atlantic salmon parr, reared in two different rearing systems. The gut of salmon reared in a recirculating laboratory aquarium had unique differences from the fish reared in cage cultures in open freshwater loch (Dehler, Secombes, and Martin 2017). A meta-analysis by Sullam et al. (2012) also investigated environmental factors, and they suggested that salinity, trophic level and possibly host phylogeny were the most important determinants for the gut community. Host

factors, like genetics, age, gender, species, and inter-individual differences have also been discussed as factors affecting the gut microbial community in fish (Wang et al. 2018). Host species was shown to be a strong determinant for intestinal microbiota in different cohabitating freshwater larvae (Li et al. 2012). Differences were also discovered in gut microbiota between male and female largemouth bronze gudgeon (*Coreius guichenoti*) (Li et al. 2016), which indicates that selection forces in the host also affect the microbial composition. This was investigated in cod larvae where they observed considerable changes in community structure with increasing age. Bakke et al. (2015) concluded that selection in the host structures the microbial communities associated with developing cod larvae. However, key factors that structure the gut microbiota of a developing fish larvae is not, yet, fully understood.

1.1.2 Microbiota associated with fish skin

Establishment and functionality of the fish skin microbiota

The fish skin microbiota is poorly studied, and there are limited research investigating how and when the skin microbiota is established. However, it is known that fish is continuously surrounded by microbes in their aquatic environment and have ideal mucosal surfaces for colonization of bacteria, where the skin is particularly exposed. Due to this close relationship, it would be reasonable to assume that the skin microbiota would reflect the community found in the aquatic environment. However, in spite of this close relation, studies have discovered that the skin microbiota differs from the microbial communities in the surrounding aquatic environment (Chiarello et al. 2015). Others have described the fish skin microbiota as resilient (Larsen et al. 2015), suggesting that they have developed adherence mechanisms and some ability to withstand the host defense system, in addition to a competitive relationship to other bacteria (Karlsen et al. 2017).

Research on the functionality of fish skin microbiota is also poor, and the present knowledge is restricted to vertebrate gut microbiota (Minniti et al. 2017). However, due to the skin's close interactions with the surrounding microbes, it is believed that it has an important role in the first line of defense against pathogens. This first line of defense consists of the mucus that creates a barrier, which ensures mechanical and chemical protection against the external environment and pathogens. It is believed that the microbiota aids in these mucus barrier functions

(Merrifield and Rodiles), by antagonistic activity and competition for adhesion sites and nutrients (Balcazar et al. 2007). It has also been reported that a shift in the skin microbiota caused by for example environmental factors may lead to breaches in the barrier, and dominance of pathogenic bacteria, which compromise the health of the fish (Lokesh and Kiron 2016).

Microbial composition in fish skin and the factors affecting their structure

Based on previous studies, the estimated bacterial density on fish skin is between 10^2 to 10^4 bacteria per cm skin. However, this is based on culture-dependent studies, which has shown to grossly underestimate bacterial densities, since not every bacterium is cultivable under lab conditions (Austin 2006). It is however demonstrated that the dominating phyla in skin associated microbiota is *Proteobacteria*, followed by *Actinobacteria, Bacteroidetes*, and *Firmicutes* (Chiarello et al. 2015; Lowrey et al. 2015; Larsen et al. 2013). However high variations at species level have been reported in many of the same studies. Chiarello et al. 2015 also discovered high variations in skin associated microbiota between individuals of the same species and between body parts. Nevertheless, only a limited number of studies have focused on the complexity of the bacterial community on fish skin.

Several factors have been reported to influence the bacterial composition of the gut microbiota in fish. Factors affecting the skin associated microbiota of fish has however not been as thoroughly investigated. Yet, several external and host-related factors, have been reported as determinants of the density and composition of the fish skin. These factors include host species specificity, environment, season and mucus composition (Larsen et al. 2013; Merrifield and Rodiles 2015). A shift in the microbial composition in skin and gut was observed as a result of lowered pH in the study of Sylvain et al. (2016). Furthermore, host genotype and gender have shown to be strong influencers, which results in significant intra-species variations (Boutin et al. 2014). Different diets have also been related to community changes in the fish skin, through changes of the mucus composition. Stress can also cause changes in mucus and subsequently changes in bacteria community. Minniti et al. (2017) investigated the bacterial communities in the skin of farmed Atlantic salmon, before and after different types of handling. They observed significant variabilities in the skin microbiota between individuals before fish handling, which in turn shifted to more similar bacterial communities after handling. This shows that different types of stress aids in altering the microbial composition.

Understanding the role of environmental bacteria in the colonization of skin mucus, and the part it plays in the mucosal barrier function is extremely important. Nevertheless, these problems are poorly understood and unexplored today and is yet to be discovered (Lescak and Milligan-Myhre 2017).

1.2 Gnotobiotic studies

The importance of the commensal bacteria to host mucosal development, immune response and function in fish has received more attention in recent years. However, most of the information available has focused on the molecular mechanisms that underlie pathogenic host-microbial relationships, and less is known about the mechanisms for the commensals bacteria (Rawls, Samuel, and I Gordon 2004). Germ-free (GF) or gnotobiotic models make excellent tools for investigating the interaction between a host and its microbiota (Merrifield and Rodiles 2015). The term germ-free is used to describe an animal completely deprived of microbes, including bacteria, viruses, fungi, protozoa, and parasites. Germ-free animals that are colonized with a selection of one or multiple known bacterial species are referred to as gnotobiotic (Al-Asmakh and Zadjali 2015).

Gnotobiotic systems were first developed for different mammals, such as mice and rats. However, several successful reports on generating germ-free fish have been described for many species, such as platy fish (*Xiphophorus maculatus*) (Baker, Ferguson, and TenBroeck 1942), Atlantic halibut (*Hippoglossus hippoglossus*) (Verner-Jeffreys, Shields, and Birkbeck 2003), turbot (*Scopthalmus maximus*) (Munro, Barbour, and Birkbeck 1995), different types of salmonids (Trust 1974), zebrafish (*Danio rerio*) (Rawls, Samuel, and I Gordon 2004; Pham et al. 2008) and Atlantic cod (*Gadus Morhua*) (Forberg, Arukwe, and Vadstein 2011a). Gnotobiotic techniques are rather easy to conduct on fish compared with mammals, due to their development *ex utero* and ability to sterilize the surface of the eggs shortly after fertilization (Lescak and Milligan-Myhre 2017). Generally, the first step to generate germ-free fish involves surface disinfection of the egg and subsequently hatching in an GF environment. The main advantage of using these systems is the high degree of control one can exert on the microbial environment of the fish. One of the biggest challenge has been developing sterile diets that also support growth and survival (Melancon et al. 2017).

The development of GF zebrafish has had a major impact on the use of fish as model organisms for gnotobiotic studies (Rawls, Samuel, and I Gordon 2004; Lescak and Milligan-Myhre 2017). It has made it possible to compare the phenotype of GF zebrafish larvae with conventionally reared fish. This has revealed important aspects of the microbiota as a necessary component for normal physiology of the host. Germ-free zebrafish have shown to have impaired neutrophil migration to injury sites (Kanther et al. 2014), impaired level of larval resistance to viral infections (Galindo-Villegas et al. 2012), lacking expression of innate immune genes and changes gut epithelial cell turnover (Rawls, Samuel, and I Gordon 2004). Nevertheless, if the fish is colonized with its natural microbiota, it regains its immune functions (Rawls, Samuel, and I Gordon 2004). However, the results generated from one fish species may not be easily transferable to another. Germ-free seabass larvae did not show any of the abnormalities mentioned above (Rekecki et al. 2009), and cod larvae showed high survival and no significant differences in growth compared to larvae with conventional microbiota (Forberg, Arukwe, and Vadstein 2011b; Rekecki et al. 2009). These differences suggest that the mechanisms underlying host responses may be different between species. This highlights the need for more gnotobiotic research in multiple fish species (Vestrum et al. 2018).

Mice have traditionally been used to study host-microbe interactions. However, studies using inbred mouse models have restrictions such as genetic constraints, inability to observe microbe-host interactions in a living organism and making artificial conditions similar to the natural habitat. All these limitations indicates the need for a better model system that allows the examination of how microbial communities are affected and shaped by natural host genetic variation both statistically and in lab-reared versus wild strains (Lescak and Milligan-Myhre 2017). Fish comprise nearly half of all the vertebrate diversity, with over 28 000 characterized fish species. They have extensive variations in physiology, ecology, natural history, and facilitate physiological and immunological characteristics common to other vertebrates, including humans (Lescak and Milligan-Myhre 2017). Fish have short lifecycles, a high number of offspring and diversified properties which can easily be manipulated (Butt and Volkoff 2019).

Most of the research on host-microbe interactions in teleost's has primarily focused on germ-free zebrafish (Lescak and Milligan-Myhre 2017). Due to its rapid external development, optical transparency and large brood size, they make excellent models for germ-free studies (Melancon et al. 2017). Understanding the role of the microbiota on skin barrier function in salmon fry is highly relevant for aquaculture issues. Together with being the most important

fish species in Norwegian Aquaculture, it presents major advantages as a model for germ-free protocols. The fry is large upon hatching, and the yolk sac stage can last up to two months at standard temperatures at 6-7 °C (Webb et al. 2007). This means that large fry can be produced and kept over a long period of time, without external feeding.

1.3 Hypothesis and aims

The hypothesis of this project is that different bacterial strains have various abilities to colonize the mucosal surfaces of Atlantic salmon yolk-sac fry. The aims of this study are

- To classify five bacterial strains, previously isolated from salmon fry skin and gut, and characterized their growth on non-selective general growth medium and mucin medium.
- Investigate if any of the bacterial strains are pathogenic to the salmon fry, by examining the survival of the fish.
- Investigate if there are differences in the colonization densities of the bacterial strains between salmon fry skin and gut.
- Investigate the strains' ability to colonize the salmon fry gut and skin mucus as assessed after three days of exposure

The overall goal of this thesis is to improve the understanding of the initial colonization of developing fish fry.

2 Materials and Methods

This study was a part of a research project at NTNU Department of Biotechnology and Food Science called “Microbial contributions to the Atlantic salmon (*Salmo salar*) skin mucosal barrier”. The study consisted of two parts; the classification and characterization of bacterial strains isolated from salmon fry, and a gnotobiotic fish experiment to assess the initial colonization of these bacteria on salmon fry skin and gut.

2.1 Characterization of bacterial strains isolated from salmon fry

2.1.1 Selection of bacterial strains

A strain collection of approximately 80 bacterial strains, isolated from salmon fry skin and gut, had previously been collected at SalMar’s hatchery at Follafoss and established in the research group ”*Analysis and control of microbial system*” (ACMS). The isolates were identified at the genus level based on a short sequence (400 bp) of their 16S rRNA-gene. Five bacterial strains were selected based on their taxonomic differences, to work with, in this experiment (Table 2.1). Preliminary analysis performed in the ACMS group, based on short 16S rDNA sequences (400 bp) showed that the five strains represented the genus *Bacillus, Pedobacter, Arthrobacter, Janthinobacterium* and *Psychrobacter.*

Table 2.1: Collection of bacterial strains isolated from Atlantic salmon fry.

ID	**Phylum**	**Class**	**Genus**	**Isolated from salmon fry skin/gut**	**Gram +/-**
S1	Firmicutes	Bacilli	*Bacillus*	Skin and gut	+
S2	Bacteriodetes	Sphingobacteria	*Pedobacter*	Only skin	-
S3	Actinobacteria	Actinobacteria	*Arthrobacter*	Skin and gut	+
S4	Proteobacteria	Betaproteobacteria	*Janthinobacterium*	Only skin	-
S5	Proteobacteria	Gammaproteobacteria	*Psychrobacter*	Only gut	-

2.1.2 Taxonomic identification of the strains using PCR and Sanger sequencing

The strains S1-S5 were further classified by using PCR and Sanger sequencing, to sequence almost the complete 16S rRNA gene.

2.1.2.1 Harvesting of bacteria and DNA extraction

A bacterial culture was prepared in 3 ml liquid medium, Tryptic soy broth (TSB) (Appendix A), for each of the five strains S1-S5. The bacteria were harvested by centrifuging at 5000 rpm for 5 minutes, the supernatant was removed, and the pellet was used as starting material for the DNA extraction. Total DNA was extracted using the DNeasy Powersoil DNA Isolation Kit (Qiagen), according to the manufacturer's protocol (Appendix B)

2.1.2.2 Polymerase chain reaction (PCR)

Almost the complete 16S rRNA gene (1490 bp) from the extracted bacterial samples were amplified using polymerase chain reaction (PCR) and the primers Eub8F and 1492yR (Table 2.2). A master mix was made according to Table 2.3, and a volume of 24 µl was aliquoted to PCR tubes. A volume of 1 µl DNA extract was used as a template and added to the PCR tubes, making a total volume of 25 µl. PCR amplification was performed using the T100^{TM} Thermal Cycler (BioRad) with the following cycle conditions: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, 72 ° for 60 seconds, and a final elongation step at 72 °C for 30 minutes (Table. 2.4)

Table 2.2: Primers (Sigma-Aldrich) used in this study for qPCR, Sanger sequencing and PCR amplification as specified in the table.

Primer name	**Sequence (5'-3')**	**Application**
Eub8F	5'- AGA GTT TGA TCM TGG CTC AG -3'	PCR
984yR	5'- GTA AGG TTC YTC CGC GT -3'	Sanger sequencing
518R	5'- ATT ACC GCG GCT GCT GG -3'	Sanger sequencing
1492yR	5'- GGT TAC CTT GTT ACG ACT T -3'	PCR
RT-966F	5´- GCA ACG GCM RGA ACC TTA CCT A - 3´	qPCR
RT-1089R	5´- CSG GAC TTA ACC SAA CAT YTC A - 3´	qPCR

Table 2.3: Components for making a 24 µl master mix for PCR.

Components	**Volume**
Taq-buffer (10 x)	2.5 µl
dNTP (10 mM each)	0.5 µl
Primer Eub8F (10 µM)	0.75 µl
Primer 1492R (10 µM)	0.75 µl
Taq polymerase	0.125 µl
Filtrated H_2O	19.375 µl
Total	24 µl

Table 2.4: Cycling conditions used during the amplification of almost the complete bacterial 16S rRNA gene. Step 2-4 was repeated 35 times.

Step	**Reaction**	**Temperature**	**Time**	
1	Denaturation	95 °C	3 min.	
2	Denaturation	95 °C	30 sec.	x35
3	Annealing	60 °C	30 sec.	
4	Elongation	72 °C	60 sec.	
5	Final step and elongation	72 °C	30 min.	

2.1.2.3 Agarose gel electrophoresis

The quality and quantity of the PCR products were verified by agarose gel electrophoresis, on a 1% agarose gel in 1X TAE buffer. A volume of 50 ml of the gel was mixed with 2.5 µl GelRed (final concentration of 50 µM) (Qiagen) for subsequent visualization of the DNA in the gel and poured into a gel chamber. Wells were made using a comb, and the gel was left to polymerize for 30 minutes. Loading dye (1 µl) (Thermo Scientific) was mixed together with 4 µl PCR product and added to a well. A GeneRuler 1kb Pluss ladder (Thermo Scientific), a pure *Vibrio sp. (*RD5-30) strain (positive control) and a non-template sample (negative control) were also added. The gel was run in a 50 x TAE buffer solution at 120 volts for 75 minutes. Subsequently, DNA bands in the gel were visualized in a UV light cabinet.

2.1.2.4 Sanger sequencing

Sanger sequencing was performed to sequence almost the complete 16S rRNA gene for each of the five strains. First, the PCR product was purified using the Qiaquick purification kit (Qiagen), following the manufacturer's protocol (Appendix C). Three sequencing primers (518R, 984yR, and 1492yR) (Table 2.2) were used to cover almost the complete gene. A volume of 5 µl template and 5 µl primer (5 mM) were mixed together and sent for sequencing at Eurofins Genomics.

Taxonomic classification was performed based on the results received from the Sanger sequencing. The sequencing results were presented as chromatograms, where “peaks” and colors represented the different bases in the sequence. Primer sequences and regions of poor quality in the 5’- and 3’- ends of the sequences were removed. For each strain, the sequences obtained for the three sequencing reactions were aligned, using the Clone manager software (Sci-Ed 2016) covering almost the complete 16S rRNA gene. Finally, the sequences were analyzed by using the Ribosomal database project (RDP), classification tool (Wang et al. 2007).

2.1.3 Growth curves for the bacterial strains with TSB and mucin medium

Growth curves were made for the five bacterial isolates S1-S5 with non-selective, general growth medium and mucin medium. Mucin is the main component of mucus, and the isolates ability to grow on this was investigated.

The isolates were revived from glycerol stocks, plated on tryptic soy agar (TSA) (Appendix A) and incubated for two days at 22°C. A single colony from each strain were transferred to 3 ml liquid medium (TSB) and incubated for 48 hours at 22 °C with shaking. These cultures were used to prepare 1 % sub-cultures, which were used as starting material for the OD_{600}-measurements. A TECAN Spark® 20M microplate reader and a 96 well plate were used for the culturing an automatic OD- measurements. A total volume of 150 µl of each sub-culture was aliquoted in wells, with three replicates. The outer wells were not used due to the increased chance of evaporation. All the empty wells were filled with liquid media, to detect any potential contaminations. The following growth conditions were used during the OD measurements: shaking for 120 seconds, followed by a loop which alternated between absorbance measurements at 600 nm and one hour shaking. The actions inside the loop will take place repeatedly for the duration of the loop, which lasted 72 hours in this experiment. The temperature was set to 22 ° during the cultivation.

The same procedure was applied with mucin medium, to test the strains ability to grow on mucin. Liquid mucin medium was prepared with 5.25g M9 broth (VWR) and 2 g mucin (Sigma Aldrich) in 500 ml MQ water (Appendix A). The medium was autoclaved at 121 °C for 15 minutes before the addition of 1 ml magnesium sulfate (1M). The cultures were incubated over four days at 22°C with shaking. Subsequently, 1 % sub-cultures were made and used as starting material in the 96 well plate and TECAN Spark® plate reader. The same conditions were used as for the TSB medium, except that the duration of the loop was 96 hours, to ensure that the strains would reach stationary phase.

The data obtained from the cultivation experiment in the plate reader were used to make growth curves. The exponential phase of the growth curves together with Equation 2.1 and 2.2 were used to calculate the generation time.

$$\mu = \frac{lnOD_2 - lnOD_1}{t_2 - t_1} \qquad (2.1)$$

$$Generation\ time = \frac{ln2}{\mu} \qquad (2.2)$$

2.2 Gnotobiotic fish experiment

2.2.1 Generating germfree salmon fry

Salmon eggs were delivered from Aquagen and immediately acclimatized at 6-7 °C in the dark. Upon arrival, the eggs were transferred to large petri dishes containing 100 ml autoclaved (121 °C, 15 min.) salmon growth medium (SGM) (Appendix D). To obtain germ-free (GF) fish, a double disinfection procedure was applied for the eggs. In the first disinfection procedure, eggs were immersed in sterile SGM containing antibiotics (Appendix E), filter-sterilized and incubated for 24 h at 7 °C. The second procedure was performed 24 hours after the first. A solution of 100 mg/l available iodine was prepared by adding 500 µl Buffodine in 50 ml of SGM. The eggs were exposed to the iodine disinfectant solution for 30 minutes. During the disinfection, eggs were gently agitated to ensure that all eggs had equal contact with the disinfected agent. Following disinfection, eggs were rinsed 4 times with sterile SGM. All disinfection procedures were performed under a laminar flow hood, using UV-irradiated equipment.

Surface disinfected embryos were distributed to 500 ml sterile tissue culture flasks containing 100 ml sterile SGM and incubated at a density of 150 eggs l^{-1}. Conventionally raised (CVR) fish, i.e. fish containing unknown bacteria potentially originated from the hatchery were used as a control group. Eggs of the control group underwent the same incubation procedure but without the antibiotic and surface disinfection treatment. All the fish flasks (GF and CVR) were kept in the dark with temperatures around 6-7°C, during the whole experiment. A total of 60 % SGM in the flasks were changed three times a week, for maintaining good water quality. A sterility check was also performed one-week post-hatching. Three different liquid media (Nutrient Broth, Brain heart infusion BHI, and Saboraud dextrose broth SD) (Appendix F) and TSA plates were inoculated with 100 µl SGM, from each GF flask. The samples were incubated at both room temperature and at 6-7 °C. A control bottle (CVR) was also tested in the same way.

2.2.2 Bacterial mono-association of the germ-free salmon fry

The colonization of a germ-free (GF) organism with a microbe of a single species is referred to as mono-association (Melancon et al. 2017). This was used in this experiment, to investigate the initial colonization of individual bacterial isolates (S1-S5) on salmon fry skin, gut and rearing water. Germ-free (GF) and conventionally raised (CVR) fish were transferred from tissue culture flasks to 6-well plates, filled with 8 ml SGM in each well. The fish were transferred by lifting them by their yolk sac, with a serological pipette. Subsequently, the rearing water of the GF and CVR fish in the well plates were inoculated with single bacterial isolates (S1-S5), and left exposed for three days. There were 12 replicates prepared for each treatment, and as a control group, 12 GF fish and 12 CVR fish were not inoculated with any of the bacteria (Figure 2.1).

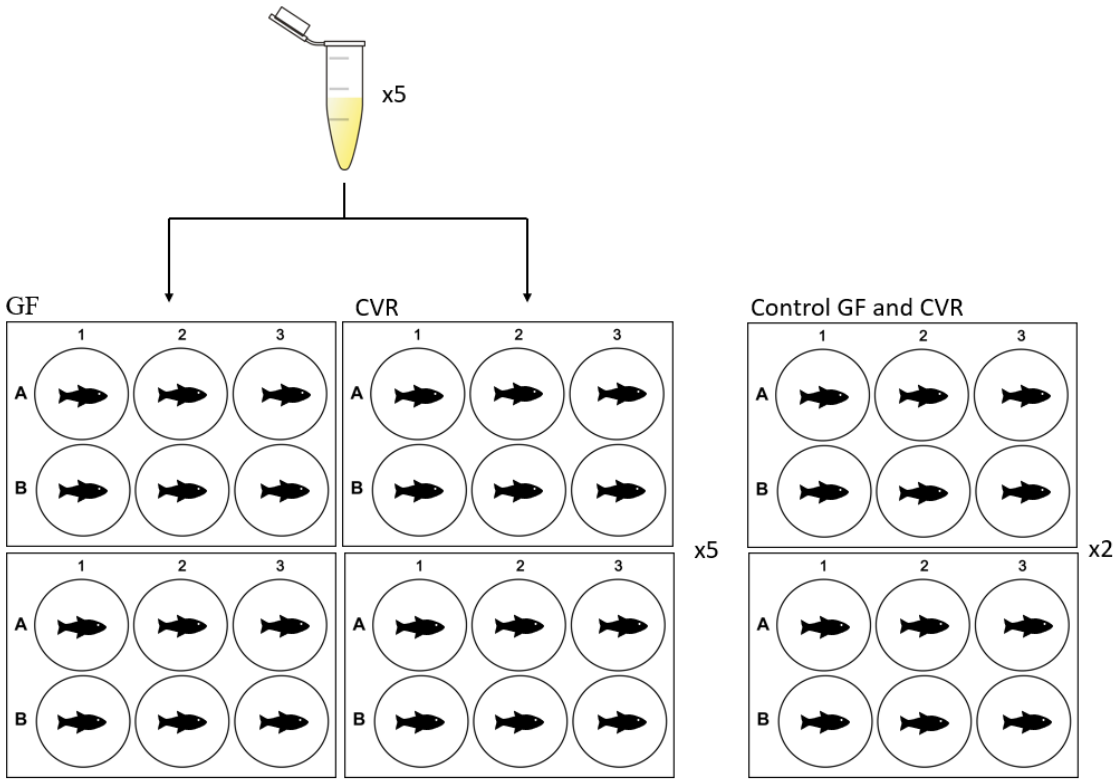

Figure 2.1: Experimental design of the gnotobiotic fish experiment. 6 well plates, containing salmon growth medium (SGM) and salmon fry, were used in the mono-association with bacteria. There were 12 replicate fish for each bacterium, both germ-free (GF) and for conventionally raise (CVR) fish. 2 types of control were used, with no addition of bacteria (Control GF and CVR), with 12 replicate fish each (Eppendorf tube originated from clker.com (2019))

Bacteria were introduced approximately 1-week post-hatching, by which time the salmon fry's mouth and intestine had opened. A liquid culture (TSB) of each bacterium was prepared two days before the exposure day, in 13 ml tubes at 22°C with shaking. The bacterial suspensions were harvested by centrifuging at 2000 rpm for 3 minutes and resuspended in 6 ml of SGM. This washing procedure was repeated two times. The bacterial solutions were kept on ice, and the OD_{660} was measured with a spectrophotometer, for all the samples. Samples with OD_{660} values over 0.3 were diluted with SGM to reach an appropriate concentration between 0.1-0.3. The density of the bacterial suspensions was determined by using the McFarland standard (Equation 2.3). Bacteria were aliquoted to achieve a density of 10^5 colony forming units (CFU) ml^{-1} in each well.

$$\text{Bacterial Density (CFU ml}^{-1}) = OD_{660} \cdot 1{,}2 \cdot 10^9 \qquad (2.3)$$

The fish were kept for three days at 6-7°C, in the dark. Survival of the fish was reported every day of the experiment.

2.2.3 Sampling of the gnotobiotic salmon fry

This was a short-term experiment, where the fish was sampled three days after exposure. The initial colonization of the five bacterial strains was assessed using one culture-dependant (CFU) and one culture-independent method (qPCR). Plate counts on TSA and serial dilutions were used to estimate the colony forming units (CFU) in the salmon fry skin, gut and rearing water, after exposure to the bacteria. Whole individual fish were also collected from both GF and CVR wells, exposed to bacteria, together with fish from GF control wells (i.e. containing no bacteria), to quantify the bacterial load using qPCR. The whole fish were snap-frozen in liquid nitrogen and stored at -80°C. All fish were euthanized using Ethyl 3-aminobenzoate methane sulfonate (5.2g/l) (Sigma).

2.2.3.1 CFU counts

For each bacterium, three mono-associated fish were collected, and the gut was dissected out. The dissection was performed under a microscope with pre-sterilized forceps. The yolk sac was removed, and the whole gut of the fish was transferred to sterile screw cap, pre-loaded with 150 µl 1.4mm zirconium oxide beads (Precellys) and 250 µl SGM. The rest of the fish were placed in sterile cryotubes containing 150 µl 1.4mm zirconium oxide beads (Precellys) and 500 µl SGM, which were further used as skin samples. The samples were homogenized by shaking (vortex adapter for 2 ml tubes) for 5 minutes. The gut and skin homogenates were serially diluted three time (i.e. 10^{-1}, 10^{-2}, 10^{-3}) in triplicates. A volume of 80 µl from each dilution was aliquoted on TSA plates and spread, using glass beads. The undiluted samples were also plated in triplicates. Samples of the rearing water for each individual fish were also collected. A volume of 80 µl of undiluted and diluted (10^{-1} and 10^{-2}) water samples, were plated in the same procedure as the fish samples, but with no replicates. The plates were incubated at room temperature for 72 hours.

2.2.3.2 Real-time PCR

To quantify the number of bacteria in whole salmon fry samples, real-time PCR (qPCR) was conducted. Fish exposed to strain S1 was however excluded from the qPCR, due to the low amount of CFU's observed in the salmon fry skin, gut and water (see section 3.2.1). Real-time PCR was used to estimate the bacterial load in germ-free salmon fry, mono-associated with the bacterial strains S2-S5. Conventionally raised (CVR) salmon fry mono-associated with strain S4 were also included, together with CVR and GF fish controls (i.e. no added bacteria). The GF control was included to investigate the possibility of co-amplification of salmon DNA. Prior to the qPCR, PCR was conducted on a few selected samples, including a pure *Vibrio* sp. isolate RD5-30 (Fjellheim et al. 2010) (positive control) and a non-template sample (negative control). Whole individual fish samples were collected from -80 °C and thawed. The fish were divided into smaller pieces with a scalpel, under sterile conditions, and used as starting material.

DNA extraction and PCR

Total DNA was extracted from the samples using the Ultradeep Microbiome Prep kit (Molzym). The extraction was performed according to the manufacturer's protocol (Appendix G), with minor alterations. Instead of a thermomixer at step 6 and 7, the sample was transferred continuously between a heat block and the vortex.

PCR and agarose gel electrophoresis were conducted on a few selected samples to investigate the quality of the qPCR cycling conditions and the quantity of product obtained in the DNA extraction. A region of the 16S rRNA gene of the DNA extracts was amplified by using broad coverage primers RT996F and RT1089 (sequences specified in Table 2.2). A master mix was made according to table 2.5, and a volume of 24 µl was distributed to PCR tubes. The DNA extracts were used as templates, and 1 µl was added to the PCR tubes, making a total volume of 25 µl. PCR amplification was performed using the T100^{TM} Thermal Cycler (BioRad) with the following cycle conditions: initial denaturation at 98 °C for 1 min, followed by 38 cycles of 98 °C for 15 seconds, 55 °C for 20 seconds, 72 ° for 20 seconds, and a final elongation step at 72 °C for 5 minutes.

qPCR

Real-time PCR (qPCR) can be used for quantifying bacterial DNA. The quantification is performed by measuring a fluorescent signal from amplified DNA, which is proportional to the amount of DNA product. SYBR®Green binds to double bonds in the DNA and can be used as a fluorescent probe in qPCR. When the fluorescent signal from the sample is higher than the background fluorescent, a cycle threshold (C_t) is determined. The C_t value is directly proportional to the amount of DNA template and can be used to calculate DNA copy numbers. To quantify samples with an unknown concentration, a standard curve for bacteria with known sequences and concentrations are usually generated (Gunduz 2007).

qPCR was performed to quantify the 16S rRNA copies in all the whole salmon yolk-sac fry samples. First, a standard curve with known DNA concentrations was generated. DNA extracted from a *Vibrio sp. (*RD5-30) was amplified by using the primer set RT996F and RT1089 (Table 2.2) The PCR reaction was performed with Phusion Hot Start Polymerase II (Thermo scientific) together with the components represented in Table 2.5. The PCR product was purified using the QIAquick PCR purification kit (Qiagen), following the manufacturer's protocol (Appendix C). The DNA concentration was measured with Qubit 3 Fluorometer

(Invitrogen, Thermo Scientific). The purified *Vibrio sp. (*RD5-30) (0,606 ng μl^{-1}) was used to prepare a 5-fold dilution series and used to generate the standard curve.

Prior to the qPCR reaction, all samples were diluted 1/10. The qPCR was performed in triplicates with a total volume of 25 µl per reaction. The samples were prepared in a 96 well plate (Thermo Scientific) containing SYBR®Green master mix (Thermo Scientific), 5 µM of each primer (RT996F and RT1089R) and 5 µl template. The qPCR was run in QuantStudio (AppliedBiosystems), with the following cycle conditions: pre-incubation 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 the amplification of the PCR product, with the following conditions: 95 °C (15 sec.), 60 °C (1 min.), 95°C (1 sec.) and finally a cool down stage at 37 °C (30 sec.).

Table 2.5: Components for making a 24 µl master mix in PCR.

Components	**Supplier**	**Amountx1**
PCR grade water		16,6875 µL
5x Phusion buffer HF (7,5 mM $MgCl_2$)		5,0 µL
RT-966F (10 µM)	Sigma Aldrich	0,75 µL
RT-1089 (10 µM)	Sigma Aldrich	0,75 µL
dNTP (10 mM each)		0,625µL
Phusion Hot Start DNA polymerase		0,1875µL
Total		24 µL

The data obtained from the qPCR reaction was processed by using QuantStudio Design and Analysis Software v1.5.0 (AppliedBiosystems). The copy number (CN) of the standard curve samples were calculated, using Equation 2.4, with DNA length of 123 base pairs and DNA concentration of the diluted *Vibrio sp.* (RD5-30) samples. The standard curve was generated by using the C_t values from the 5-fold dilution series of the DNA extracted from the *Vibrio sp. (*RD5-30), by excluding the two highest DNA concentrations.

$$CN_{stock}\,\frac{(molecules)}{\mu l} = \frac{DNA_{conc}\left(\frac{g}{\mu l}\right) x 6.022 x 10^{23}\left(\frac{molecules}{mol}\right)}{DNA_{length} x 660\left(\frac{g}{mol}\right)} \qquad (2.4)$$

3 Results

These results are a part of the project called "*The microbial contribution to Atlantic salmon skin mucosal barrier*". The main goal was to investigate the initial colonization of single bacteria isolates on developing salmon fry by conducting a gnotobiotic experiment. Germ-free salmon fry was mono associated with five different bacterial strains, representing both skin and gut bacteria. Prior to the gnotobiotic fish experiment, the strains were classified based on the complete 16S rRNA gene and characterized based on their growth rates on general and mucin medium.

3.1 Classification and characterization of the bacterial isolates

A strain collection consisting of bacterial isolates from salmon fry skin and gut had previously been created and classified based on a short sequence (400 bp) of the 16S rRNA gene. A selection of five strains (S1-S5) were chosen for this study and subsequently classified and characterized, based on growth. Almost the complete 16S rRNA gene was sequenced for all the strains, in order to try to classify the isolates on species level. Growth curves were also made with general (TSB) and mucin medium, to determine their growth rate and investigate their ability to grow on mucin.

3.1.1 Classification and determination of the 16S rRNA gene sequences

PCR was performed on extracted DNA from the five bacterial strains S1-S5, which resulted in amplification products of the expected size (approximately 1490 bp), representing almost the complete 16S rRNA gene. No bands were observed for the negative DNA extraction control (kit blank) or the non-template control, indicating that there were no DNA contaminations (Figure 3.1).

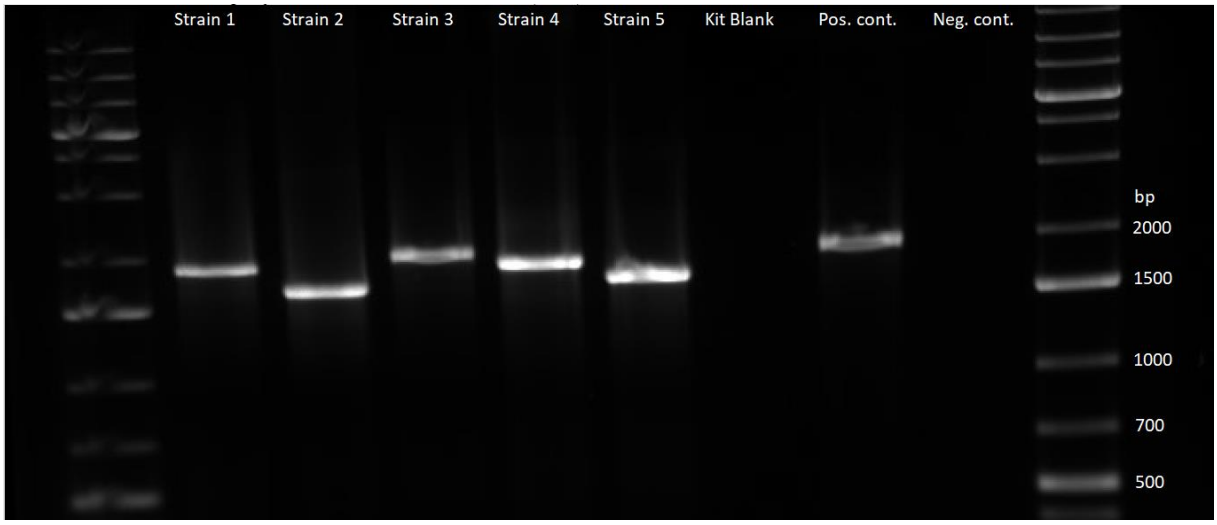

Figure 3.1: Agarose gel of PCR products representing nearly the complete 16S rRNA gene amplified from DNA extracted from the bacterial isolates (S1-S5). The amplification was performed using Taq polymerase. A kit blank (negative control for the DNA extraction kit), a positive (DNA representing a *Vibrio* isolate) and negative control were also included.

Subsequently, three sequencing primers were used to sequence the PCR product, of each bacterium. The resulting sequences were of good quality and were assembled to represent almost the complete 16S rRNA gene sequence (Appendix H). To classify the bacterial strains, the classifier tool in the ribosomal database project (RDP) was used. All the strains were classified at the genus level (Table 3.1)

For each of the five bacterial isolates, the most closely related type strains were identified using the RDP SeqMatch tool. SeqMatch score (S_ab) between every closely related sequence, were reported for each match in the RDP tool. The scores represent the number of unique 7-base oligomers, shared between the sample sequence and a given RDP sequence, divided by the lowest number of unique oligos in either of the two sequences (Wang et al. 2007). The closely related species and their following S_ab scores are presented in Table 3.1. The 16S rRNA sequences of S2 and S3 were found to be closely related to the sequences of "type strain" *Pedobacter aquatilis*, *P. jejuensis,* and *P. kyungheensis* and to *Arthrobacter psychrochitiniphilus*, *A. cryoconite* and *A. livingstonensis*, respectively. However, the S_ab scores were low, indicating that S2 and S3 probably do not represent any "type strain" in the database. The 16S rRNA sequence of S1 was found to be identical to the sequences for the "type strain" *Bacillus altitudinis*, *B. stratosphericus* and *B. aerophilus*. The same was proposed

for S4 and S5 which were found to be identical to sequences of "type strain" *Janthinobacterium lividum* and *Psychrobacter cibarius*, respectively.

For the rest of the paper, the strains S1-S5 will be referred to as *Bacillus sp*., *Pedobacter sp*., *Arthrobacter sp*., *J. lividum* and *P. cibarius*, respectively.

Table 3.1: Taxonomy and most similar "type strain" for the five strains (S1-S5) as inferred from the Ribosomal Database Project (RDP).

Strain ID	RDP classifier taxonomy (class)	"Type strain" closest match (S_ab score*)
S1	*Bacillus (*Bacilli*)*	*Bacillus altitudinis* (1.000*)*
		B. stratosphericus (1.000)
		B. aerophilus (1.000)
S2	*Pedobacter* (Sphingobacteria)	*Pedobacter aquatilis* (0.855)
		P. jejuensis (0.863)
		P. kyungheensis (0.890)
S3	*Arthrobacter* (Actinobacteria)	*Arthrobacter psychrochitiniphilus* (0.907)
		A. cryoconite (0.910)
		A. livingstonensis (0.912)
S4	*Janthinobacterium* (Betaproteobacteria)	*Janthinobacterium lividum* (1.000)
S5	*Psychrobacter* (Gammaproteobacteria)	*Psychrobacter cibarius* (1.000)

* A seqmatch score (S_ab) were reported between every closely related sequence, which represents the number of unique 7-base oligomers shared between the sample sequence and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences (Wang et al. 2007).

3.1.2 Characterization of growth on general and mucin media

Non-selective, general growth medium (TSB) and mucin medium were used to investigate the growth of the five bacterial strains. The mucin medium was used to investigate the strains abilities to grow on mucin as a sole source of carbon.

The growth curves generated from cultivation in TSB are presented in Figure 3.2. The maximum OD_{600} was between 1.3 and 1.6 for all the strains, which indicates that they have similar abilities to utilize and grow on TSB. The largest variation was observed between *Arthrobacter sp*. and *Psychrobacter cibarius* with maximum OD_{600} of 1.32 and 1.66, respectively. Variation was also observed in the length of the lag-phase between the five strains. Longer lag phase indicates, that longer time is needed to adapt to new growth conditions before growth can begin. *P. cibarius* and *Bacillus sp*. started their exponential growth at approximately 2 and 5 hours, respectively. The *Pedobacter sp.*, *Janthinobacterium lividum* and *Arthrobacter sp.* started their exponential growth later, at approximately 10, 17 and 18 hours, respectively.

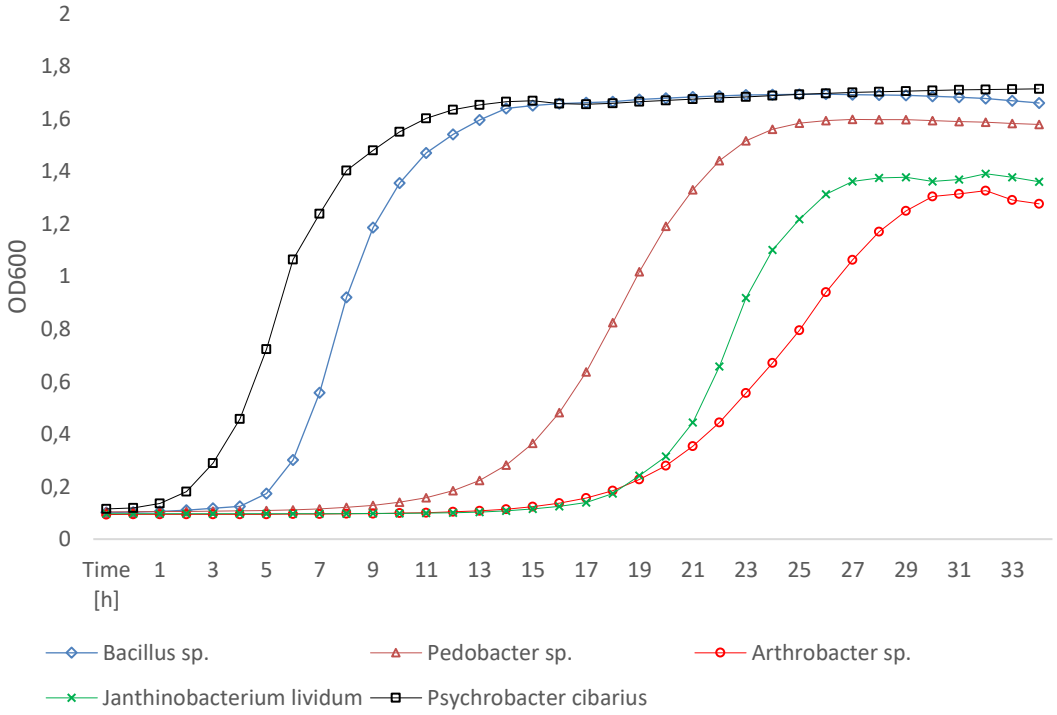

Figure 3.2: Growth curves for the five bacterial strains, cultivated on rich general growth medium, TSB. OD was measured at 600 nm every hour. The strains were cultured in a 96-well plate in a TECAN Spark® plate reader, with 3 replicate wells for each strain. The growth rates represent the average of the three replicate wells.

The growth curves generated from cultivation in mucin medium are presented in Figure 3.3. The results show that all strains were able to utilize mucin as a carbon source. This indicates that all the strains are mucin-degradable bacteria, which can survive with mucin as the sole source of carbon. The maximum OD_{600} was between 0.5 and 0.7 for all the strains, which indicate that they have approximately similar abilities to utilize and grow on mucin medium. However, the lowest maximum OD_{600} was observed for *Arthrobacter sp.*, and the trend of the curve was very different from the other curves. This may indicate that *Arthrobacter* is less capable of growing on mucin media, than the other strains. For *J. lividum*, large variations in OD_{600} measurements was observed throughout the incubation period. It was also observed that this culture was significantly denser compared to the others, due to the production of some type of extracellular polymeric substances. This may have affected the resulting OD_{600}-measurements of the *J. lividum.*

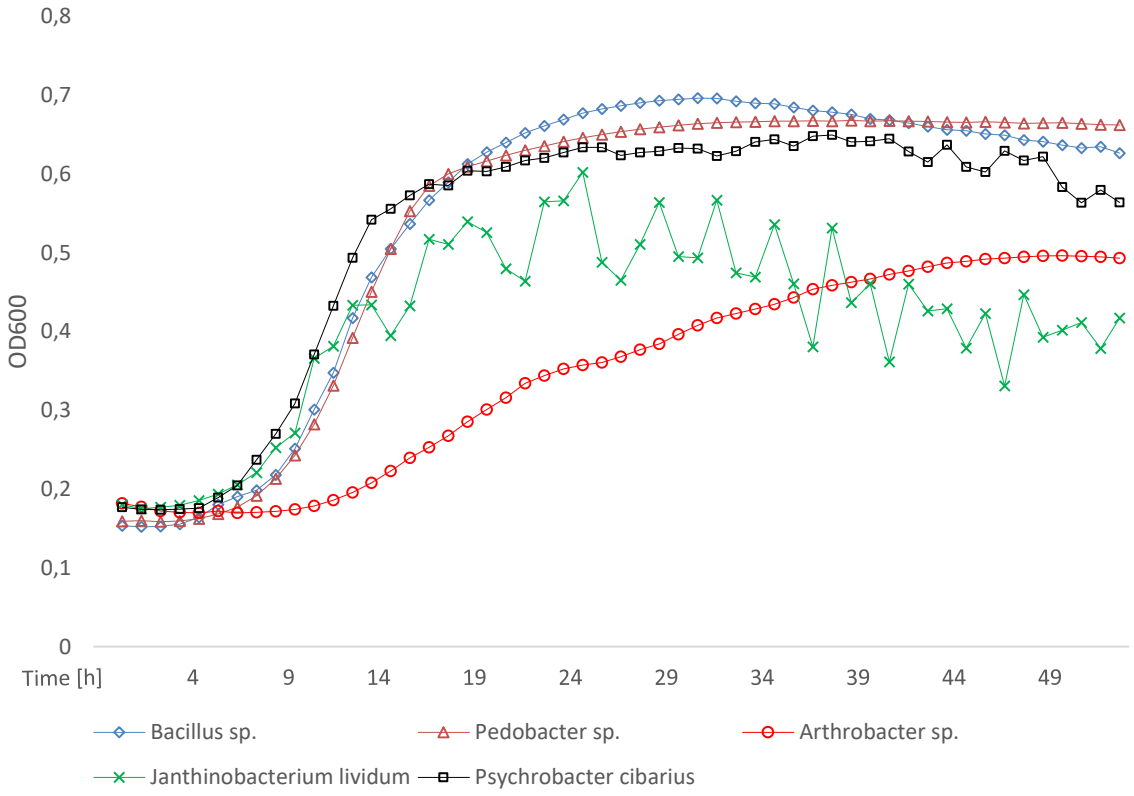

Figure 3.3: Growth curves for the five bacterial strains, cultivated on mucin medium. OD was measured at 600 nm every hour. The strains were cultured in a 96-well plate in a TECAN Spark® plate reader, with 3 replicate wells for each strain. The growth rates represent the average of the three replicate wells.

Subsequently, the exponential phase of the growth curves was identified and used to calculate the generation time for each bacterium (Table 3.2). The generation times for the five strains grown in TSB were relatively diverse. Short generation time indicates rapid growth in contrast to long generation time which indicates slow growth. The fastest generation time was observed for *P. cibarius* (1.24) followed by *Bacillus sp*. (1.84 h), while the slowest generation time was seen for strain *Arthrobacter sp*. (3.69h). The fastest and slowest growing bacteria were also observed to have the shortest and longest lag phase, respectively (Figure 3.2).

The generation times for the five strains on mucin medium was also relatively diverse. The fastest generation times were observed for *P. cibarius* (4.54 h), *Bacillus sp*. (4.63 h) and *Pedobacter sp*. (4.78 h), which were relatively similar. The slowest generation time was seen for the *Arthrobacter sp*. (12.27 h), as it also was observed when grown in TSB. This, together with the variations observed in maximum OD_{660} and lag phase, indicates that the strains have different abilities to utilize the mucin as a carbon source.

Table 3.2: Generation time and maximum OD_{600} for the five bacterial strains S1-S5 based on growth in TSB and mucin-media

	TSB		Mucin	
Strain	Generation time [h]*	Max OD	Generation time [h]*	Max OD
Bacillus sp.	1.84	1.65	4.63	0.69
Pedobacter sp.	2.91	1.59	4.78	0.62
Arthrobacter sp.	3.69	1.32	12.27	0.49
Janthinobacterium lividum	2.29	1.37	9.43	0.60
Psychrobacter cibarius	1.24	1.66	4.54	0.62

* in the exponential phase

3.2 Gnotobiotic fish experiment

A gnotobiotic system was set up with germ-free salmon yolk-sac fry. The fish were transferred from culture flasks to well-plates, and mono-associated with bacteria, to investigate whether the strains affected the survival of the fish. A sterility check of the rearing water in the fish flasks was performed, to verify that the fish were germ-free. There was no visible growth in the liquid media or on TSA plates. There was, however, one exception where one culture flask showed growth in all the media. This flask was excluded from the rest of the experiment. The survival was 100 % during the incubation period, which indicates that none of the bacterial strains were pathogenic to the salmon fry.

3.2.1 Quantification of bacterial colonization of gnotobiotic salmon fry using CFU

Colony forming units (CFU) counts were conducted to investigate the initial colonization of the fives strains, in the salmon gut, skin and rearing water. For the rearing water inoculated with single bacterial strains, only the expected colony morphologies were observed on agar plates, indicating that there were no contamination. Based on the CFUs, the final bacterial concentration in the rearing water, after three days, was found to be 8, $2.8 \cdot 10^5$, $6.8 \cdot 10^3$, $8.1 \cdot 10^3$, $8.1 \cdot 10^3$ CFU ml^{-1} for the strains *Bacillus sp.*, *Pedobacter sp.*, *Arthrobacter sp*, *Janthinobacterium lividum* and *Psychrobacter cibarius*, respectively (Table 3.3).

Table 3.3: Average CFU counts in germ-free salmon yolk-sac fry, mono-associated with five bacterial strains S1-S5. CFU was registered in fish skin, gut and rearing water after three days of exposure to the bacteria. N.d stands for not detected.

	CFU fish *		CFU water per µl
	Per fish skin	Per fish gut	
Bacillus sp.	n. d	n. d	0,008
Pedobacter sp.	43,33 ± 4,0	n. d	287,5
Arthrobacter sp.	2,5 ± 0,7	n. d	6,8
J. lividum	30 ± 2,7	n. d	8,1
P. cibarius	3,33 ± 0,7	n. d	8,1

*3 replicate fish per strain

The initial density of bacteria in the rearing water was intended to be 10^5 CFU ml^{-1}. However, the final CFU counts were much lower than expected. Similar bacterial densities were observed in the rearing water containing *Arthrobacter sp*., *J. lividum and P. cibarius*. CFU counts were much higher for the water inoculated with *Pedobacter sp*., which might indicate that this strain is better at colonizing the water than the others. Extremely few CFUs (0.008) were observed for the rearing water with the *Bacillus* sp., which suggest that this strain is not able to colonize the water at all.

The initial colonization of the strains in the salmon fry gut- and skin mucus was also assessed, and the counts indicated that there was a large difference between the colonization density between these two surfaces (Table 3.3). Surprisingly, the counts showed no CFUs present in the gut for any of the strains. The density was higher in the skin, but still indicated only a few bacteria (from zero to around 40 CFUs) per individual. The skin samples showed expected colony morphologies on agar plates, for all the strains, indicating no contaminations, except for *Bacillus* sp., which was not present at all. The strains *Pedobacter sp*. and *J. lividum* had the highest CFU counts. However, calculating the CFU in fish per CFU in the water, only the *J. lividum* differs from the other strains (Figure 3.4). This indicates that *J. lividum,* may be better at colonizing the exterior of salmon fry, than the other strains.

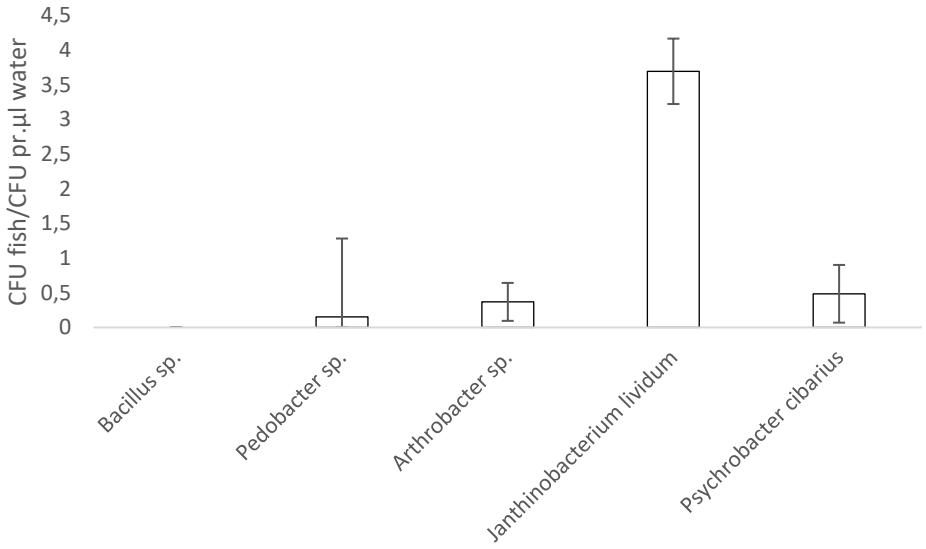

Figure: 3.4 The average number of CFU's from three fish skin samples per CFU found in per µl water. This represents the initial colonization for the five bacteria *Bacillus* sp., *Pedobacter* sp., *Arthrobacter sp., J. lividum*, and *P. cibarius*. The error bars represent the standard deviation between three fish skin samples for each bacterium.

3.2.2 Quantification of bacterial loads in gnotobiotic salmon fry using qPCR

Real-time PCR (qPCR) was performed to quantify the 16S rDNA copy number in whole salmon fry samples. This was used to estimate the bacterial amount in germ-free salmon fry, mono associated with the bacterial strains: *Pedobacter* sp., *Arthrobacter sp.*, *Janthinobacterium lividum*, and *Psychrobacter cibarius*. Conventionally raised (CVR) salmon fry mono-associated with *J. lividum* were also included, together with CVR and GF fish controls (no added bacteria).

3.2.2.1 Standard curve of *Vibrio* DNA and PCR amplification efficiency

A standard curve was created in order to calculate the 16S rDNA copy number (CN) of the bacteria associated fish. The standard curve was generated by using DNA extracted from an isolated *Vibrio* sp. isolate RD5-30, with known DNA concentrations. The primer pair RT996F and RT1089R (Table 2.2) was used to amplify a DNA fragment consisting of 123 base pairs from the 16S rRNA gene of the *Vibrio* (RD5-30). The amount of purified DNA in the PCR product was measured and shown to be 0.606 ng/µL. According to Equation 2.4, this corresponds to $4{,}495 \cdot 10^{9}$ copies per µl. Subsequently, a 5-fold dilution series was made from the amplified PCR product of the *Vibrio* (RD5-30). The dilution series ranges from 0.1212 to $1{,}9392 \cdot 10^{-4}$ ng/µL. The log of the copy numbers for each concentration in the dilution series was plotted against the cycle threshold (C_t) values. Linear regression was conducted, and the resulting standard curve can be found in Appendix I. A slope with -3.32 in a standard curve indicates 100 % amplification efficiency. It was, however, observed that the C_t values for the two most concentrated samples were lower than expected, possibly due to inhibition caused by too high template concentrations. So, these samples were excluded from the standard curve, and the resulting linear regression is shown in Figure 3.5. The resulting slope was 3.329, which indicates an amplification efficiency of 99.71 %.

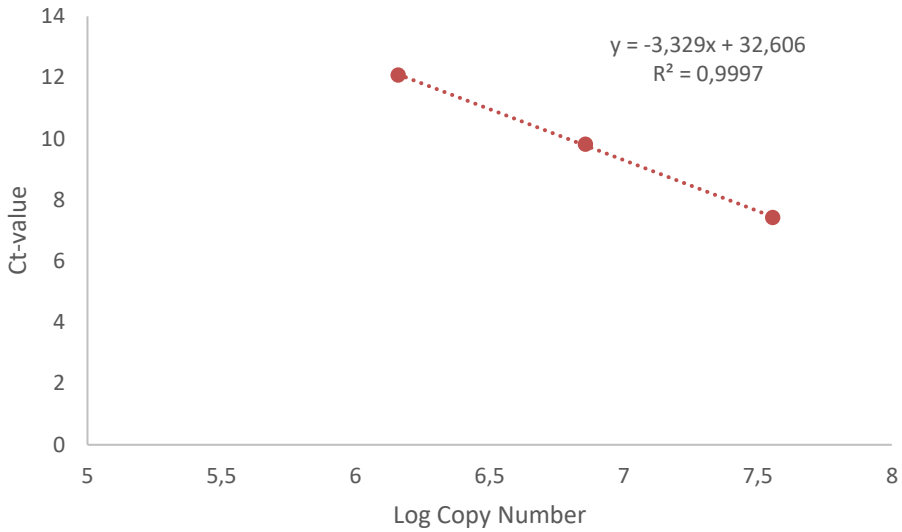

Figure 3.5: Standard curve showing Ct values as a function of Log copy number. The standard curve is created by using a dilution series of a 123 base pair long PCR product of the 16S rDNA gene, from a *Vibrio* (RD5-30) strain as a template in qPCR.

3.2.2.2 Quantification of the 16S rDNA copies in the salmon yolk sac samples

The standard curves and the C_t values were further used to estimate the quantity of the 16S rDNA copies in the salmon yolk-sac fry samples amplified in the qPCR reaction. Amplification curves representing all the samples are presented in Figure 3.6-A. By visual inspection of the curves, the amplification of the GF samples occurs almost at the same time as the bacteria-associated samples. This is more clearly demonstrated in figure 3.6-B which includes only the standard, non-template and GF samples.

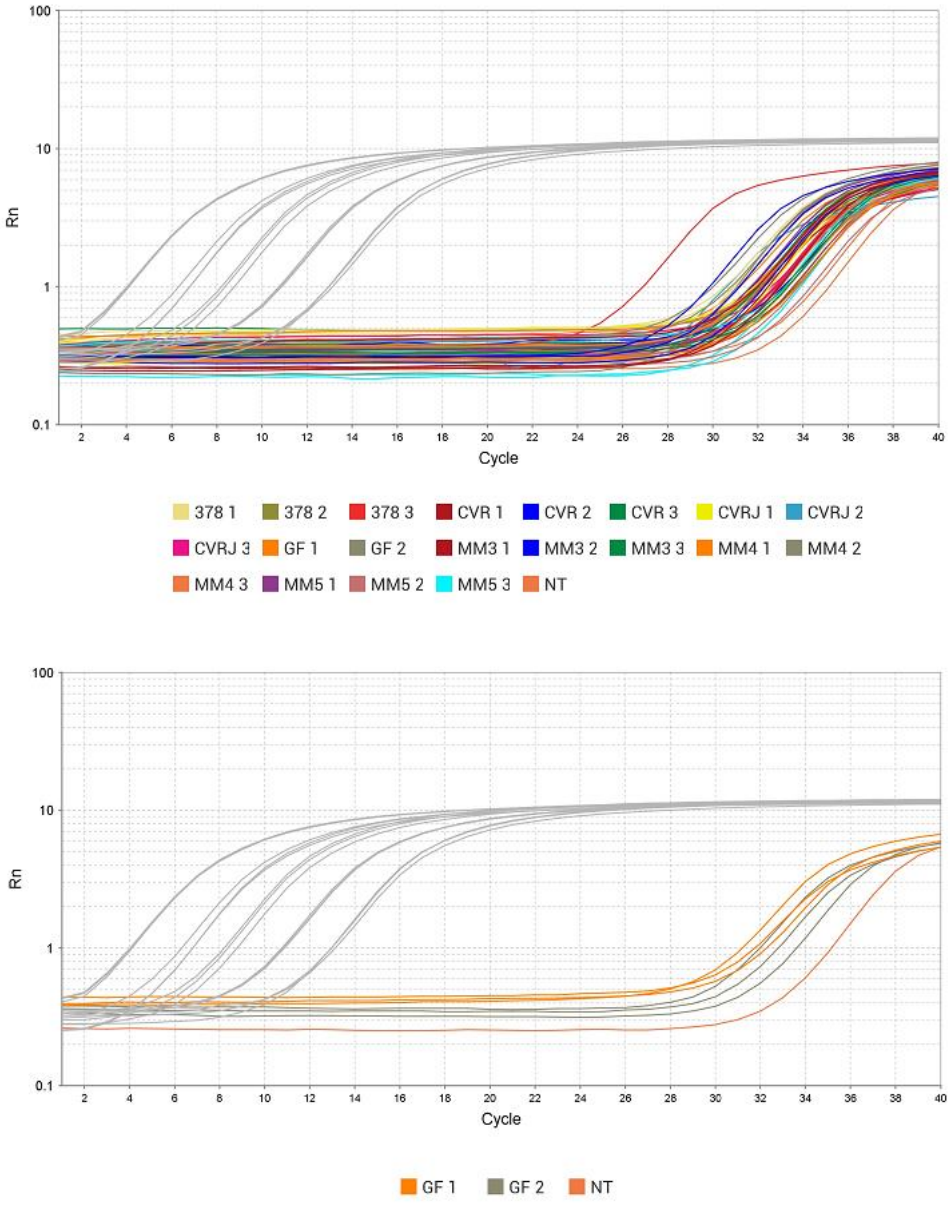

Figure 3.6: A) Amplification curves generated from qPCR reactions with fish associated with bacteria including standard, germ-free (GF) and non-template (NT) control samples. The first amplification curve to the right (grey) represents the standard curve and the remaining curves are the amplified samples of the 16S rDNA which includes GF fish mono-associated with bacteria (378, MM3, MM4, MM5), CVR fish exposed to *J. lividum* (CVRJ), CVR and GF fish with no added bacteria (CVR and GF) and non-template samples (NT). B) Amplification curve which only represents the standard curve, GF and NT samples.

An average copy number for the samples run in triplicate reactions were calculated based on the standard curve (Figure 3.7). The copy numbers varied between 43 to 137 per fish. The copy number of fish exposed to different strains were observed to be extremely low and similar to that found for the GF control. This indicates that there might have been a co-amplification of the salmon DNA, making it impossible to detect these low number of bacteria in the templates, which may be dominated by salmon DNA. The large error bars indicate large variations between the samples.

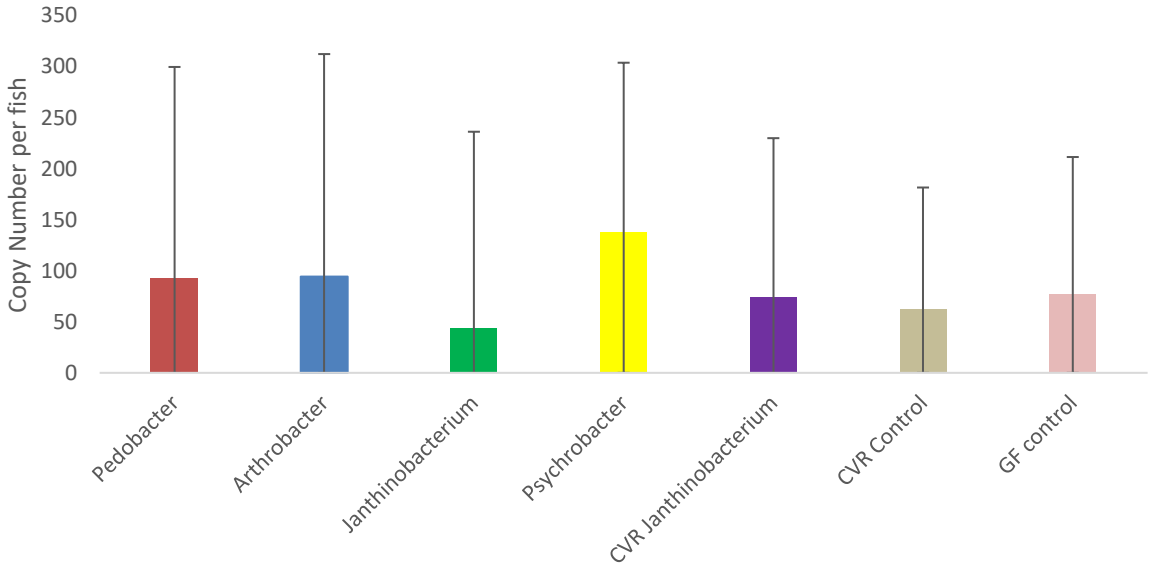

Figure 3.7: Average copy number of the 16S rDNA gene per fish as estimated by qPCR for samples representing salmon fry, mono-associated with 5 bacterial strains. A CVR and GF control is also included. For each sample, the average Ct value for three replicate qPCR reactions was determined and was used to determine the copy number. The error bars represent the standard deviation of three replicate fish samples, each run in triplicate qPCR reactions.

4 Discussion

4.1 Classification of the bacterial isolates and their previous association with fish

All five strains were characterized by sequencing of almost the complete 16S rDNA gene, to get the most accurate classification as possible. Strain S1 was classified as a *Bacillus*. The *Bacillus* is a member of the phylum Firmicutes, which are described as gram-positive, rod-shaped and obligate aerobic or facultative anaerobic. Several *Bacillus* species have been associated with fish, and some strains have even been identified as probiotic in different fish species (Adorian et al. 2019). However, the closest related type strain based on 16S rRNA gene sequence similarity was *Bacillus altitudinis, B. stratosphericus* and *B. aerophilus*. These are usually found to be abundant in air and has been observed in great altitudes (Shivaji et al. 2006). These have not previously been associated with fish or aquatic environments, except for the *B. altitudes*, which has been reported in diverse habitats such as freshwater, lakes and soil (Vettath et al. 2017). This may be an indication that the *Bacillus sp.* strain used in this experiment might represent a contamination originated from air, and not the salmon fry, when the strain collection was created.

Strain S2 was classified as *Pedobacter. Pedobacter* is a member of the phylum Bacteroidetes within the class of Sphingobacteria. These bacteria are described as gram-negative and rod-shaped and has often been associated with soil environments. The closets related type strain based on the 16S rRNA gene sequence was found to be *Pedobacter aquatilis, P. jejuensis* and *P. kyungheensis.* These strains have been isolated from drinking water and different types of soil (Kook, Park, and Yi 2014; Gallego, Garcia, and Ventosa 2006; Yang et al. 2012), and has not typically been associated with fish before. Nevertheless, the similarity between S2 and these strains were low (Table 3.1) which indicates that S2 represents a separate strain or species.

Strain S3 was classified at the genus levels as *Arthrobacter*. *Arthrobacter* is a member of the class Actinobacteria, which are described as gram-positive and obligate aerobic. These are usually found in soil environments (Ganzert et al. 2011). The closets related type strain based on the 16S rRNA gene sequence was found to be *Arthrobacter psychrochitiniphilus, A. cryoconite* and *A. livingstonensis*, which has been found in Antarctic environments and glaciers

(Ganzert et al. 2011; Margesin et al. 2012; Wang et al. 2009). No studies have associated these strains with fish, but *A. psychrochitiniphilus* have previously been found in the gut of slaughtered and stored Atlantic horse mackerel (*Trachurus trachurus*) (Alfaro and Hernandez 2013). However, other *Arthrobacter* species have been associated with fish gut communities, especially in freshwater fish (Jami et al. 2015; Nayak 2010; Ringø, Sperstad, Myklebust, Refstie, et al. 2006). Nevertheless, the similarity between S3 and these strains were low (Table 3.1) which indicates that S3 represents a separate strain or species.

Strain S4 was classified at the genus level as *Janthinobacterium*, which is a member of the class of betaproteobacteria. These strains are described as gram-negative, motile and aerobic, which is common in soil and cold-water temperature regions (Valdes et al. 2015). The strain was found to be identical to type strain *Janthinobacterium lividum,* based on the 16S rRNA gene sequence. *J. lividum* have received attention due to its production of a violet pigment called violacein (VIO) (Pantanella et al. 2007b). Violacein is a component which has shown to have anti-bacterial, anti-fungal and anti-cancer properties (Matz et al. 2004; Johnson, Tymiak, and Bolgar 1990; Andrighetti-Fröhner et al. 2003). The biological function of the violacein for *J. lividum* is unknown, but it has been proposed as a component that provides protection against bacterial pathogens (Valdes et al. 2015). It has also been shown that *J. lividum* produced extracellular polymeric substances (EPS) that result in biofilm formation, as a response to environmental stress (Pantanella et al. 2007a). This together with the anti-bacterial properties, have been proposed as main factors for VIO-producing bacteria's survival in an ecological competition between other organisms for nutrients (Masuelli et al. 2016; Pantanella et al. 2007b).

J. lividum has also been found to be present on skin microbiota of some amphibians, where the present and production of violacein provides protection against fungal pathogens. This bacterial-amphibian relationship has been classified as symbiotic (mutualistic), where the bacteria are provided with food and shelter, while the host is protected against pathogens (Harris et al. 2009). Studies have also revealed that the introduction of *J. lividum* as a probiotic to the skin of frogs and salamanders have resulted in lowered morbidity and mortality caused by pathogens (Harris et al. 2009; Brucker et al. 2008). Interestingly, *J. lividum* has also been discovered to be a typical member of the human skin microbiota (Grice et al. 2008), and because of its potential use as a probiotic in amphibians, it has also been proposed as a probiotic for humans (Ramsey et al. 2015). This is a strong indication that the *J. lividum* strain may also be present on fish skin as a commensal bacterium, providing beneficial protection against pathogens.

Strain S5 was classified at the genus levels as *Psychrobacter*, belonging to the family of Moraxellaceae, within the class of Gammaproteobacteria. They are described as a gram-negative, aerobic, non-motile coccoid bacteria. Several studies have associated *Psychrobacter* with fish gut microbiota, such as the distal gut of Arctic charr (*Salvelinus alpinus L.*) (Ringø, Sperstad, Myklebust, Mayhew, et al. 2006), the alimentary tract of Atlantic salmon (Bakke-McKellep et al. 2007) and the gut of Atlantic cod (Ringø, Sperstad, Myklebust, Refstie, et al. 2006). The strain identified in this study were found to be identical, in terms of 16S rRNA gene sequences, to the type strain *Psychrobacter cibarius*. This strain has been associated with the gut of Atlantic cod, where they discovered 13 isolates, which showed a 96 % similarity to *P. cibarius*, based on 16S rRNA sequencing. This indicates that extremely closely related species are typical fish gut bacteria, which may suggest that *P. cibarius* also may have this role. However, there exist limited studies on *P. cibarius*, and only one additional study has been published, where the strain was isolated from jeotgal, a traditional Korean fermented seafood (Jung et al. 2005).

4.2 The strains ability to grow in mucin medium with mucin as a sole carbon source

Under batch cultures, a typical growth curve will show four distinct phases. The first phase is called the lag-phase, which represent the delay before the exponential phase. This phase consists of adaption to new growth conditions, where the bacteria is preparing for cell division. When the bacteria are ready to start cell division, it enters the exponential phase. The bacterial cells start dividing at a constant rate, which is dependent on the available carbon and energy sources present in the medium. When the conditions become unfavorable for growth, the bacteria will stop replication and enter the stationary phase. Finally, it enters the death phase, when the cell loses its viability (Rolfe et al. 2012). All these phases were relatively easy to observe from the growth curves generated in TSB and mucin medium, with some exceptions. The growth curve for the *J. lividum* was not comparable to the others, due to variable OD_{600} -measurements. The reason for this is probably the production of EPS, creating a dense layer which will affect the results. The *J. lividum*, as described earlier have been associated with high formation of biofilm (Valdes et al. 2015), and EPS production was observed during cultivation in this study.

Differences were observed between the strain's growth curves, which revealed characteristics regarding their growth. Especially, *Psychrobacter cibarius*, which was observed to have the fastest growth rate and the shortest lag-phase in both TSB and mucin medium (see section 3.1.2). The opposite was observed for the strain *Arthrobacter sp.*, which had the slowest growth rate and longest lag-phase, in both media. This may suggest that the *P. cibarius* can easily adapt to new growth conditions and utilize the sources faster and therefore, be favorable in many environments, compared to the *Arthrobacter sp*. The growth rates found on TSB showed that the generation times between the strains varied to some extent. However, the utilization of the component in the medium was believed to be similar, since they grew to approximately the same maximum OD. The resulting growth curves on mucin medium showed that all the strains were able to utilize the mucin and can, therefore, be categorized as mucin degradable bacteria. The degradation of mucin has often been reviewed as a pathogenic factor since it results in loss of the protective mucus layer. However, mucin has also been shown to be an important carbon- and energy source for mammalian gut bacteria (Derrien et al. 2004). The mucin degrading bacteria digest the mucin with the help from enzymes which can degrade the oligosaccharides chains. These provide nutrient for the other resident bacteria, which can utilize the degraded monosaccharides or amino acids (Hoskins and Boulding 1981). Mucin is the main component in mucus, which has been suggested to play a role in the mucosal surface barrier function (Cone 2009). The ability to grow and utilize mucin can, therefore, be a beneficial factor for the residing microbiota, and therefore be beneficial to the host. However, the growth rates were significantly reduced compared to the growth on TSB, which may indicate that the strains used in this study, are not very efficient in the presence of mucin as sole carbon source. However, the concentration of carbon in these two media was not investigated, and since the growth usually depends on the amount of available carbon, the end concentration of cells (measured as OD_{600}), cannot be compared directly and may give inconclusive results. The use of mucin medium can also be problematic, due to the impurity of the mucin powder. For some mucin powders, it is difficult to establish that there are no other traces of other carbon sources. However, if they were not able to utilize the mucin, you would expect a much lower OD_{600} in the stationary phase, because of the low amount of carbon to grow on.

4.3 Gnotobiotic fish experiment

4.3.1 Evaluation of the gnotobiotic system

Germ-free salmon fry was reared in bottles and then transferred to 6 well plates, where the mono-association with bacteria was performed. This experimental system was used successfully in a gnotobiotic experiment. Only one out of seven rearing bottles showed signs of bacterial contamination, which was probably introduced through one of the many manipulations which are required for cleaning and maintenance (water change and removal of debris).

This was a short-term experiment, with 5 different defined and one undefined (CVR) microbial condition that were tested regarding their effect on the survival of salmon yolk-sac fry. The length of the experiment was chosen to be three days so the initial colonization of the strains could be assessed, without having to change the water in the wells and risking contamination. The gnotobiotic experiment was conducted in 6-well plates, stocked with one salmon fry in each well. This experimental design was selected based on the simplicity of the detection of surviving fry after exposure to bacteria. In a rearing bottle containing more than one fish, it is difficult to separate the major determinant of death. When a fish dies, the body will quickly putrefy and dissolve, which may pollute the water, and risk the health of the other fish. So, by using only one fish per well, it was easier to control if the mortality of the fish came from the strain or other sources.

The selected bacterial strains had previously been isolated from salmon fry gut and skin but had not previously been tested under gnotobiotic conditions. Mono-gnotobiotic conditions are artificial regarding “natural conditions” of fry rearing, and the effect of bacterial strains added as pure cultures may not be representative of the effect of the microbes in the natural environment. Yet, it is a tool allowing us to further investigate the effect on specific bacterial additives, without the complications of an already existing microbiota (Forberg, Arukwe, and Vadstein 2011a).

4.3.2 Evaluation of methods for determining the bacterial amount in salmon fry

The determination of bacterial loads is among the most fundamental procedures in microbiology (Hazan et al. 2012). Multiple different methods are commonly used, which comes with different advantageous and disadvantageous. One of the most commonly used methods is colony forming units (CFU) counting on agar plates, which has the capacity to count any bacterial densities, by using dilutions. Another advantage is that only viable bacteria is being counted, due to the exclusion of dead bacteria and debris (Sieuwerts et al. 2008). However, the CFU counting also has limitations. One of the biggest limitations is that all bacteria are not cultivable, which often results in an underestimation of the number of bacteria (Hazan et al. 2012). However, this was not an issue in this experiment, since it was previously established that all five strains were cultivable on general growth media. Yet, another problem was seen under the counting of CFU's. When the abundance of bacteria is lower than 30 CFU's, the result will not be precise nor reliable. This was observed for the CFUs in all the undiluted skin samples, which were lower than 30 (between 0-14 CFU's), making the result less reliable.

The second method used for quantification of the bacterial load was qPCR. This was conducted on whole salmon samples, instead of only skin or gut. This was due to the low number of bacteria observed with the CFU method and the fact that no bacteria were found in the gut. Culture-independent studies of microbial communities have been revolutionizing in the understanding of microbiology and the revelation of the interactions between microbes and their host (Gunduz 2007). These molecular-based methods are powerful but come with several limitations. These limitations include choices related to sample collection, sample storing and preservation, DNA extraction, amplification primers, sequencing technology, etc. (Salter et al. 2014). An additional problem is the introduction of contaminating microbial DNA during the sample preparation. Possible sources of DNA contamination include PCR grade water, PCR reagents and DNA extraction kit. The presence of contaminating DNA is extremely problematic in experiments working with samples containing low bacterial numbers. In these cases, the low amount of initial bacterial DNA may efficiently be overrun by the contaminating DNA and generate misleading result (Glassing et al. 2016). The fish samples used in this experiment was expected to contain extremely low bacterial loads, as seen for the CFU counting analysis. This may be one of the reasons why the qPCR result obtained from this study was not as expected. The amplification occurred around approximately the same PCR cycle for the non-template control (NTC), germ-free (GF) samples and samples representing fish colonized with bacteria.

This result suggests that there is approximately the same amount of bacterial DNA in all the samples, even the ones who should not contain any DNA template. This may indicate that there was a problem with DNA contamination. Contamination is usually found to originate from DNA extraction kit's and the PCR reagents (Glassing et al. 2016), which may suggest that the contamination originated from either the SYBR®Green mix or the DNA extraction kit used in this study.

Quantification of samples containing large amounts of eukaryotic DNA and small amounts of bacterial DNA has shown to be challenging (Bakke et al. 2011). This was also probably a challenge in this qPCR reaction, because of the expected low number of bacteria in the fish samples. The amplification curves of the reactions representing the GF samples, occurred at a similar time as the fish-bacteria samples, indicating that there might have been a co-amplification of salmon DNA. This gives us reason to believe that the large amount of salmon DNA may have outcompeted the low number of bacterial DNA present in the fish samples. This may be a consequence of some homology between the qPCR primers and salmon rRNA gene sequences (personal communication with Ingrid Bakke). However, the amplification of the NT control and the GF sample occurred at very similar time points, which makes it difficult to conclude if there was a problem with the amplification of salmon DNA, or DNA contamination, or both.

Another challenge observed with qPCR, and any PCR-based method is inhibitory components. Inhibitory components in qPCR reactions create complete or partial inhibition of amplification of the target DNA fragment. Consequences of this include lowered detection of accuracy or increased number of false negatives (Wang, Qi, et al. 2017). One solution to this problem has been to dilute the DNA extracts, which can lower the concentration of co-extracted inhibitory factors, and thus improve the qPCR amplification (Schneider, Enkerli, and Widmer 2009). The amplification efficiency obtained in this study was observed to yield an overestimation of the samples used in the standard curves. This was however avoided by only selecting the most diluted samples for the standard curve.

The results obtained from the qPCR reaction indicates that it is not possible to determine the abundance of bacteria in these salmon fry samples. This is probably due to the low number of bacterial cells and the large number of host DNA and possibly DNA contamination which may have overrun the amplification of the low bacterial DNA load.

4.3.3 None of the strains showed to be pathogenic to the salmon fry

Investigating the potential pathogenic properties of the strains against the salmon fry was important, especially since we were most interested in looking at typical commensal bacterial strains. At the yolk-sac stage, the fish fry is rather vulnerable, because of a poor developed functional immune system and is protected against bacterial infection by non-specific mechanisms and factors (Uribe et al. 2011; Lønning, Kjørsvik, and Falk-petersen 1988). This is especially observed for marine larvae, such as the cod, who hatches at less advanced stages of development than for example salmon fry (Lønning, Kjørsvik, and Falk-petersen 1988). The salmon fry egg is characterized as demersal, which usually hatches at a more advanced stage of development (Webb et al. 2007). This makes the salmon fry more robust and developed than other fish species, and therefore suitable for aquaculture, where high-quality offspring is extremely important. The number of surviving salmon fry was registered every day during this experiment. A 100 % survival was observed during the incubation period, which indicates that none of the strains are pathogenic to the fish.

4.3.4 Initial bacterial colonization in the skin vs. gut in salmon fry

One of the aims was to investigate if there was any difference in the initial bacterial colonization between the skin and gut of salmon fry. The results revealed that there was no CFU's detected for any of the five strains in the salmon fry gut, after three days of exposure. This indicates that there was no colonization of the gut at this stage of development. This was unexpected since the fish was exposed until approximately 10 days post-hatching (dph), at a time where the salmon fry should have opened its mouth. This was demonstrated by Sahlmann et al. (2015), which showed that the Atlantic salmon's mouth was open at 7 dph. This gives us reasons to speculate if the gut was suitable as an attachment site for bacteria at this early stage. However, this contradicts with the fact that the salmon fry has shown to be well developed at hatching, compared to other fish species, and the gut at this stage should be able to provide a nutrient-rich surface where bacteria can thrive. This opens up for more questioning, about whether or not the gut is available for the bacteria despite the mouth opening. This could possibly be explained by the fact that salmon is a freshwater species at this stage, and therefore don't have an active uptake of water. This is different in marine fish larvae, which must compensate for the gradual dehydration. The water uptake of marine larvae begins in an early stage, and the drinking rate has been observed to be 6 nLh^{-1} per larvae in the yolk sac stage of Atlantic cod

(Ringø and Birkbeck 1999). It has also been reported that the clearance of bacteria was 10-100 times the drinking rate, which indicates that the larvae have an early active uptake of bacteria (Reitan, Natvik, and Vadstein 1998). The drinking rate of freshwater fish species has been reported to be much lower than for marine species (Perrott et al. 1992), which may be a reason why the gut has not been colonized with any of the bacteria present in the rearing water. This may be an indication that the gut of freshwater fish fry is colonized at a later stage than for marine larvae.

The initial colonization of the skin was observed to be higher compared to the gut, which may suggest that the skin is colonized by bacteria at an earlier stage than the gut of salmon yolk-sac fry. This can probably be explained by the immediate close contact between the fish skin and the surrounding microbes, and that the skin is more available to the microbes than the gut.

4.3.5 Differences in the initial colonization of salmon fry for each bacterium

The hypothesis for this experiment was that different bacterial strains have various abilities to colonize the skin and gut of salmon fry. This was demonstrated, by assessing the initial adherence and colonization of each bacterium in the skin- and gut mucus. However, no strains were observed in the gut.

Very low numbers of CFU's were detected in the rearing water during the fish experiment for the *Bacillus sp*. strain. This is an indication that the strain might not be able to maintain viable under aquatic conditions, suggesting that the strain is not a typical aquatic bacterial species. This strengthens the suspicion that this strain might be a contamination, originated from air and not the salmon fry, which would, furthermore, explain why the strain was neither present in the skin or gut of the fish.

The *Pedobacter sp*. was observed to colonize the rearing water better than the other strains (Table 3.3), which may suggest that it is common in aquatic environments. The strain was also found to colonize the skin of the salmon fry; however, the number of bacteria present was very low, according to the observed CFU's. This may suggest that the *Pedobacter sp.* is good at colonizing the water, but not so good at colonizing the fish skin mucus. However, the fish was only exposed to the strain for three days, which may have not been enough time for the strain to attach and grow on the surface. Because of the short exposure time, it is difficult to say if this strain is a typical skin bacterium in salmon fry. Nevertheless, the strain was isolated from

salmon fry skin, which gives a good indication that it might be part of the bacterial community on the skin, but maybe at a later developing stage.

The *Arthrobacter sp.* strain was observed to colonize the water less than the other strains, except for the *Bacillus sp*. (Table 3.3). This strain was also observed to have the slowest generation time and the longest lag phase on both TSB and mucin media (Figure 3.2 and Figure 3.3), which may suggest that the strain require more time than the other strains to establish in a new environment. The generation time on mucin media was found to be approximately 12 hours. The long generation time together with the short exposure period, may explain the low number of CFU's found in the salmon fry skin. This may suggest that the strain is not part of the initial adherence to the skin mucus and that it require more than three days to potentially establish in the fish skin mucosal surface.

The *J. lividum* strain was observed to colonize the skin of the fish better than all the other strains (Figure 3.4), which may suggest that the *J. lividum* is a good colonizer of the skin in early development stages of salmon fry. The skin of humans, amphibians and fish are very diverse in structure and composition (Lowrey et al. 2015). Yet, *J. lividum* has been associated with all these skin mucosal surfaces. One possible explanation for this can be related to its capnophilic behavior. A capnophilic strain is characterized by favorizing high concentrations of carbon dioxide, typically with an optimum at 5 % for *J. lividum*. One study investigated the mechanisms underlying the capnophilic trait in *J. lividum*, and found genes encoding for products that aids in the carbon fixation pathways and enzymes of the glyoxylate cycle. Based on these discoveries, they suggested that the CO_2 secretion of the amphibian skin is a signal molecule that guides the colonization of *J. lividum* (Valdes et al. 2015). Cutaneous respiration occurs in a wide variety of organisms, including amphibians, fish and to a lesser extent in mammals, including humans (Piiper and Scheid 1992). This may explain why this specific species can appear in these distinct surfaces. It has been showed that newly hatched Atlantic salmon fry has poorly developed gills and that the skin area to mass ratio is high. This makes the cutaneous respiration the most important surface for gas exchange, and the skin surface provides 95 % of the total area available for respiration in newly hatched Atlantic salmon (Wells and Pinder 1996). This together with the high production of EPS observed when grown in liquid media may be an explanation for the colonization success observed in the skin of salmon fry. The production of EPS creates an adhesive surface, which may easily stick to different surfaces, like the mucosal surface of the fish skin (de Alexandre Sebastião, Pilarski, and Lemos 2013).

All these results and findings give a good indicates that the *J. lividum* may be a typical commensal skin bacterium for salmon fry. This is very interesting, especially considering the anti-bacterial, anti-fungal and anti-cancer properties associated with this strain previously. Maybe *J. lividum* can be a potential probiotic strain, that can strengthen the protection against pathogens in developing fish fry. However, more research is needed to reveal the true role of *J. lividum* as a commensal skin bacterium in salmon fry.

The *Psychrobacter cibarius* was also observed in the skin of the fry (CFU counts) during this experiment. However, the number of CFU was very low (3.33±0.7). This could be explained by the fact that the strain was only isolated from the salmon fry gut, which may indicate that the *P. cibarius* may be a more typical gut colonizer. This correlates with the previous findings, associating closely related species to *P. cibarius* with the gut of Atlantic cod, which might strengthen the possibility for this strain to be a part of the commensal bacteria in salmon fry gut. However, there is limited knowledge regarding this strain and whether this is a typical commensal bacterium in salmon fry gut is yet to be discovered.

4.4 Future work

Bacteria will always be present in significant concentrations in any commercial fish larvae rearing system. The potentially harmful effect of bacteria on fish larvae have long been the focus of aquaculture industry. However, more research is now focusing on the commensal bacteria that lives on the mucosal surfaces of fish, and how these interact with their host. One challenge that microbiologist faces today, is how to control these microbial communities associated with developing larvae and fry, and how to make them more beneficial to the developing fish (Ringø and Birkbeck 1999). There exists limited research regarding this topic and also very little about the initial colonization of bacteria in salmon fry gut and even less about the skin. The results from this experiment show that different bacterial strains have various abilities to colonize mucosal surfaces of the salmon fry, and that maybe the initial adherence and colonization with the skin occurs faster than in the gut. However, this was a short-term experiment, where the fish were only exposed to the bacteria for three days. Future work should include a long-term experiment, where the fish is exposed to the strains for a longer time period. This could reveal the strains ability to grow and establish in the skin and to determine when the initial colonization of the gut of salmon fry occurs. Also, it would be interesting to inoculate the fish with a mixture of all the five strains to investigate which strain

that would do best in the colonization of the fish under a potential “tougher” condition. However, these conditions cannot be compared to the “natural” conditions in an aquaculture system. Therefore, it would also be interesting to investigate the first colonization and establishment of skin- and gut microbiota during the salmon yolk-sac fry stage under conventionally, uncontrolled microbial conditions. Another compelling finding was also discovered for one of the strains *J. lividum*. This strain appeared to be good at colonizing the skin of the salmon fry, which is interesting since it previously has been shown to have anti-bacterial, anti-fungal and anti-cancer properties. It would be interesting to further investigate this strain and its potential as a probiotic strain for fish fry.

5 Conclusion

Because of the large variation in physiology, natural history and ecology, fish can be used as model organisms to investigate a large number of factors that are relevant for host-microbe interactions. Studies on fish with economic and cultural significance, like salmonids, have the potential to improve aquaculture and contribute to our understanding of how the fish interact with their surrounding microbes. Today, most of the studies on host-microbe interaction have been focusing on mammals, with a special interest for the intestine. However, fish represent "ancient vertebrates", which means that it is possible to investigate for example host responses that are conserved between fish and mammals (Lescak and Milligan-Myhre 2017). Yet, there is limited research on the microbial communities and their function in fish larvae, especially for the community found on skin. This project is a contribution to increasing the knowledge of this topic, and the resulting conclusions are:

- All the strains were classified at the genus or species level as *Bacillus sp., Pedobacter sp., Arthrobacter sp., Janthinobacterium lividum* and *Psychrobacter cibarius*. All the strains had the ability to grow on mucin medium and were characterized as mucin-degradable bacteria.
- None of the strains affected the survival of the salmon fry, which indicates that they were not pathogenic to the fish.
- No bacteria were observed in the gut after three days of exposure, which may suggest that the skin is colonized to a greater extent than the gut in early developing salmon fry. This may indicate that the gut of salmon fry is "unavailable" to the bacteria at this developing stage, despite that the mouth had opened, which may be correlated with the fact that freshwater fish do not have an active uptake of water.
- *J. lividum* was observed to be "better" at colonizing the skin of the salmon fry, than the other strains. This is very interesting since *J. lividum* have beneficial traits and has been suggested as probiotic for both amphibians and humans. The *Bacillus sp.* strain was not found in either the skin or the gut, and in very small loads in the rearing water, which may indicate that this might represent an airborne contamination which did not originate from the salmon fry.
- The overall bacterial densities in the skin were lower than expected, which may indicate that most bacteria require a longer time to colonize the mucosal surfaces of fish.

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Appendix A

Recipe for different solid and liquid media

Tryptic soy agar (TSA) medium (1 liter)

Tryptic soy broth	30 g
Agar	15 g
Distilled water	1 L

→ Autoclave 121 °C, 15 minutes

Tryptic soy broth (TSB) medium

TSB	30 g
Distilled water	1 L

→ Autoclave 121 °C, 15 minutes

Mucin medium

M9 broth	5.25 g
Mucin	2 g
Distilled water	500 ml

Autoclave at 121 °C for 15 minutes and add 1 ml magnesium sulfate (1M).

Appendix B

DNeasy Powersoil DNA isolation kit (Qiagen) was used to extract DNA from pure bacterial strains

Experienced User Protocol
Please wear gloves at all times

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

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

Thank you for choosing the PowerSoil® DNA Isolation Kit.

Technical Information: Toll free 1-800-606-6246, or 1-760-929-9911 Email: technical@mobio.com **Website: www.mobio.com**

Appendix C

QIAquick PCR purification kit (Qiagen) was used to purify PCR products of pure bacterial strains S1-S5

Quick-Start Protocol

QIAquick® PCR Purification Kit

The QIAquick PCR Purification Kit (cat. nos. 28104 and 28106) can be stored at room temperature (15–25°C) for up to 12 months.

For more information, please refer to the *QIAquick Spin Handbook, March 2008*, which can be found at: www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I. Do not add pH indicator I to buffer aliquots.

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in ▲ a provided 2 ml collection tube or into ● a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.
3. To bind DNA, apply the sample to the QIAquick column and ▲ centrifuge for 30–60 s or ● apply vacuum to the manifold until all the samples have passed through the column. ▲ Discard flow-through and place the QIAquick column back in the same tube.

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4. To wash, add 0.75 ml Buffer PE to the QIAquick column ▲ centrifuge for 30–60 s or ● apply vacuum. ▲ Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Appendix D

Recipe for the synthetic freshwater used as rearing water for the germ-free salmon fry. This protocol is created by Sol Gómez de la Torre Canny.

Salmon Gnotobiotic media (SGM)

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

Salt Stocks

$MgSO_4 \bullet 7H_2O$ 100X

Dissolve 12.3 g in 1 l. Autoclave.

KCl 100X

Dissolve 0.4 g in 1 l. Autoclave.

$NaHCO_3$ 100X

Dissolve 9.6 g in 1 l. Autoclave.

$CaSO_4 \bullet 2H_2O$ 5X

Dissolve 0.3 g in 1 L. Filter sterilize.

SGM prep

$MgSO_4 \bullet 7H_2O$ 100X		10 ml
KCl 100X		10 ml
$NaHCO_3$ 100X		10 ml
$CaSO_4 \bullet 2H_2O$ 5X	200 ml	
Miiq H_2O		700 ml

		1000 ml

Prepare in pre-autoclaved 1 L glass bottles.

Autoclave and store in fish room.

Appendix E

Antibiotic cocktail used in the disinfection procedure to obtain germ-free salmon fry. The recipe and procedure are created by Sol Gómez de la Torre Canny.

AB-GSM

Sol Gómez de la Torre Canny

Antibiotic Cocktail Preparation

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.

Kanamycin (Kan)

(420311-5, VWR)
Stock: 50 mg/ml in H_2O
Dissolve 1000mg of powder in 20 ml of filtered/autoclaved mqH_2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

PenicillinG (PenG)

(A1837.0025, VWR)
Stock: 100 mg/ml in H_2O
Dissolve 5000mg of powder in 50 ml of filtered/autoclaved mqH_2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

Ampicillin (Amp)

(171254-5, VWR)
Stock: 100 mg/ml in H_2O
Dissolve 5000mg of powder in 50 ml of filtered/autoclaved mqH_2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

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 mqH_2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

Amphotericin B (Fun)

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

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 mqH_2O (19,2 ml of EtOH + qs 20 ml mqH_2O=
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

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

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

Appendix F

Recipe for different media used to investigate the sterility of the germ-free salmon fry

Nutrient Broth (NB)

NB	8g
Distilled water	1 L

→ Autoclave 121°C, 15 minutes

Brain Heart infusion (BHI)

BHI	37 g
Distilled water	1 L

→ Autoclave 121°C, 15 minutes

Saboraud- 2% dextrose broth (SD)

SD	30 g
Distilled water	1 L

→ Autoclave 121°C, 15 minutes

Appendix G

Ultradeep microbiome prep kit (Molzym) was used to extract DNA from whole salmon samples associated with different strains of bacteria

Ultra-Deep Microbiome Prep
Sample Pre-Treatment*

Molzym

Protocol 2, part A: Tissue Sample Preparation

Kit 1: Buffers & Consumables (+18 to +25°C)
Kit 2: Enzymes & Reagents (-15 to -25°C)

- Transport the specimen under conditions avoiding contamination to the laboratory for analysis.
- Per specimen, place a *Sample tube* (*ST*, Kit 1,) in a rack and mark. Pipette 180µl of buffer *PKB* (Kit 1) into the *Sample tube* (*ST*).
- Transfer the specimen to a sterile support (e.g., Petri dish). Cut the specimen (~0.5x0.5cm) into small pieces by using a sterile scalpel.
- Transfer the cut specimen to the *Sample tube* (*ST*) filled with buffer *PKB*. **Add 20µl of *Proteinase K*** (Kit 2), **vortex for 15s.**
  Incubate at 56°C, 10min, 1,000rpm (thermomixer).
  → 400 rpm + 3min 1000 rpm
- Fill up to 1ml with the transport solution, if available, or with buffer *TSB* (use the measure line of the tube).

Continue with the instructions of the scheme Ultra-Deep Microbiome Prep / Protocol 2, part B: DNA Isolation (page 5, short manual).

Ultra-Deep Microbiome Prep
Protocol 2, part B: DNA Isolation*

Molzym

Kit 1: Buffers & Consumables (+18 to +25°C)
Kit 2: Enzymes & Reagents (-15 to -25°C)

Arrange bottles according to the sequence of steps as below:
CM – DB1 – RS – RL – RP – CS – AB – WB –70% Ethanol – Deionized Water

Continued from Ultra-Deep Microbiome Prep / Tissue Sample Preparation, part A (page 2, short manual).

Per sample:

Removal of Human DNA

1. Add **250µl buffer *CM***, vortex for 15s.
   Let stand at room temperature (+18 to +25°C) for 5min.
2. Briefly centrifuge.
   Add **250µl buffer *DB1***.
   Add **10µl *MolDNase B*** (Kit 2), vortex for 15s.
   Incubate at room temperature (+18 to +25°C) for 15min.
3. Centrifuge at ≥12,000x*g*, 10min.
   Remove supernatant by pipetting and discard.
4. Resuspend pellet in **1ml buffer *RS*** by pipetting.
5. Centrifuge at ≥12,000x*g*, 5min.
   Remove supernatant by pipetting.
   (Optional: freeze pellet at -15 to -25°C for storage).

Lysis of Pathogens

6. Resuspend pellet in **80µl buffer *RL***, briefly centrifuge tube.
   Add **20µl *BugLysis*** (Kit 2).
   Add **1.4µl *ß-mercaptoethanol*** (Kit 2), vortex for 15s.
   Take care not to inhale.
   Incubate at 37°C, 30min, 1,000rpm (thermomixer).
7. Briefly centrifuge.
   Add **150µl buffer *RP***.
   Add **20µl *Proteinase K*** (Kit 2), vortex for 15s.
   Incubate at 56°C, 10 min, 1,000 rpm (thermomixer).

Continue on page 6

Ultra-Deep Microbiome Prep
Protocol 2, part B: DNA Isolation*

Molzym

During 10 min incubation:

Kit 1: Buffers & Consumables

Unpack *Spin columns* (*SC*), 2 ml *Collection tubes* (*CT*) and 1.5 ml *Elution tubes* (*ET*), label; heat ***Deionized Water*** (100µl each sample) vial to **70°C** (thermomixer).

Lysis of Pathogens

8. Briefly centrifuge.
   Add **250µl buffer *CS***, vortex for 15s.

9. Briefly centrifuge.
   Add **250µl buffer *AB***, vortex for 15s.

DNA Purification

10. Briefly centrifuge to clear lid.
    Pipette lysate into a *Spin column.*
    Pipette the fluid phase in the column.
    Avoid transfer of any unresolved particles!
    Centrifuge: ≥12,000x*g*, 30 to 60s.

11. Remove column and place in a new 2 ml *Collection tube.*
    Add **400µl buffer *WB***.
    Centrifuge: ≥12,000x*g*, 30 to 60s.

12. Remove column and place in a new 2 ml *Collection tube.*
    Add **400µl *70% Ethanol***.
    Centrifuge: ≥12,000x*g*, 3min.

DNA Elution

13. Carefully remove column and place in a 1.5 ml *Elution tube*.

14. Add **100µl *Deionized Water*** heated to 70°C.
    Incubate at room temperature (+18 to +25°C) for 1min .
    Centrifuge: ≥12,000x*g*, 1min.
    Discard column, close lid of *Elution tube*.

15. Store eluted DNA (1.5 ml *Elution tube*) at -15 to -25°C.

Appendix H

The 16S rRNA gene sequences of the five bacterial strains S1-S5 obtained from PCR and Sanger sequences.

Bacillus sp. (S1)

```
   1  aggacgaacg ctggcggcgt gcctaataca tgcaagtcga gcggacagaa gggagcttgc tcccggatgt
  71  tagcggcgga cgggtgagta acacgtgggt aacctgcctg taagactggg ataactccgg gaaaccggag
 141  ctaataccgg atagttcctt gaaccgcatg gttcaaggat gaaagacggt ttcggctgtc acttacagat
 211  ggacccgcgg cgcattagct agttggtgag gtaacggctc accaaggcga cgatgcgtag ccgacctgag
 281  agggtgatcg gccacactgg gactgagaca cggcccagac tcctacggga ggcagcagta gggaatcttc
 351  cgcaatggac gaaagtctga cggagcaacg ccgcgtgagt gatgaaggtt ttcggatcgt aaagctctgt
 421  tgttagggaa gaacaagtgc aagagtaact gcttgcacct tgacggtacc taaccagaaa gccacggcta
 491  actacgtgcc agcagccgcg gtaatacgta ggtggcaagc gttgtccgga attattgggc gtaaagggct
 561  cgcaggcggt ttcttaagtc tgatgtgaaa gcccccggct caaccgggga gggtcattgg aaactgggaa
 631  acttgagtgc agaagaggag agtggaattc cacgtgtagc ggtgaaatgc gtagagatgt ggaggaacac
 701  cagtggcgaa ggcgactctc tggtctgtaa ctgacgctga ggagcgaaag cgtggggagc gaacaggatt
 771  agataccctg gtagtccacg ccgtaaacga tgagtgctaa gtgttagggg gtttccgccc cttagtgctg
 841  cagctaacgc attaagcact ccgcctgggg agtacggtcg caagactgaa actcaaagga attgacgggg
 911  gcccgcacaa gcggtggagc atgtggttta attcgaagca acgcgaagaa ccttaccagg tcttgacatc
 981  ctctgacaac cctagagata gggctttccc ttcggggaca gagtgacagg tggtgcatgg ttgtcgtcag
1051  ctcgtgtcgt gagatgttgg gttaagtccc gcaacgagcg caacccttga tcttagttgc cagcattcag
1121  ttgggcactc taaggtgact gccggtgaca aaccggagga aggtggggat gacgtcaaat catcatgccc
1191  cttatgacct gggctacaca cgtgctacaa tggacagaac aaagggctgc gagaccgcaa ggtttagcca
1261  atcccacaaa tctgttctca gttcggatcg cagtctgcaa ctcgactgcg tgaagctgga atcgctagta
1331  atcgcggatc agcatgccgc ggtgaatacg ttcccgggcc ttgtacacac cgcccgtcac accacgagag
1401  tttgcaacac ccgaagtcgg tgaggtaacc tt
```

Pedobacter sp. (S2)

```
   1  gatgaacgct agcggcaggc ctaatacatg caagtcgagg ggtagagtta gcttgctagc ttgagaccgg
  71  cgcacgggtg cgtaacgcgt atgcaaccta ccttaatcag ggggatagcc cgaagaaatt cggattaaca
 141  ccgcataaaa tcacagaata gcattattca atgatcaaat atttatagga ttaagatggg catgcgtgtc
 211  attagctagt tggcggggta acggcccacc aaggcgacga tgactagggg atctgagagg atgacccccc
 281  acactggtac tgagacacgg accagactcc tacgggaggc agcagtaagg aatattggtc aatggaggca
 351  actctgaacc agccatgccg cgtgcaggaa gactgcccta tgggttgtaa actgctttta tctgggaata
 421  aacctattta cgtgtaagta gctgaatgta ccagaagaat aaggatcggc taactccgtg ccagcagccg
 491  cggtaatacg gaggatccaa gcgttatccg gatttattgg gtttaaaggg tgcgtaggcg gcctgttaag
 561  tcagaggtga aagacggtag ctcaactatc gcagtgcctt tgatactgat gggcttgaat ggactagagg
 631  taggcggaat gagacaagta gcggtgaaat gcatagatat gtctcagaac accgattgcg aaggcagctt
 701  actatggttt tattgacgct gaggcacgaa agcgtgggga tcaaacagga ttagataccc tggtagtcca
 771  cgccctaaac gatgaacact cgctgttggc gatacacagt cagcggctaa gcgaaagcgt taagtgttcc
 841  acctggggag tacgctcgca agagtgaaac tcaaaggaat ngacgggggc ccgcacaagc ggaggagcat
 911  gtggtttaat tcgatgatac gcgaggaacc ttacccgggc ttgaaagtta gtgaattatt tagagataaa
 981  taagtgagca atcacacgaa actaggtgct gcatggctgt cgtcagctcg tgccgtgagg tgttgggtta
1051  agtcccgcaa cgagcgcaac ccctatgttt agttgccagc acgtcaaggt ggggactcta aacagactgc
1121  ctgtgcaaac agagaggaag gaggggacga cgtcaagtca tcatggccct tacgtccggg gctacacacg
1191  tgctacaatg gatggtacag agggcagcta gctggcaaca gtatgcgaat ctcacaaagc cattcacagt
1261  tcggattggg gtctgcaact cgaccccatg aagttggatt cgctagtaat cgcgtatcag caatgacgcg
1331  gtgaatacgt tcccgggcct tgtacacacc gcccgtcaag ccatggaagt tgggggtacc taaagtatgt
1401  aaccgtaagg agcgtcatag ggtaatccct
```

Arthrobacter sp. (S3)

```
   1  gatgaacgct ggcggcgtgc ttaacacatg caagtcgaac gatgaacccc gcttgcgggg ggattagtgg
  71  cgaacgggtg agtaacacgt gagtaacctg cccttaactc tgggataagc ctgggaaact gggtctaata
 141  ctggatattg acttttcacc gcatggtggt tggttgaaag atttttttggt tttggatgga ctcgcggcct
 211  atcagcttgt tggtgaggta atggctcacc aaggcgacga cgggtagccg gcctgagagg gtgaccggcc
 281  acactgggac tgagacacgg cccagactcc tacgggaggc agcagtgggg aatattgcac aatgggcgaa
 351  agcctgatgc agcgacgccg cgtgagggat gacggccttc gggttgtaaa cctctttcag tagggaacaa
 421  ggccagtgtt tatctggttg agggtacttg cagaagaagc gccggctaac tacgtgccag cagccgcggt
 491  aatacgtagg gcgcaagcgt tatccggaat tattgggcgt aaagagctcg taggcggttt gtcgcgtctg
 561  ccgtgaaagt ccgggggctca actccggatc tgcggtgggt acgggcagac tagagtgatg taggggagac
 631  tggaattcct ggtgtagcgg tgaaatgcgc agatatcagg aggaacaccg atggcgaagg caggtctctg
 701  ggcattaact gacgctgagg agcgaaagca tggggagcga acaggattag ataccctggt agtccatgcc
 771  gtaaacgttg ggcactaggt gtgggggaca ttccacgttt tccgcgccgt agctaacgca ttaagtgccc
 841  cgcctgggga gtacggccgc aaggctaaaa ctcaaaggaa ttgacggggg cccgcacaag cggcggagca
 911  tgcggattaa ttcgatgcaa cgcgaagaac cttaccaagg cttgacatga accggaaaca cctagagata
 981  ggtgccccac ttgtggtcgg tttacaggtg gtgcatggtt gtcgtcagct cgtgtcgtga gatgttgggt
1051  taagtcccgc aacgagcgca accctcgttc catgttgcca gcgggttatg ccggggactc atgggagact
1121  gccggggtca actcggagga aggtggggac gacgtcaaat catcatgccc cttatgtctt gggcttcacg
1191  catgctacaa tggccggtac aatgggttgc gatactgtga ggtggagcta atcccaaaaa gccggtctca
1261  gttcggattg gggtctgcaa ctcgacccca tgaagtcgga gtcgctagta atcgcagatc agcaacgctg
1331  cggtgaatac gttcccgggc cttgtacaca ccgcccgtca agtcacgaaa gttggtaaca cccgaagccc
1401  atggcctaac ccgtttacgg gagggagtgg t
```

Janthinobacterium lividum (S4)

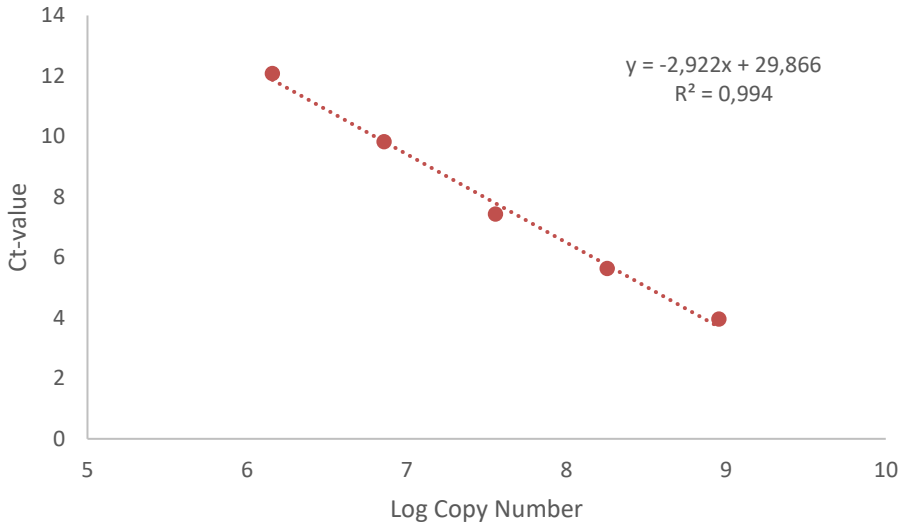
```
   1  attgaacgct ggcggcatgc cttacacatg caagtcgaac ggcagcacgg agcttgctct ggtggcgagt
  71  ggcgaacggg tgagtaatat atcggaacgt accctggagt gggggataac gtagcgaaag ttacgctaat
 141  accgcatacg atctaaggat gaaagtgggg gatcgcaaga cctcatgctc gtggagcggc cgatatctga
 211  ttagctagtt ggtagggtaa aagcctacca aggcatcgat cagtagctgg tctgagagga cgaccagcca
 281  cactggaact gagacacggt ccagactcct acgggaggca gcagtgggga attttggaca atgggcgaaa
 351  gcctgatcca gcaatgccgc gtgagtgaag aaggccttcg ggttgtaaag ctcttttgtc agggaagaaa
 421  cggtgagagc taatatctct tgctaatgac ggtacctgaa gaataagcac cggctaacta cgtgccagca
 491  gccgcggtaa tacgtagggt gcaagcgtta atcggaatta ctgggcgtaa agcgtgcgca ggcggttttg
 561  taagtctgat gtgaaatccc cgggctcaac ctgggaattg cattggagac tgcaaggcta gaatctggca
 631  gaggggggta gaattccacg tgtagcagtg aaatgcgtag atatgtggag gaacaccgat ggcgaaggca
 701  gccccctggg tcaagattga cgctcatgca cgaaagcgtg gggagcaaac aggattagat accctggtag
 771  tccacgccct aaacgatgtc tactagttgt cgggtcttaa ttgacttggt aacgcagcta acgcgtgaag
 841  tagaccgcct ggggagtacg gtcgcaagat taaaactcaa aggaatngac ggggacccgc acaagcggtg
 911  gatgatgtgg attaattcga tgcaacgcga aaaaccttac ctacccttga catggctgga atcctcgaga
 981  gattgaggag tgctcgaaag agaaccagta cacaggtgct gcatggctgt cgtcagctcg tgtcgtgaga
1051  tgttgggtta agtcccgcaa cgagcgcaac ccttgtcatt agttgctacg aaagggcact ctaatgagac
1121  tgccggtgac aaaccggagg aaggtgggga tgacgtcaag tcctcatggc ccttatgggt agggcttcac
1191  acgtcataca atggtacata cagagcgccg ccaacccgcg agggggagct aatcgcagaa agtgtatcgt
1261  agtccggatt gtagtctgca actcgactgc atgaagttgg aatcgctagt aatcgcggat cagcatgtcg
1331  cggtgaatac gttcccgggt cttgtacaca ccgcccgtca caccatggga gcgggtttta ccagaagtag
1401  gtagcttaac cgcaaggagg gcgctac
```

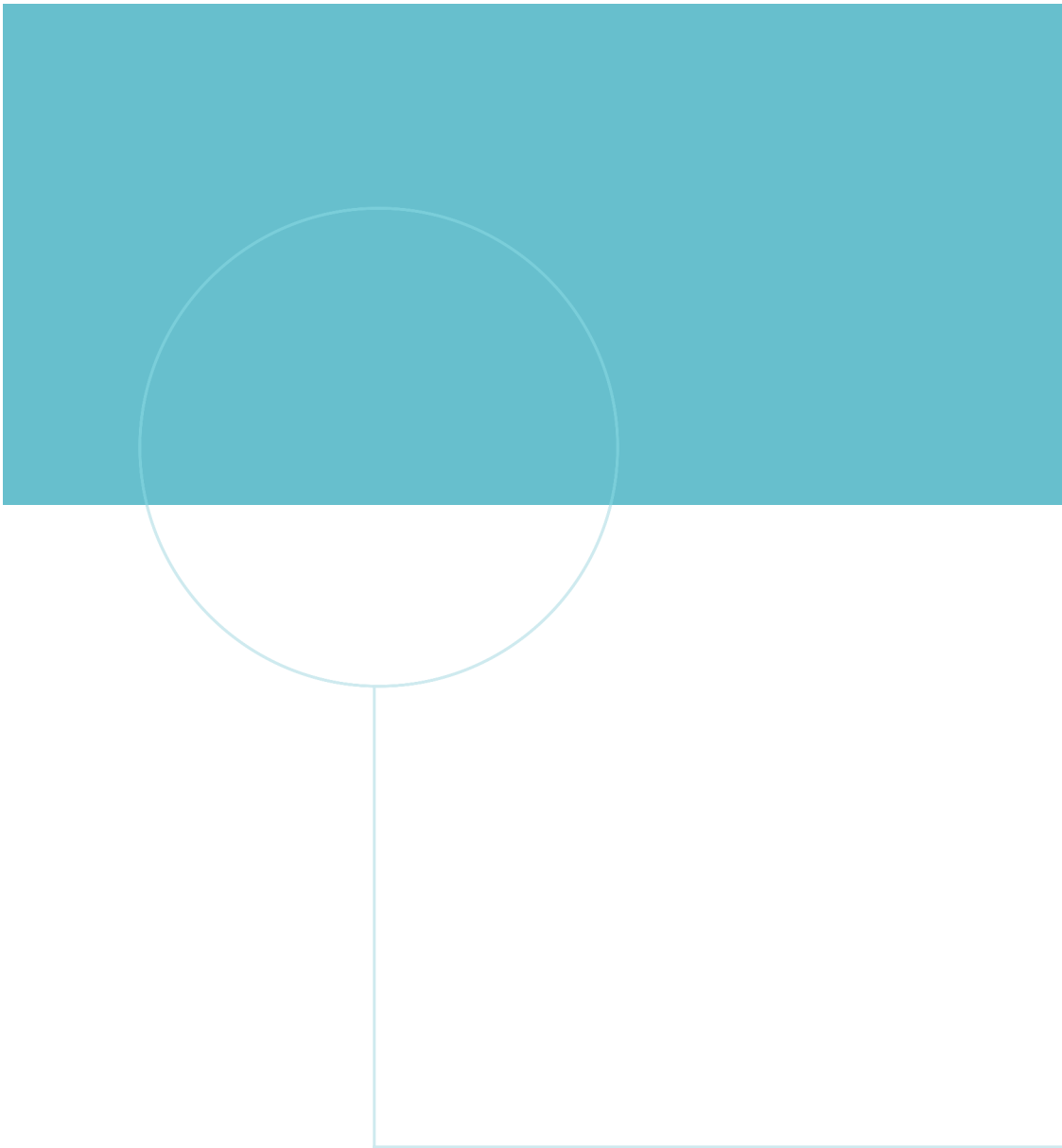
Psychrobacter cibarius (S5)

```
   1  attgaacgct ggcggcaggc ttaacacatg caagtcgagc ggtaacagga gaagcttgct tctcgctgac
  71  gagcggcgga cgggtgagta atacttagga atctacctag tagtggggga tagcacgggg aaactcgtat
 141  taataccgca tacgacctac gggagaaagg gggcagttta ctgctctcgc tattagatga gcctaagtcg
 211  gattagctag atggtggggt aaaggcctac catggcgacg atctgtagct ggtctgagag gatgatcagc
 281  cacaccggga ctgagacacg gcccggactc ctacgggagg cagcagtggg gaatattgga caatggggga
 351  aaccctgatc cagccatgcc gcgtgtgtga agaaggcctt ttggttgtaa agcactttaa gcagtgaaga
 421  agactccatg gttaataccc atggacgatg acattagctg cagaataagc accggctaac tctgtgccag
 491  cagccgcggt aatacagagg gtgcaagcgt taatcggaat tactgggcgt aaagggagcg taggtggctc
 561  tataagtcag atgtgaaatc ccgggcttta acctgggaac tgcatctgaa actgtagagc tagagtatgt
 631  gagaggaagg tagaattcca ggtgtagcgg tgaaatgcgt agagatctgg aggaataccg atggcgaagg
 701  cagccttctg gcataatact gacactgagg ctcgaaagcg tgggtagcaa acaggattag ataccctggt
 771  agtccacgcc gtaaacgatg tctactagtc gttgggtccc ttgaggactt agtgacgcag ctaacgcaat
 841  aagtagaccg cctggggagt acggccgcaa ggttaaaact caaatgaatn gacgggggcc cgcacaagcg
 911  gtggagcatg tggtttaatt cgatgcaacg cgaagaacct tacctggtct tgacatatct agaatcctgc
 981  agagatgcgg gagtgccttc gggaattaga atacaggtgc tgcatggctg tcgtcagctc gtgtcgtgag
1051  atgttgggtt aagtcccgca acgagcgcaa cccttgtcct tagttaccag cgggttaagc cgggaactct
1121  aaggatactg ccagtgacaa actggaggaa ggcggggacg acgtcaagtc atcatggccc ttacgaccag
1191  ggctacacac gtgctacaat ggtaggtaca gagggcagct acacagcgat gtgatgcgaa tctcaaaaag
1261  cctatcgtag tccagattgg agtctgcaac tcgactccat gaagtaggaa tcgctagtaa tcgcggatca
1331  gaatgccgcg gtgaatacgt tcccgggcct tgtacacacc gcccgtcaca ccatgggagt tgattgcacc
1401  agaagtggat agcttaacct tcgg
```

Appendix I

A standard curve showing Ct values as a function of Log copy number. The standard curve is created by using a dilution series of a 123 base pair long PCR product of the 16S rDNA gene, from a *Vibrio* (RD5-30) strain as a template in qPCR. The amplification efficiency was too high, indicating that the samples were not diluted enough. This was solved by only using the three most dilutes samples as shown in the result section.





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