Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

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Exploring the Role of Egg Microbiota in the Initial Colonization of Yolk-Sac Fry of Atlantic Salmon (*Salmo salar*)

Master's thesis in Sustainable Chemical and Biochemical Engineering
Supervisor: Ingrid Bakke
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ABSTRACT

Microbial colonization of the skin, gut, and gills is known to impact the fish's development and overall health. Yet, limited research has focused on understanding the impact of the egg microbiota on the fish after hatching. Traditionally, the surrounding water has been assumed to be the primary source of the initial colonization, particularly in aquaculture, where the eggs undergo disinfection prior to hatching. However, the potential contribution of egg microbiota to the initial colonization of fry during hatching has been overlooked.

This thesis aimed to understand the role of the egg microbiota for a newly hatched yolk-sac fry. Germ-free salmon eggs were colonized with salmon skin mucus, yolk-sac fry rearing water or were kept germ-free until hatching. On the day of hatching, the rearing waters were exchanged with either smolt-production RAS water or start-feeding RAS water. Water, eggs, and yolk-sac fry were sampled to investigate the microbial communities in the different experimental groups, employing Illumina sequencing of the V3-V4 region of 16S rDNA amplicons.

Variations in fish and water microbiota were observed between the treatment groups and with a consistent abundance of *Oxalobacteraceae* and *Flavobacteriaceae* across all groups. Skin mucus-exposed groups had a higher presence of *Comamonadaceae*, while *Pseudomonadaceae* was more common in the groups exposed to yolk-sac fry rearing water during the egg stage and the untreated group. High survival rates indicated favorable conditions, but concerns were raised regarding bacterial contaminants in some germ-free rearing flasks.

Moreover, the microbial treatments exposed to the eggs significantly influenced the microbiota of newly hatched yolk-sac fry. Yolk-sac fry exposed to skin mucus or yolk-sac fry rearing water during the egg stage exhibited distinct microbiotas compared to those without microbial treatments. Interestingly, skin mucus-exposed fry showed less sensitivity to post-hatching microbial treatments, whereas yolk-sac fry exposed to rearing water were significantly influenced by such treatments.

These findings enhance our understanding of the factors influencing initial microbial colonization of fish and emphasize the importance of considering not only the surrounding water but also the egg-associated microbial community when studying the early life stages of fish. Moreover, the results suggest that introducing microbial treatments at the egg stage could be a feasible strategy for enhancing the survival rate and promoting the overall health of farmed Atlantic salmon.

SAMMENDRAG

Mikrobiell kolonisering av skinn, tarm og gjeller er kjent for å påvirke fiskens utvikling og helse. Det finnes imidlertid lite forskning som fokuserer på å forstå eggmikrobiotaens bidrag til kolonisering av yngel etter klekking. Tradisjonelt sett har vannet blitt antatt til å være hovedkilden til den første koloniseringen av fisk, spesielt innen akvakultur der eggene desinfiseres før klekking. Imidlertid så har det mulige bidraget fra eggmikrobiotaen på yngel blitt oversett.

Målet med denne masteroppgaven var å forstå rollen til eggmikrobiotaen på nylig klekkede plommesekkyngel. Bakteriefrie lakseegg ble kolonisert enten med skinnmucus fra en eldre laks, vann fra en tank med plommesekkyngel, eller forble bakteriefrie frem til klekking. På klekkedagen ble vannet i flaskene enten byttet ut med vann fra et RAS-anlegg for smoltproduksjon eller startfôring av lakse-yngel. Prøver av vann, egg og plommesekkyngel ble tatt for å undersøke de mikrobielle samfunnene i de ulike gruppene ved hjelp av Illumina-sekvensering av V3-V4-regionen av 16S rDNA-amplikon.

Variasjoner i fisken og vannets mikrobiota ble observert mellom behandlingsgruppene, men *Oxalobacteraceae* og *Flavobacteriaceae* familiene ble funnet på tvers av alle behandlinsgruppene. Gruppene eksponert for lakseskinnmucus hadde en høyere forekomst av *Comamonadaceae*, mens *Pseudomonadaceae* var mer vanlig i gruppene eksponert for plommesekkyngelvann og de ubehandlede gruppene. Høy overlevelsesrate indikerte gunstige forhold, men bakteriell kontaminering ble funnet i noen av de bakteriefrie gruppene.

Funnene i oppgaven synliggjør en signifikant forskjell mellom mikrobiotaen til plommesekkyngelen som ble eksponert for behandlingene sammenlignet med de uten mikrobiell behandling på egg stadiet. Videre viste plommesekkyngelen som var eksponert for lakseskinnmucus mindre følsomhet overfor mikrobielle behandlinger etter klekking, mens plommesekkyngel som var eksponert for plommesekkyngelvann var betydelig påvirket av mikrobiell behandling etter klekking.

Disse funnene bidrar til forståelse om faktorene som påvirker den første mikrobielle koloniseringen av fisk og understreker betydningen av å ikke bare vurdere det omkringliggende vannet, men også eggmikrobiotaen når man studerer de tidlige livsstadiene til fisk. Videre antyder resultatene at mikrobielle behandlinger på eggstadiet kan være en mulig strategi for å forbedre overlevelsesraten og fremme helsen til oppdrettslaks.

CONTENTS

ABBREVIATIONS

- **ACMS** Analysis and Control of Microbial Systems
- **ASV** Exploratory Data Analysis
- **bp** Base pair
- **DNase** Deoxyribonuclease
- **dph** Days post-hatching
- **GF** Germ-free (control group used in the experiment)
- **M** Mucus (microbial treatment used in the experiment)
- **nt** Nucleotide
- **NTNU** Norwegian University for Science and Technology
- **rRNA** Ribosomal RNA
- **rRNA** Ribosomal ribonucleic acid
- **SIMPER** Similarity Percentage
- **V3** Variable region 3
- **V4** Variable region 4
- **wph** Week(s) post-hatching
- **PCoA** Principal Coordinate Analysis
- **PCR** Polymerase Chain Reaction
- **RDP** Ribosomal Database Project
- **S** Smolt water (microbial treatment used in the experiment)
- **SF** Start-feeding water (microbial treatment used in the experiment)
- **PERMANOVA** Non-parametric multivariable analysis of variance
- **SGM** Sterile Gnotobiotic Media
- **YW** Yolk-sac fry rearing water (microbial treatment used in the experiment)

CHAPTER

ONE

INTRODUCTION

Atlantic salmon is a popular species for farming and fisheries in the world. It is a member of the *Salmonidae* family and can be found on both sides of the Atlantic Ocean; in north-eastern North America and in Europe[1]. The global supply of seafood is turning towards aquaculture as the wild catch supply is stagnant in many regions and for many important species. Since 1999, the total supply of salmonids has been dominated by farmed salmonids; in 2021, the total global supply of farmed salmon exceeded 2.82 million tons, and the amount of wild-caught was about 25% of the amount of farmed salmon. The aquaculture industry has now reached a level where biological boundaries are being pushed, and the growth can no longer be driven only by industry and regulators. To continue growing the production while reducing its impact, advancements in technology, development of pharmaceuticals, adoption of non-pharmaceutical methods, enhanced industry regulations, and inter-company collaborations are required[2].

1.1 The Life Cycle of Atlantic Salmon

Most Atlantic salmon are anadromous; meaning they spend their lives in both fresh and salt water. Some, however, spend their entire lives in freshwater rivers, while others make short migrations to brackish water or stay close to the rivers. The spawning season typically takes place between September and February in freshwater rivers. Female salmon lay their eggs in nests in the gravel, and an adult male or mature juvenile fertilizes them. Northern populations often spawn earlier than southern ones due to the colder temperature which makes egg development slower[3]. Each female lays around 1,500 to 1,800 eggs per kilogram of body weight, and the eggs hatch after 480 to 520-degree days (water temperature times the number of days)[4]. In the spring, yolk-sac fry, or alevins, hatch. They carry a small sac of egg yolk that they utilize for nutrition for approximately 290-degree days. The alevins are then ready to emerge from the gravel and start their first feeding as fry[1]. Depending on the latitude, environmental conditions, and genetics, the juveniles remain in freshwater for 1 to 8 years before undergoing physiological and morphological changes during a process called smoltification. Smoltification prepares the salom for the transition from freshwater to marine environments[3].

Once in the sea, the post-smolts face new challenges, including adapting to different food sources and avoiding predators. They remain in the ocean for 1 to 5 years, undergoing further growth and development, before returning to their freshwater rivers for spawning. Although some salmon may return to spawn multiple times, the majority only survive to do so once or twice. The spawning period represents the highest mortality risk for the salmon, as many individuals die from predators, disease, or exhaustion[3].

1.2 Atlantic Salmon Farming

The lifecycle of farmed Atlantic salmon is often shorter compared to wild salmon; they are bred to grow faster, mature later, and resist diseases better[2]. In Norway, salmon producers buy eggs from specialized breeders, whilst some produce their own eggs. The breeders produce fish eggs with genetics that provide robust and healthy fish[5]. Prior to being sent to the salmon farms, the eggs undergo surface disinfection using iodine disinfectant to eliminate any potential pathogens[4].

At the salmon farms, the eggs are kept in a hatchery under optimal conditions until they hatch and have consumed their yolk sac. Approximately 50 days after hatching, the yolk-sac fry are transferred to a bigger tank to initiate the start-feeding phase and begin the smoltification phase[4]. When the fry reaches a body length of 2 to 2.5 cm, they develop distinct dark "fingermarks" on their sides and are referred to as "parr". Once the parr has undergone the smoltification and reached a body weight of 50 - 100 g, they are ready to be transferred to sea cages[1]. However, since the removal of the weight limits for smolts in the freshwater stage in Norway in 2016, it has become more common to prolong the freshwater stage to minimize exposure to sea lice and other diseases[6]. While the main periods for transferring smolt to sea in Norway are spring and fall, the smolt transfer occurs throughout the year. The fish spend 12 to 24 months in seawater cages before being harvested. Following the harvest, the area is left fallow for two to six months before the next generation is introduced in the same location[2].

In 2020, the global production of farmed Atlantic salmon reached 2.7 million tons, with Norway accounting for over 37% of the production, closely followed by Chile with a 27% share[7]. Despite Norway's strong production rate, the mortality rate has shown a worrisome increase in the last few years. In 2022, 56.7 million (16.1%) salmon in the seawater phase and an additional 35.6 million from the freshwater phase were reported as mortalities. The freshwater stage numbers are excluding the mortality of eggs and juveniles from 0 to 3 grams as they are responsible for 45% of the total mortality rate, and numbers in percentages in this phase are not available due to production routines. Various factors contribute to the high mortality rate, including infectious diseases, poor environmental conditions, trauma related to injuries, and lack of physiological adaptation. Especially worrying is the increase in bacterial diseases[8].

1.3 Disease Challenges in Atlantic Salmon Farming

In correlation to an increase in the mortality rate in the Atlantic salmon farming industry in Norway, there has also been an increase in registered infections. The increasing incidences are recorded both for known fish diseases, but also for diseases related to more opportunistic environmental bacteria. The reasons for the changes are complex. The increase in the fish pathogen bacteria can be linked to the spread of infection from reservoirs with farmed or wild fish. Consequently, it is crucial to maintain an overview to prevent the introduction of diseased fish into the marine environment. The increased incidences caused by more opportunistic bacteria may result from reduced resistance in the fish, changes in the bacterial properties, or changes in infection dynamics influenced by external conditions[6].

Issues with pathogens can arise as early as in the hatchery phase. Fish eggs are particularly susceptible to infection by *Saprolegnia* species, which contribute to significant losses [9, 10, 11, 12]. Saprolegniosis can be seen as a thin, cotton-like layer covering the fish or egg's surface epidermis. *Saprolegnia* is present in all waterbodies in the world and is spread through zoospores. *Saprolegnia* is therefore commonly present in the water sources of hatcheries. The zoospores colonize and multiply within biofilms in pipes and tanks, often evading detection. Hence, the fish are continuously exposed to these spores. However, *Saprolegnia* infection only occurs when the fish or eggs are weakened or have damaged skin and mucus. However, the infection may spread to healthy eggs and fish and result in major losses if not removed[13]. Today, formalin is used to avoid *Saprolegnia* outbreaks, however, the use of formalin in aquaculture is controversial and is currently under consideration in the EU system and may thus become regulated or forbidden in the next few years[6].

Yersinosis, caused by the bacterium *Yersinia ruckeri*, is another common disease in hatcheries. It is believed to be introduced during the hatchery phase but often does not cause disease until later. While antibacterial drugs have been used to treat yersiniosis, a water-based injection vaccine has become widespread the recent years due to its effectiveness[8].

Several other bacteria-caused diseases are common for farmed Atlantic salmon, such as Furunculosis, Vibriosis, Winter ulcer, Mycobacteriosis, Bacterial Kidney Disease, and Pasteurellosis. Viral diseases, fungal diseases, and parasite diseases are also common. Among these, the salmon louse *Lepeophtheirus salmonis* is one of the largest problems in the aquaculture industry for salmon in the seawater phase. These lice feed on the skin, mucus, and blood of the fish, causing lesions that can lead to secondary infections and osmoregulatory problems[6].

Approaches to solving the disease problems have mainly been focused on disinfection procedures, vaccines, and the cautious handling of the fish[8, 14]. Disinfection of the inflow water is a common approach for different systems to control the bacterial densities in rearing tanks. However, this approach primarily targets external sources of microbes and neglects the internal processes of bacterial growth. The

bacterial densities in the rearing tanks are influenced not only by the bacterial densities in the process water but by the bacterial growth in the rearing system and the overall supply of organic substances from live food and microalgae. Consequently, a more comprehensive understanding of the interactions between rearing technology and microbial ecology is needed to develop strategies that create environments preventing the growth of opportunistic bacteria[14].

1.4 Fish Microbiota: Interactions and Implications

Similar to other animals, fish establishes a symbiotic relationship with their microbial community. This symbiotic relationship plays an important role in the physiological functions of the host[15] and is necessary for the development of immune and digestive system development[16, 17]. The skin, gills, and gastrointestinal tract are recognized as the major sites for microbial colonization in fish, harboring diverse microbial communities that contribute to the host's mucosal barrier defenses[18]. Through competition for adhesion sites and nutrition, they may limit or reduce the abundance of pathogens[19].

Studies focusing on zebrafish have provided insights into the complex and dynamic interaction between the host and its gut microbiota[16]. A study by Rawls Et al.[16] revealed that zebrafish share several responses with mammals in response to gut microbiota and that these responses exhibit microbial specificity. It was discovered that different bacterial species had distinct effects on gene expression in intestinal development and immune function. For example, colonization with the bacterium *Aeromonas veronii* was found to induce the expression of genes involved in gut maturation, while colonization with *Vibrio anguillarum* had no effect on these genes. Similarly, colonization with *Pseudonomas aeruginoas* induced the expression of immune-related genes, while colonization with *Edwardsiella tarda* did not. Moreover, the gut microbiota has been found to play an important role in the development of the nervous system in zebrafish, and disruption of the gut microbiota during early development may have long-term effects on neurobehavioral functions[20]. These findings suggest that the interaction between the host and its gut microbiota in fish is complex and dynamic, and that microbial specificity plays an important role in shaping host immunity and maintaining a healthy gut ecosystem.

Particularly for the gut, there have been numerous studies of environmental, nutritional, and genetic causes of microbiome diversity. However, the skin and gills may also be affected by diet, environmental conditions (e.g. seasonality, salinity, geographic location); mutualistic relationships with other hosts, and host genotypes[18].

Studies have demonstrated that the skin microbiota of healthy zebrafish contains several bacterial species that are capable of producing compounds with antimicrobial properties. In one particular study, some of these identified bacterial species were introduced to the skin microbiota of other zebrafish, and a group of 10 bacterial species was sufficient to protect zebrafish from the pathogen *Flavobacterium*

columnare. Furthermore, they found that stress can significantly impact the diversity and composition of the skin microbiota, which in turn affects the fish's susceptibility to infection[21].

Similarly, a study of rainbow trout also revealed that germ-free infected larvae were susceptible to *Flavobacterium columnare*, while conventionally raised larvae were not. In this study, germ-free larvae were recolonized larvae with 11 different bacterial species, and two of these species were sufficient to provide full protection against the infection[22].

The comprehensive research conducted on zebrafish has laid the groundwork for further investigations into the microbiota of various fish species. With the advancements in Next Generation Sequencing technologies and their reduced costs, numerous studies focusing on the fish microbiota have emerged. These studies have shed light on the diverse array of bacterial species that inhabit different fish species.

Within the gastrointestinal tract of fish, several phyla have been consistently observed, including *Firmicutes*, *Proteobacteria*, *Bacteriodetes*, *Actinobacteria*, and *Fusobacteria*. Conversely, the skin microbiota is mainly composed of *Proteobacteria*, while the gills are primarily dominated by *Gammaproteobacteria*. The bacterial community in the reproductive organs of the fish displays variability, influenced by factors such as sex and reproductive status of the fish [18].

The understanding of microbiota in fish species important to aquaculture is still evolving. A recent study has revealed that stage-specific microbial signatures are found at the phylum level for Atlantic salmon[15]. Notably, *Proteobacteria* was reported to be the most abundant phylum in eggs, while newly hatched fry exhibit a significant abundance of *Actinobacteria*, *Firmicutes*, *Tenericutes*, *Spirochaetes*, and *Deinococcus-Thermus*. In the early intestines of the early freshwater stages *Proteobacteria* was dominant while later in the freshwater stage *Firmicutes* and *Bacteroides* were the dominant phyla. However, *Proteobacteria* regained its dominance of relative dominance upon the fish's transition to seawater. These stage-specific phyla can be used as indicators for the salmon's developmental stages. Upon confirming the functions of these indicators, selected members of a specific phylum could be utilized through microbial manipulation to improve the growth and health of farmed fish. While there are several other studies that focus on the gut microbiota of Atlantic salmon[23, 24, 25], and on the microbial change when moved from freshwater to seawater[26, 27], limited information is available regarding the microbiota during the egg and larval stages and the functional roles of different phyla in the microbiota.

The fish larvae are known to be germ-free in their eggs[28], and it is anticipated that the diverse microbiota that grows on the egg's surface mimics that of the surrounding water. However, as soon as the eggs hatch, the sterile larvae become colonized by the microbiota from the surrounding microbes[28]. In contrast to mammals, the transfer of bacteria from parent to offspring is assumed to be less significant for fish. However, species differences have been observed in terms

of bacterial colonization of fish eggs between cod and halibut[29]. Such species-specific assemblages on the chorion of the egg could be a result of differential attraction to surface receptors[29], or possibly from vertical transmission of microbiota components to eggs during oviposition[28]. In fact, a dominant bacterial strain, *Pseudonomas sp.*, found in the juvenile gastrointestinal tract, has also been identified on fish eggs but not in the surrounding water or food of Salmo coho[30, 28]. This bacterial strain is commonly observed as a dominant genus in the gut microbiota of mature fish[31, 32, 33], which suggests that vertical transmission may be possible[28]. Despite identifying bacterial species on fish eggs, the extent of influence exerted by the egg microbiota on the initial colonization of yolk-sac fry remains unclear.

The colonization steps of fish, including the potential for vertical transmission (Step 5), are illustrated in Figure 1.4.1.

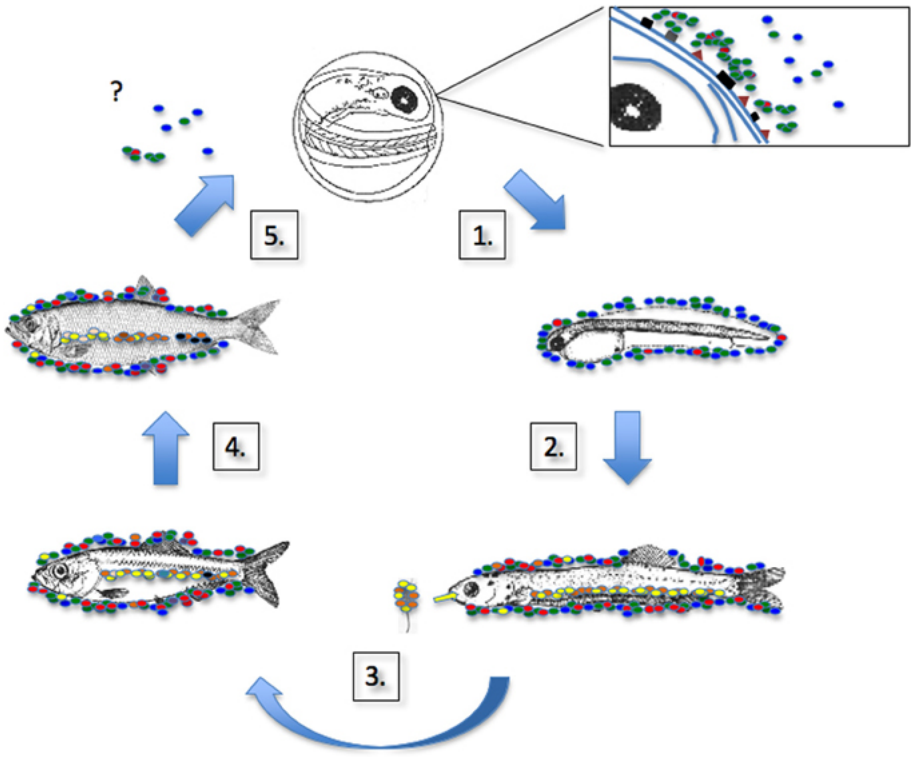


Figure 1.4.1: Fish microbiota development. The figure shows a schematic generalized overview of microbial colonization of fish. (1) Bacteria initially colonize the chorion of the egg. (2) Upon hatching, the larva becomes colonized by surrounding microbes. (3) Early digestive tract colonization occurs when the fish starts feeding, and the bacterial taxa strongly resemble those of the associated food. (4) The microbiome develops and matures. (5) The adult microbiome is a diverse assemblage of microbial taxa which might be transferred to the eggs through vertical transmission. Figure obtained from Llewllyn M.S. et. al[28].

1.5 Gnotobiotic Models

Gnotobiotic or "germ-free" models have been proven to be powerful tools in unraveling the effects of the microbiota on host organisms. In gnotobiotic studies, animals are raised in a germ-free environment and then selectively colonized with known bacteria. By comparing the germ-free models to conventionally raised animals it is possible to study the effect of a single microbe or more complex communities on the host responses[34].

Gnotobiotic fish models offer several advantages over other animal models. A single female fish may release from 80 to several thousand eggs at once, depending on the species. This abundance of offspring enables statistically significant observations within a genetically related host population. Furthermore, the fish embryos are enveloped in a chemically resistant chorion, which permits quick and thorough surface disinfection of the eggs. Additionally, since many fish develop their adaptive immune system relatively late, studying the innate immune response to the microbiota in fish larvae and juveniles can be conducted independently of the influence of the adaptive immune response[34].

The field of microbiota research in fish began with pioneering studies focused on generating germ-free zebrafish, which provided crucial insights into the interactions between surrounding microbes and the host's microbes[16, 35]. These early studies have laid the foundation of the knowledge of the interactions between microbes and their hosts[34]. Subsequent studies examining the microbiota of both farmed and wild fish species have further expanded our knowledge, shedding light on the microbial contributions to survival[36], growth[23], immunity, and pathogenesis in fish[37, 38].

While gnotobiotic models have been established for several food fish species, including Atlantic halibut, Nile tilapia, Atlantic cod, Dover sole, Sea bass, and some Salmonid species[39], there are relatively few models for species utilized in aquaculture production. However, recently a gnotobiotic model system for Atlantic salmon (*Salmo salar*) was developed by Gómez de la Torre Canny[39]. This model system was successfully applied to both wild and farmed Atlantic salmon, with a relatively high success rate of germ-free fish derivation (87.8%). In this study, they also found that the presence of certain microbes was associated with changes in the mucosal barrier function and adipose tissue accumulation in the fish. Fish with a more diverse microbiota exhibited a thicker mucosal layer compared to those with a less diverse microbiota.

1.6 Aims and Objectives

Today, it is known that bacterial colonization of skin, gut, and gills happens quickly after hatching and that it is important for the development of the fish. However, little is known about how the egg microbiota influences the initial colonization of the fry when it hatches. In the aquaculture industry, eggs undergo disinfection prior to hatching, eliminating both beneficial and pathogenic bacteria.

This raises the question of whether the initial colonization of newly hatched fry can be influenced by introducing beneficial microbiota. Understanding the role of the egg microbiota in the colonization of larvae is crucial for enhancing the fish's immune system and promoting their development. Therefore, this study aims to investigate how the timing of the microbial colonization affects the microbiota of the newly hatched fry, with the following key objectives:

1. Characterize the microbiota of the rearing water and yolk-sac fry in the early yolk-sac fry stage.
2. Investigate if microbial treatments on eggs can affect the post-hatching fish microbiota.
3. Determine if post-hatching microbial treatments have a greater influence on the fish microbiota compared to the potential effects contributed by the egg microbiota

CHAPTER

TWO

METHODS

2.1 Atlantic Salmon Experiment

The Atlantic salmon experiment aimed to study the initial colonization of yolk-sac fry was conducted during November and December 2022. A comprehensive description of the experiment's methodology can be found in the associated specialization project[40]. While the Atlantic salmon experiment for the specialization project had to be terminated due to bacterial contamination in the rearing flasks, the experiment was repeated for this master's thesis with an enhanced concentration of the antibiotic solution (from 22.5 mg/L to 37.5 mg/L of oxalinic acid, Appendix A) for the disinfection procedure. The experimental design remained consistent with the previous study.

To investigate the extent to which the microbiota of eggs and water influenced the microbiota of newly hatched yolk-sac fry, salmon eggs were exposed to different microbial communities. Following hatching, the yolk-sac fry were carefully rinsed, and the rearing water was exchanged with new water sources. Samples of eggs, yolk-sac fry, and rearing water were collected and frozen for later analysis of the microbial community composition using Illumina sequencing of the V3-V4 region of the 16S rRNA gene.

2.1.1 Experimental Design

The salmon eggs were subjected to surface disinfection upon arrival using antibiotics (Appendix A) and Buffodine. Subsequently, they were then distributed into 500 mL tissue flasks, with each flask containing 100 mL of sterile Salmon Gnotobiotic Medium (SGM). A total of 18 flasks were prepared, with 14 eggs per flask. To investigate the sterility, water samples were taken from all the rearing flasks and incubated in growth media for three weeks to monitor for microbial growth.

The day after surface disinfection, two different microbial communities were introduced to the rearing water to colonize the eggs. One community consisted of homogenized mucus obtained from the skin of Atlantic salmon fry, while the other community was derived from the rearing water of a tank with Atlantic salmon yolk-sac fry. These microbial communities were collected from separate systems

at NTNU SeaLab. The 18 rearing flasks were divided into three groups. The first group (M) received homogenized mucus from salmon fry, the second group (YW) received water from a yolk sac fry tank, and the third group (GF) was maintained germ-free.

On the day of hatching, the rearing water was exchanged with water from either a RAS (Recirculating Aquaculture System) system for smolt production or a RAS system for the start-feeding of Atlantic salmon fry. Three replicate flasks from each group were assigned to each water source, resulting in six different groups. The rearing water from the RAS for smolt production (S) was added to three rearing flasks previously exposed to mucus microbiota (M-S), three rearing flasks previously exposed to rearing water from a yolk-sac fry tank (YW-S), and three germ-free rearing flasks (GF-S). Rearing water from the RAS for start-feeding (SF) was added to three rearing flasks previously exposed to mucus microbiota (M-SF), three rearing flasks previously exposed to rearing water from a yolk-sac fry tank (YW-S), and three germ-free rearing flasks (GF-SF).

To maintain the water quality, 60% of the rearing water of the fish flasks was exchanged three times per week. The experiment was conducted in a dark room at approximately 6°C. All procedures were performed with aseptic techniques within a laminar flow hood with UV-disinfected equipment. A more detailed description of the materials and methods is provided in the specialization project[40].

An overview of the experimental design is presented in Figure 2.1.1.

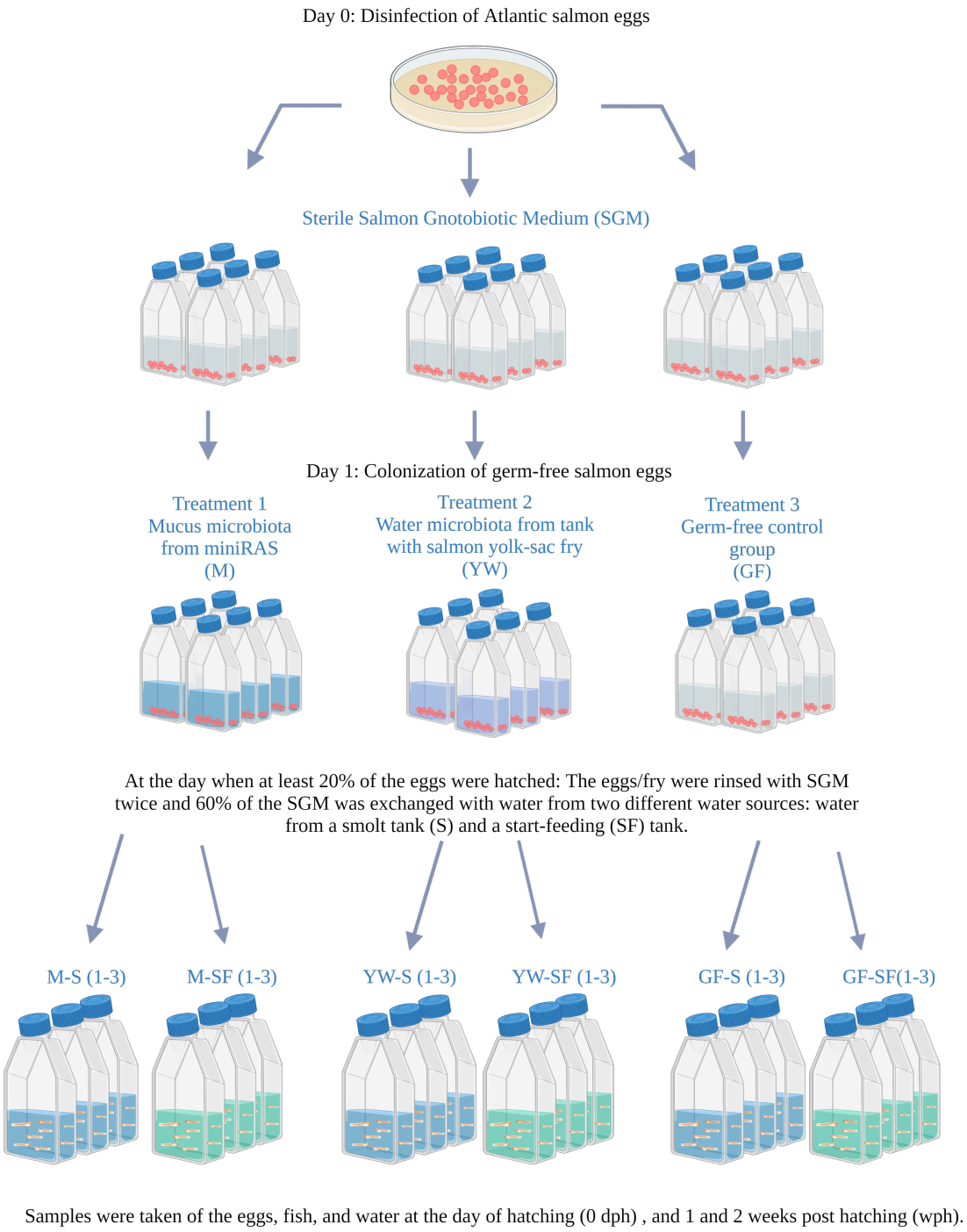


Figure 2.1.1: Overview of the experimental design for the Atlantic salmon experiment. On the day of arrival, surface disinfection of the salmon eggs was performed, followed by their distribution into three groups. Each treatment group represents a different microbial treatment: Skin mucus (M), rearing water from a yolk-sac fry tank (YW), and a germ-free control group (GF). Once 20% of the eggs had hatched, the three initial groups were further divided into six groups, incorporating two new water sources: water from a smolt tank (S) and a start feeding tank (SF) both from commercial RAS systems. The figure was created using BioRender.com

2.1.2 Sampling

There were three sampling periods throughout the experiment. The first sampling took place on the day when at least 20% of the eggs in all of the flasks had hatched (0 days post-hatching, dph). At this stage, samples of rearing water and eggs were collected. The eggs were rinsed twice with sterile SGM, after which they were immediately frozen and stored at -20°C until further processing. Additionally, 20 mL of the rearing water from each flask was individually filtrated through 0.2 µm nucleopore filter papers to collect the microbial samples. Water samples were also taken from the germ-free control flasks (GF) to verify their sterility.

The two other sampling periods took place at 1 and 2 wph. Yolk-sac fry were collected, rinsed twice with sterile SGM, and frozen as whole individuals. Furthermore, 20 mL of rearing water from each flask was again filtrated through 0.2 µm nucleopore filter papers to collect the microbial samples.

2.2 Microbial Community Analysis

2.2.1 DNA Extraction

DNA was extracted from the eggs, yolk-sac fry, and rearing water samples. The samples were transferred to Precellys tubes and homogenized with Precellys 24 tissue homogenizer (Bertin Technologies) at 6500 rpm for two cycles of 30 seconds each. Homogenization was done with 0.1 mm glass beads (Bertin Technologies) and 750 µL of the lysis buffer provided with the extraction kit. The samples were then centrifuged at 10,000 x g, and the supernatants were used for DNA extraction using the 96 MagBead DNA Kit (ZymoBIOMICS). The DNA extraction was performed following the manufacturer's protocol[41] using a KingFisher instrument (Thermo Scientific). Finally, the extracted DNA was eluted in 100 µL DNase-free water and stored at -20°C.

2.2.2 Polymerase Chain Reaction (PCR)

To amplify the v3-v4 region of the bacterial 16S rRNA gene from the DNA extracts, polymerase chain reaction (PCR) was conducted. The amplification was carried out using the forward primer ill341F_KI (5'-**TCGTCGGC AGCGTCAGATGTGTATAAGAGACAG**NNNNCCTACGGGNGG CWGCAG-3) and the reverse primer ill805R (5'**GTCTC GTGGGCTCGGAGATGTGTATAAGAGACAG**NNNNGACTACNVGGGTATCTAAKC C-3'). The target sequences are marked in bold.

The PCR amplification was performed using 1 µL of the DNA extracts as a template in a total reaction volume of 25 µL. Each PCR reaction contained 0.3 µM of each primer, 200 µM of dNTP, 0.02U Phusion hot start polymerase, and HF buffer from Thermo Scientific. The cycling conditions were as follows: an initial denaturation step at 95°C for 2 min, followed by 36 cycles of denaturation at 95°C

for 15 s, annealing at 55°C for 20 sec, extension at 72°C for 20 sec, and a final extension at 72°C for 5 min.

2.2.3 Agarose Gel Electrophoresis

The quantity and possible contamination of PCR products were assessed using agarose gel electrophoresis. A 1% agarose gel was prepared by dissolving agarose in TAE-buffer (Tris-Acetate-EDTA buffer). The agarose solution was heated until the agarose was melted and mixed with the TAE-buffer. To visualize the DNA bands, GelRed (Biotium) was added to the gel at a final concentration of 50mM. The gel solution was then poured into a gel chamber with gel combs and allowed to solidify for 20 to 30 minutes, depending on the gel thickness.

For analysis, 1 µL of the PCR products, mixed with 5 µL 6x DNA loading dye (Thermo Scientific) was carefully loaded into the wells on the agarose gel. A Generuler 1kb Plus DNA ladder (Thermo Scientific) was used as a size marker. The gel was then subjected to electrophoresis at a voltage of 100 to 120 V until sufficient separation of the PCR product band was achieved.

Following the electrophoresis run, the gel was visualized under UV light using a G:box HR GelDox (Syngene).

2.2.4 Preparation of Amplicon Library for Illumina Sequencing

The PCR products were normalized to 25 ng of PCR product and purified using a Sequal Prep Normalization plate kit (Invitrogen). The manufacturer's protocol[42] was followed, using 15 µL of the PCR products in each well.

To provide each sample with a unique index sequence combination, 14 forward sequence indexes and 24 reverse sequence indexes were used from the Nextra XT Index Kit Set A and D (Illumina).

Each indexing primer sequence (2.5 µL each) was individually added to a PCR mixture with a final concentration of 1x Phusion Hot Start DNA polymerase (Thermo Scientific), 0.25 mM dNTPs (Thermo Scientific), and 0.015 U/µL Phusion Hot start DNA polymerase (Thermo Scientific). The normalized PCR products served as the template. The samples were prepared in two 96-well plates. For the indexing PCR, the samples underwent 12 cycles of amplification using a thermocycler with the following temperature and cycling conditions: initial denaturation at 95°C for 2 min, followed by 10 cycles of 95°C for 15 s, 55°C for 20 sec, 72°C for 20 sec, and a final extension at 72°C for 5 min.

The indexed PCR products were analyzed using agarose gel electrophoresis. In cases where the yield was low, the procedure was repeated with 15 cycles.

Following the indexing PCR, the PCR products were subjected to a second round of normalization and purification using the Sequal Prep Normalization plate Kit (Invitrogen). In this step, 10 µL of each PCR product was utilized.

After normalization, the 139 indexed PCR products were combined and pooled together to generate the amplicon library. The pooled sample was then concentrated using Amicon Ultra 0.5 Centrifugal Filter units (Merck Millipore). The manufacturer's protocol[43] was followed, with one modification. To ensure optimal purity and removal of impurities, the final washing step in the protocol (Step 5) was repeated once more.

The final concentrated amplicon library was assessed using agarose gel electrophoresis.

Of the pooled concentrated amplicon library, 20 µL was mixed with 5 µL of the 6x Loading Dye (Thermo Scientific). The mixture was carefully loaded onto an agarose gel and subjected to electrophoresis. Following the electrophoresis, the gel was visualized under UV light using a G:Box HR GelDox (Syngene). The bands corresponding to the desired PCR products were then extracted from the gel using the QIAquick Gel Extraction Kit, following the manufacturer's protocol[44]. To assess the concentration and purity of the purified product measurements were performed using a NanoDrop One Spectrophotometer (Thermo Scientific). Finally, the completed amplicon library was sent to the Norwegian Sequencing Centre (NSC) for sequencing on one MiSeq lane (Illumina, San Diego, CA) with V3 reagents (Illumina)

2.2.5 Processing of Illumina Sequencing Data

The Illumina sequencing data were processed using the USEARCH pipeline version 11[45]. The fastq_mergepairs command was employed to merge sequence pairs, remove primer sequences, and filter out merged sequences shorter than 380 bp. Quality filtering was performed using the fastq_filter command, with the default expected error threshold value of 1.

To generate Amplicon Sequencing Variants (ASVs), the Unoise3 command [46] was utilized. The recommended minimum abundance threshold of 8 reads in the entire dataset was applied. Taxonomy assignment to the ASVs was performed using the sintax command [47] with the Ribosomal Database Project (RDP) 16S rRNA training set v18 as the reference data.

The resulting ASV table was manually inspected, and ASVs representing chloroplasts and archaea were excluded from the table. Subsequently, the ASV table was normalized to 26,000 reads per sample using the otutab_rare command in USEARCH.

2.3 Statistical Analyses

To assess alpha diversity, several indices were calculated using the alpha_div command in USEARCH. The observed ASV richness and Shannon's diversity were determined. Observed ASV richness represents the number of ASVs observed in each sample, while Shannon's diversity index considers both the richness and evenness of ASVs in a community. Higher values of Shannon's diversity indicate greater community diversity, while evenness reflects the equality of ASV abundances, with high evenness suggesting similar abundances among ASVs[48]. The calculated alpha diversity indices were exported to Microsoft Excel for further analysis.

To evaluate beta diversity, Bray-Curtis similarities between samples were computed using the PAST software (Version 4.0). The resulting Bray-Curtis similarity matrix was exported to Microsoft Excel for further analysis. Bray-Curtis similarities range from 0 to 1, where 0 represents completely dissimilar communities (no shared ASVs) and 1 indicates identical communities.

To visualize beta diversity, a Principal Coordinate Analysis (PCoA) was conducted using Bray-Curtis similarities. PCoA generates a low-dimensional graph based on a distance matrix, where the arrangement of data points reflects their similarity or dissimilarity. The proximity of samples in the plot indicates their degree of similarity, with closer samples indicating higher similarity to each other[49, 50].

To determine statistically significant differences between sample groups, a one-way PERMANOVA (permutational multivariable analysis of variance) using Bray-Curtis similarities was conducted. The significance threshold was set at a p-value below 0.05.

SIMPER (Similarity Percentage) analysis was performed to identify ASVs contributing the most to the dissimilarity between sample groups. SIMPER analysis determined the average dissimilarity between groups and identifies the ASVs that contribute to the most dissimilarity.

A Willcoxon rank-sum test without multiple testing correction was used to examine if there were statistically significant differences between two groups. If the p-value was below 0.05, the test concluded that there was a statistical significant difference between the two groups

CHAPTER

THREE

RESULTS

3.1 Atlantic Salmon Experiment

The Atlantic salmon experiment was designed to investigate the microbial colonization of the yolk-sac fry upon hatching. Illumina sequencing of 16S rDNA amplicons was performed on samples of rearing water, eggs, and yolk-sac fry collected at three different time points: on the day of hatching (0 dph), one week post-hatching (1 wph), and two weeks post-hatching (2 wph).

The yolk-sac fry were subjected to microbial treatments at two different stages. Initially, the eggs were colonized by exposure to skin mucus from Atlantic salmon fry (M) in their rearing water, rearing water from a yolk-sac fry tank (YW), or maintained germ-free until hatching (GF). After 20 days of rearing, the eggs hatched, and on the day of hatching, the three groups were subjected to new water sources, either from RAS of smolt production (S) or from a RAS of start-feeding of salmon fry (SF). Consequently, this experimental setup resulted in six different treatment groups (M-S, M-SF, YW-S, YW-SF, GF-S, GF-SF), each with three replicate flasks (1-3).

3.1.1 Survival Rate and Overall Performance of Eggs and Yolk-Sac Fry

The survival rate of eggs and yolk-sac fry during the Atlantic salmon experiment was monitored. A total of 252 eggs were distributed into 18 rearing flasks, each containing 14 eggs. During the five-week rearing period, only one egg was found deceased and was removed from the GF-SF2 rearing flask at 0 dph. Meanwhile, the remaining eggs in the experiment hatched successfully.

Furthermore, within the GF-S1 rearing flask, three yolk-sac fry were discovered deceased and were removed from the rearing flask at 1, 5, and 6 dph. Despite this, the remaining flasks exhibited a 100% survival rate over the whole five-week period of the experiment. Consequently, the overall survival rate for the experiment was found to be 98.6% (Table 3.1.1).

Table 3.1.1: Overview of survival rates observed in the Atlantic salmon experiment. Each treatment group consisted of three replicate flasks. The initial microbial treatments consisted of skin mucus of Atlantic salmon fry (M), rearing water from a yolk-sac fry tank (YW), or germ-free conditions until hatching (GF). The second treatment included exposure to water from either a RAS for smolt production (S) or a RAS for start-feeding of salmon fry (SF).

treatment group	**M-S**	**M-SF**	**YW-S**	**YW-SF**	**GF-S**	**GF-SF**
Total individuals	54	54	54	54	54	54
Deceased	0	0	0	0	1	3
Survival rate [%]	100	100	100	100	98	94

3.1.2 Sterility and Contamination Analysis

In the experiment, thorough measures were taken to ensure the sterility of all the rearing flasks. Following the disinfection procedure, samples of the rearing water were collected from each rearing flask to verify the sterility. These water samples underwent a three-week incubation on agar plates and in four different liquid growth media, both at room temperature and in the fish lab at 6°C. None of these samples showed any signs of bacterial growth, indicating a successful disinfection procedure.

Among the 18 rearing flasks, six were specifically designated as germ-free control flasks. These control flasks were carefully maintained in a sterile condition until the day of hatching. Before the introduction of new water sources at 0 dph, new rearing water samples were collected from the germ-free flasks to reconfirm their sterility ensuring the integrity of the experimental setup.

After a week and a half of incubating the sterility controls, bacterial growth was observed for three of the GF rearing flasks. Specifically, bacterial growth was observed in two types of liquid growth media, nutrient broth and Saboraud Dextrose broth, at room temperature. However, no signs of bacterial growth were detected in the other liquid broths at room temperature, nor on any of the agar plates or in the liquid broths incubated at 6°C.

Cell pellets from the contaminated sterility controls were subjected to 16S rDNA amplicon sequencing along with other samples from the Atlantic salmon experiment. The analysis revealed the presence of four dominant ASVs in the contaminated sterility controls classified as *Flavobacterium* and *Undibacterium*(Table 3.1.2). Among these ASVs, the sample obtained from the GF-SF2 rearing flask showed a relative abundance of 58% and 41% for two *Undibacterium* ASVs. The sample from the GF-S1 and GF-SF1 rearing flasks exhibited a relative abundance of 89% and 91% for ASVs classified as *Flavobacterium* along with 4% and 3% for *Undibacterium*, respectively.

Table 3.1.2: Overview of the ASVs identified from the sterility control samples. The taxonomy is assigned at the genus level.

ASV ID	Taxonomy	Relative abundance [%]		
		GF-S1	GF-SF1	GF-SF2
ASV6	*Flavobacterium*	77%	78%	0%
ASV29	*Flavobacterium*	12%	13%	0%
ASV2	*Undibacterium*	2%	2%	58%
ASV8	*Undibacterium*	2%	1%	41%

The water, egg, and yolk-sac fry samples of the Atlantic salmon experiment were subjected to 16S rDNA amplicon sequencing, as later described in Section 3.2. To investigate the potential impact of the contamination in the respective GF-rearing flasks and evaluate the potential contamination of other non-germ-free rearing flasks, a detailed bar plot was created to compare the relative abundance of the four contaminating ASVs across all samples in the experiment (Figure 3.1.1).

ASV6 (*Flavobacterium*), detected in the sterility controls of the GF-S1 and GF-SF1 rearing flasks, was mainly observed in the fish and water samples of the respective rearing flasks. In GF-S1 fish samples, ASV6 accounted for 23% (F1) and 23% (F2) of the relative abundance, while in GF-SF1 fish samples, it represented 13% (F1) and 10% (F2) of relative abundance. In the water samples, ASV6 had a relative abundance of 13% in GF-S1 and 0% in GF-SF1. Notably, ASV6 was only detected in one of the 2 wph samples from the respective rearing flasks (GF-SF1), with a relative abundance of 2%.

Interestingly, ASV6 was also present in selected samples from other rearing flasks belonging to the other treatment groups. Notably, the water sample collected at 0 dph from the YW-SF1 flask exhibited a remarkably high relative abundance of ASV6 (55%). Furthermore, fish samples from 1 wph in the YW-SF1 and M-S1 rearing flasks showed relatively high abundances of ASV6, accounting for 13% and 7% of the relative abundance, respectively, compared to other samples. Additionally, ASV6 was observed in a small percentage (1%) in the water originating from the start-feeding (SF) RAS that was added to the flasks at 0 dph.

Similarly, ASV29 (*Flavobacterium*), also detected in the sterility control of GF-S1 and GF-SF1 rearing flasks, was found at a lower abundance compared to ASV6. In the 1 wph samples from GF-S1, ASV29 accounted for 2%(F1) and 3%(F2) of the relative abundance in the fish samples, and 1% in the water sample. In the GF-SF1 rearing flask, ASV29 was present in one fish sample (F1) at a relative abundance of 2%. However, ASV29 was not observed in any of the 2 wph samples according to the normalized ASV table.

Interestingly, ASV29 was also detected in another rearing flask, specifically the same water sample as the ASV6 was present in (YW-S1 0 dph W), with a relative abundance of 9%.

On the other hand, ASV2 (*Undibacterium*), detected in the sterility control of the

GF-SF2 rearing flask, was present in all the samples collected from this rearing flask. At 1 wph, ASV2 exhibited a relative abundance of 8%(F1) and 5%(F2) in the fish samples and 30% in the water sample. Similarly, at 2 wph, ASV2 has relative abundances of 12%(F1) and 11%(F2) in the fish samples and 22% in the water sample.

Remarkably, ASV2 was also observed at a high relative abundance in several other rearing flasks throughout the experiment. Additionally, it was detected in the mucus sample used for egg colonization, with a relative abundance of 34% (sample M1).

ASV8 (*Undibacterium*), also detected in the sterility control of GF-SF2, showed a similar pattern to ASV2 but to a lesser extent. At 1 wph, ASV8 was found in one fish sample with relative abundance of 3% in the GF-SF rearing flask, while at 2 wph, it exhibited relative abundances of 10% in both fish samples and 17% in the water sample. ASV8 was only observed in one of the replicate flasks, specifically GF-SF1, where it accounted for 4% of the relative abundance in a fish sample from 1 wph.

Interestingly, ASV8 was found in all the samples where ASV2 was detected, except for the samples from GF-S2, where ASV8 was detected but ASV2 was not.

To summarize, contaminants ASV6 and ASV29 (*Flavobacterium*) were exclusively detected in their respective GF-S1 and GF-SF1 rearing flasks, primarily in fish and water samples at 1 wph, with minimal occurrence in replicate flasks. They were also found in a limited number of samples from other treatments groups. Conversely, ASV2 and ASV8 contaminants were present in both the 1 and 2 wph samples from the respective GF-SF2 rearing flask, with limited occurrence in the replicate flasks of the GF-SF group. However, these ASVs exhibited a high prevalence in the other treatment groups (M and YW), as well as in the skin mucus sample that was used for egg colonization. The presence of these ASVs in the GF flasks could indicate cross-contamination from other treatments groups where the same ASVs were present. Alternatively, it may indicate an unsuccessful disinfection procedure, where the bacterial growth went unnoticed during the first sterility control.

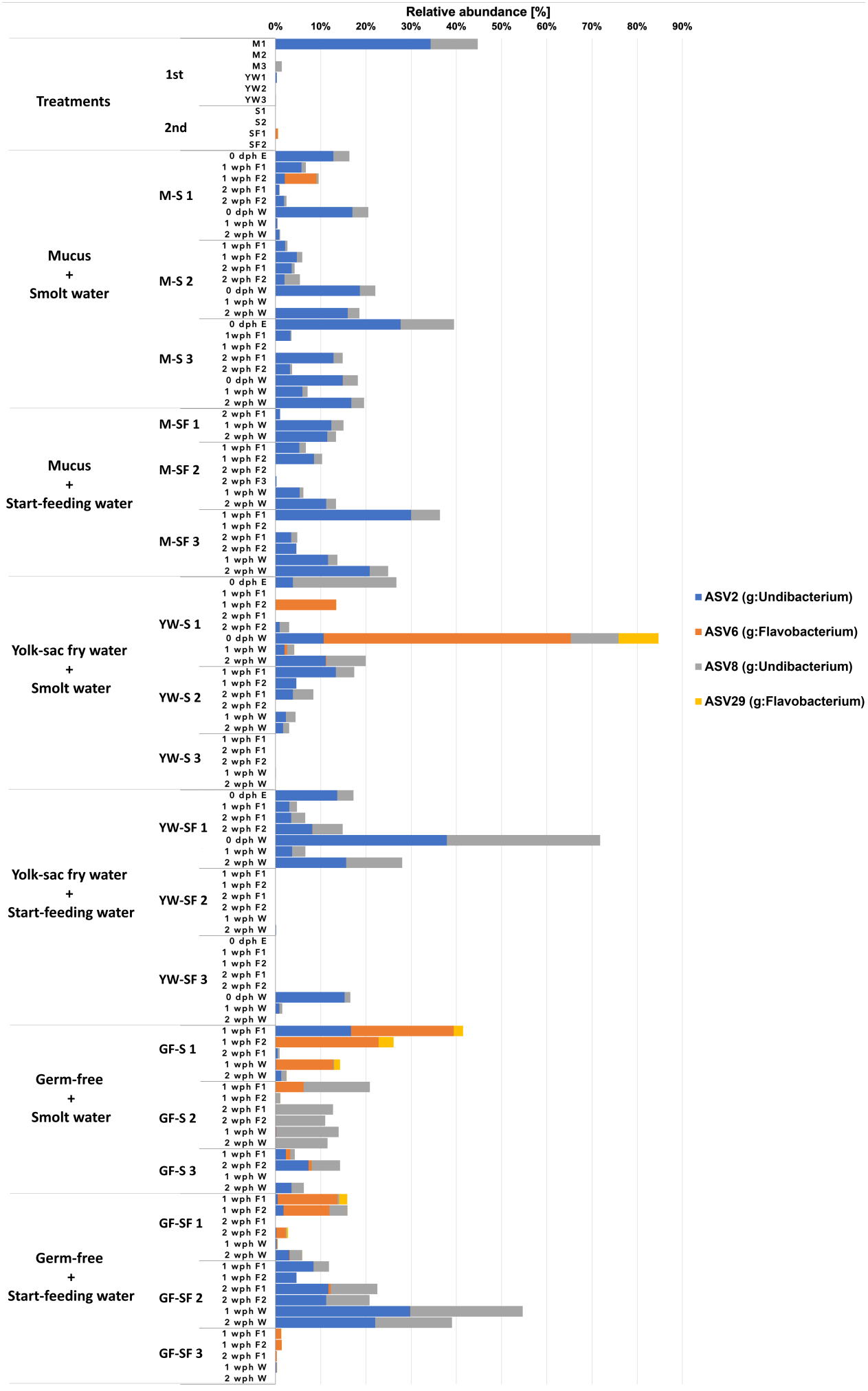

Relative abundance [%]
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
Treatments
1st
2nd
M1
M2
M3
YW1
YW2
YW3
S1
S2
SF1
SF2
Mucus + Smolt water
M-S 1
M-S 2
M-S 3
0 dph E
1 wph F1
1 wph F2
2 wph F1
2 wph F2
0 dph W
1 wph W
2 wph W
Mucus + Start-feeding water
M-SF 1
M-SF 2
M-SF 3
2 wph F3
Yolk-sac fry water + Smolt water
YW-S 1
YW-S 2
YW-S 3
Yolk-sac fry water + Start-feeding water
YW-SF 1
YW-SF 2
YW-SF 3
Germ-free + Smolt water
GF-S 1
GF-S 2
GF-S 3
Germ-free + Start-feeding water
GF-SF 1
GF-SF 2
GF-SF 3
ASV2 (g:Undibacterium)
ASV6 (g:Flavobacterium)
ASV8 (g:Undibacterium)
ASV29 (g:Flavobacterium)

Figure 3.1.1: Relative abundance of the most dominant ASVs in the contaminated sterility controls across the samples in the Atlantic salmon experiment. The four most dominant ASVs in the contaminated sterility controls were taxonomically assigned to the *Flavobacterium* (ASV6 and ASV29) and *Undibacterium* (ASV2 and ASV8) genera. The experiment consisted of different treatment groups: M-S, M-SF, YW-S, YW-SF, GF-S, and GF-SF, with sampling points at 0 dph, 1 wph, and 2 wph. The sample types are defined as eggs (E), Fish (F, 1-2 replicates), and water (W).

3.1.3 Comparison of Microbial Communities of the Microbial Treatments

The microbial communities of the samples that represented the microbial treatments were characterized using Illumina sequencing of the 16S rDNA amplicons. The microbial communities were analyzed at both Family and ASV levels (Figure 3.1.2). For the first set of treatments (M and YW), the microbial communities exhibited a high relative abundance of *Pseudomonadaceae* at the Family level (Figure 3.1.2 a). However, a notable difference was observed in the first replicate of the M treatment sample (M1) compared to the other M replicates (M2 and M3). In M1, *Pseudomonadaceae* was absent, while high relative abundances of *Flavobacteruaccae* (51%) and *Oxalobacteraccae*(45%) were observed. Within the *Flavobacteruaccae* family, one single ASV (ASV4) dominated, while the *Oxalobacteraccae* family was represented by two ASVs (ASV2 and ASV8). In contrast, the microbiota of the replicate samples M2 and M3 displayed a different wider range of ASVs but shared a high relative abundance of the *Oxalobacteraccae* family.

Considering the different community profile of the M1 replicate compared to M2 and M3, there was consideration to exclude it from the analysis due to the possibility of a sample error. However, since the microbial community of the M1 replicate resembled the microbiota of certain fish and rearing water samples, particularly the egg sample from the M-S1 rearing flask (Sample: 0 dph E, in ASV plot in Appendix G), the decision was made to retain M1 in the analysis.

Furthermore, differences were observed between the M2 and M3 replicates. At the Family level, M2 showed a higher relative abundance of *Sphingobacteriaceae*, while M3 displayed a higher abundance of *Arcobacteraceae*. At ASV level, both samples contained several ASVs within each family, but M2 exhibited a larger relative abundance of ASV56 (23%, *Pedobacter*), which was not abundant in any of the other replicate samples or treatment groups. Thus, despite originating from the same homogenized mucus sample, the three mucus replicate samples (M1-3) showed considerable differences.

Moving on to the samples of the added yolk-sac fry rearing water (YW), all three replicate samples (YW1-3) were dominated by the *Pseudomonadaceae* family, with the highest relative abundance observed in YW3 (73%). Within the *Pseudomonadaceae* family, ASV31, ASV17, and ASV22 (all *Pseudonomas*) had

the highest relative abundances. Interestingly, despite originating from the same water sample, the three YW replicates exhibit several different ASVs. Additionally, it is noteworthy that the M2 replicate sample exhibited a similar family-level composition to the YW1 replicate, but they displayed greater dissimilarity at the ASV level.

In contrast to the first microbial treatments, the second set of water treatments (S and SF) exhibited different ASV profiles (Figure 3.1.3 b). Within the water samples from the RAS of smolt production (S1 and S2), a high abundance of *Bacteriovoracaceae* family was observed, with ASV54 (*Bacteriovorax*) being the dominant member. Additionally, the large unassigned family group was dominated by ASV54 (*Parcubacteria genera incertae sedis*).

Similarly, the samples of the water from the start-feeding RAS (SF1 and SF2) demonstrated high relative abundances for ASV61 (*Parcubacteria genera incertae sedis*) which remains unclassified at the family level. The rest of the ASVs representing the replicate samples exhibited similar relative abundances.

Notably, ASV61 (*Parcubacteria genera incertae sedis*) was the only ASV with a relative abundance above 1% that was shared between the two treatment groups (S1: 3%, S2: 2%, SF1: 14%, SF2: 23%). Thus, the two microbial treatments exhibited large differences at the ASV level.

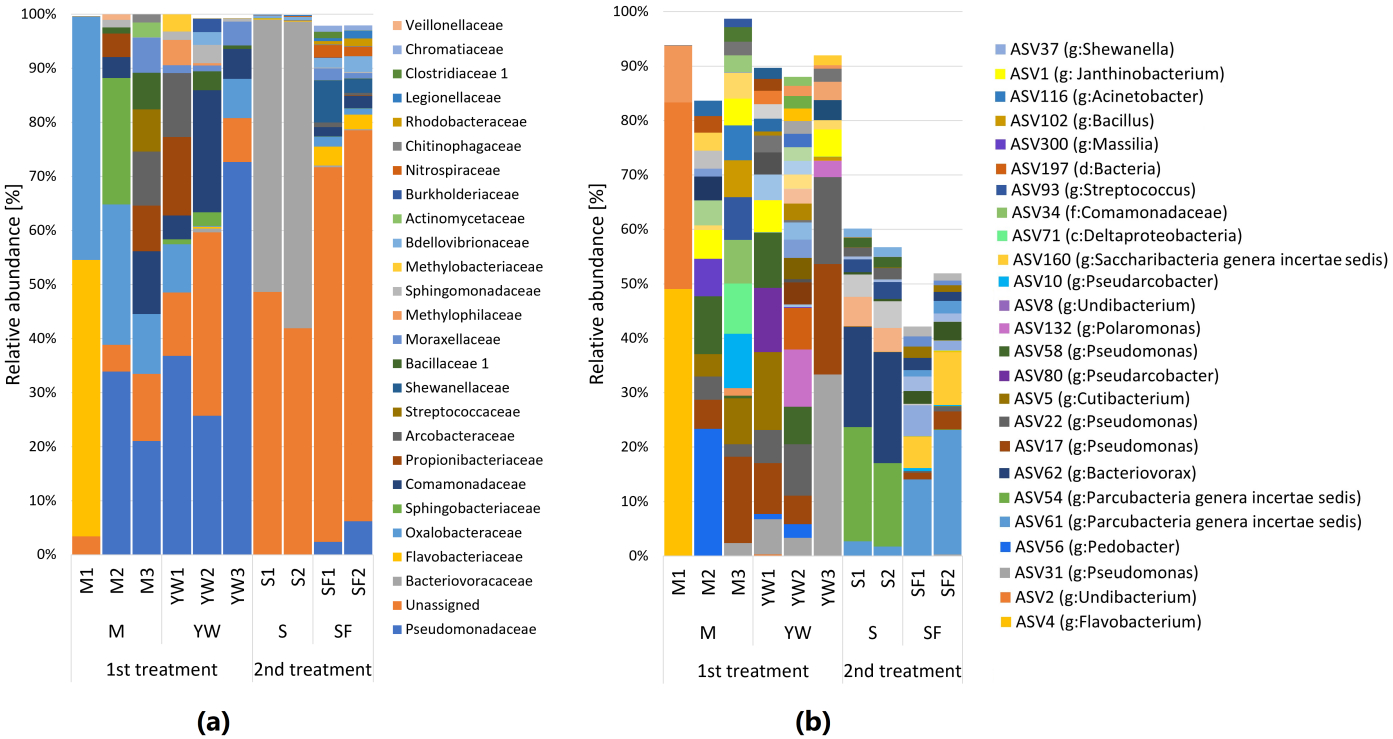


Figure 3.1.2: Microbial community composition at a) Family level and b) ASV level of the samples representing the four microbial treatments used in the Atlantic salmon experiment. The first set of treatments includes skin mucus (M) and yolk-sac fry rearing water (YW) samples, with three replicates representing each group. The second set of treatments includes water from a RAS for smolt production (S) and a RAS for start-feeding of Atlantic salmon fry (SF) samples, with two replicates representing each group. The taxonomy of the ASVs is presented in parentheses at the lowest taxonomic level obtained. Only families and ASVs with maximum abundances above 1% in at least one sample are included in the figure. Additionally, ASVs with abundances below 10% in maximum abundance in all samples are not included in the description of the ASV bar graph.

To further explore the microbial communities between the treatments, a PCoA based on Bray-Curtis similarities was performed (Figure 3.1.3).

The PCoA revealed a clear distinction between the microbial communities of the first treatments (M and YW) and the second treatments (S and SF). The M and YW samples exhibited similar microbial communities, with the exception of the M1 replicate sample. The S and SF water samples exhibited similar microbial communities between the replicate samples, but different microbial communities between the two treatments.

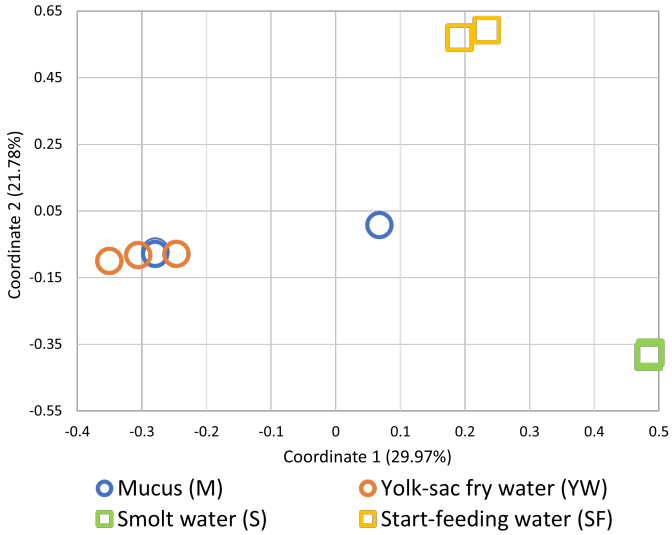


Figure 3.1.3: PCoA plot based on Bray-Curtis similarities showing the clustering of the samples from the four microbial treatments based on their microbial community composition. The plot is based on the relative abundance of bacterial ASVs in the samples of added mucus (M), yolk-sac fry water (YW), smolt water (S), and start-feeding water (SF). Each point represents a sample, and the distance between points indicates the degree of similarity in bacterial community composition between samples.

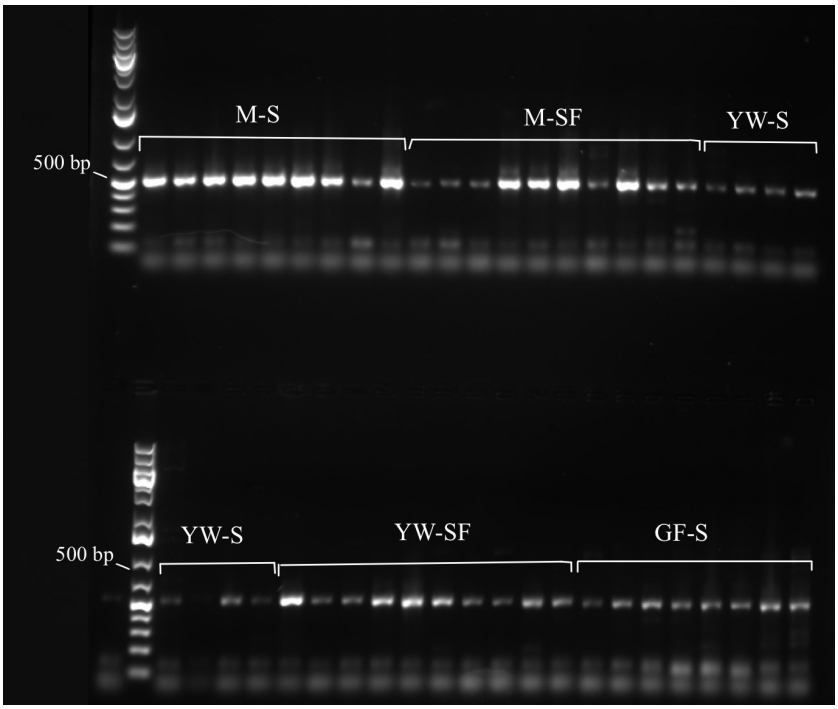
In summary, the analysis of the microbial treatments revealed that two of the replicate samples of the skin mucus (M) were highly similar to the replicate samples of the yolk-sac fry rearing water (YW) treatment, making it challenging to conclusively determine if the M samples truly represented the added skin mucus sample. Furthermore, the second set of treatments (S and SF) demonstrated distinct microbial communities, with only one abundant ASV shared between these two treatments (with a maximum relative abundance of more than 1% in at least one sample).

3.2 Microbial Community Analysis of Fish and Rearing Water Samples

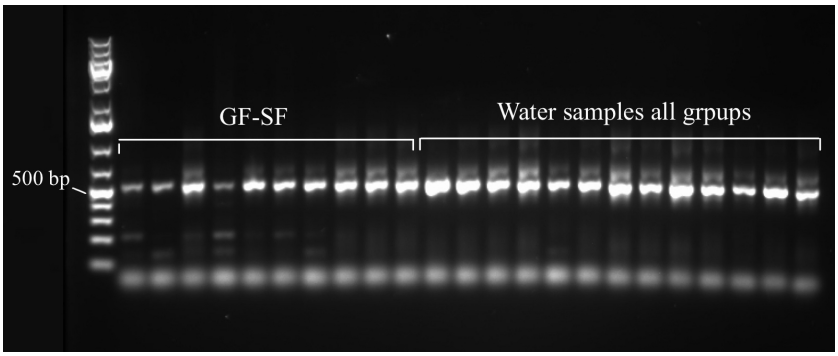
The composition of the microbial communities in the Atlantic salmon eggs, yolk-sac fry, and rearing water samples was examined using Illumina sequencing of the 16S rDNA amplicons. A total of 5 egg samples, 63 fish samples, and 42 rearing water samples were characterized. Additionally, 9 samples of the added microbial sources used as treatments, and 3 samples of bacterial cultures of the contaminated rearing flasks were characterized as previously described in Section 3.1.3.

3.2.1 PCR amplification of the V3-V4 region of the bacterial 16S rRNA gene

During the preparation of the Illumina library for sequencing, noticeable differences were observed when running PCR products on agarose gel electrophoresis. Specifically, samples that received skin mucus as their initial microbial treatment displayed a greater abundance of PCR product compared to the samples that received yolk-sac fry rearing water as their initial treatment (Figure 3.2.1). This observation suggests a potentially higher bacterial load in the added skin mucus samples. Similarly, the samples from the GF-SF treatment group exhibited a higher abundance of PCR product compared to the samples of the GF-S treatment group, indicating a higher bacterial load in the water from the start-feeding RAS compared to the water from the RAS for smolt production. Additionally, the rearing water samples yielded the highest abundance of PCR product among all samples, suggesting a substantial presence of bacteria in the rearing water.



(a)



(b)

Figure 3.2.1: Examples of agarose gel electrophoresis of the 16S rDNA PCR products for samples of fish and rearing water at 1 wph. a) PCR products of fish samples from treatment groups: M-S, M-SF, YW-S, YW-SF, and GF-S. b) PCR products of the fish samples from the GF-SF treatment group and the water samples from all six treatment groups. The expected band length was 540 nt.

3.2.2 Alpha Diversity of the Fish and Water Microbiota

In total 6,399 ASVs were identified in the ASV table that had been normalized to 26,000 reads per sample. All further analyses were based on this normalized ASV table.

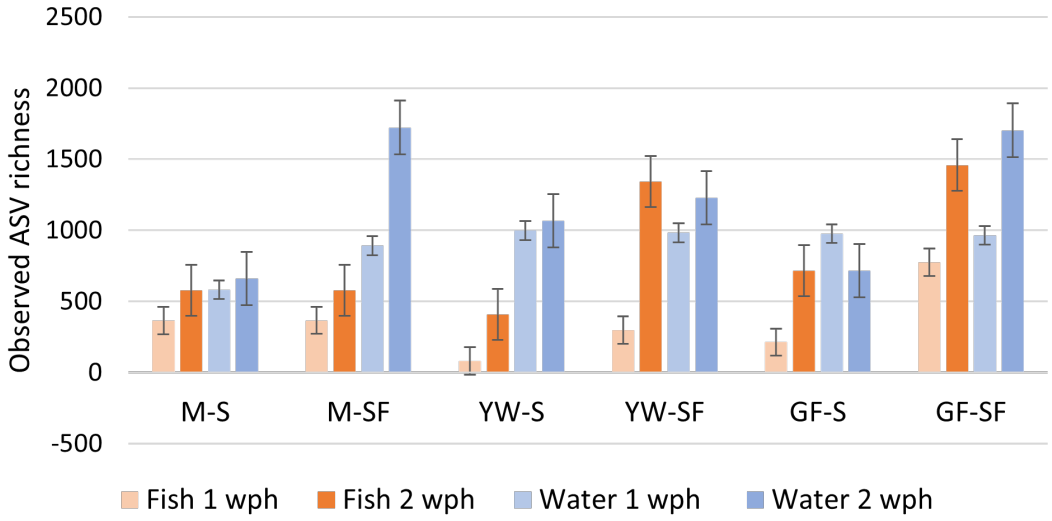
Overall, the rearing water samples showed a higher observed ASV richness and Shannon's diversity index compared to the fish samples (Figure 3.2.2). Notably, the M-SF treatment group at 2 wph exhibited significant differences in ASV richness and Shannon's diversity index between water and fish samples (Wilcoxon

Rank Sum Test without multiple testing correction, $p = 0.036$ and $p = 0.038$, respectively). However, no significant differences were observed between the other treatment groups (Wilcoxon Rank Sum Test without multiple testing correction, $p > 0.05$).

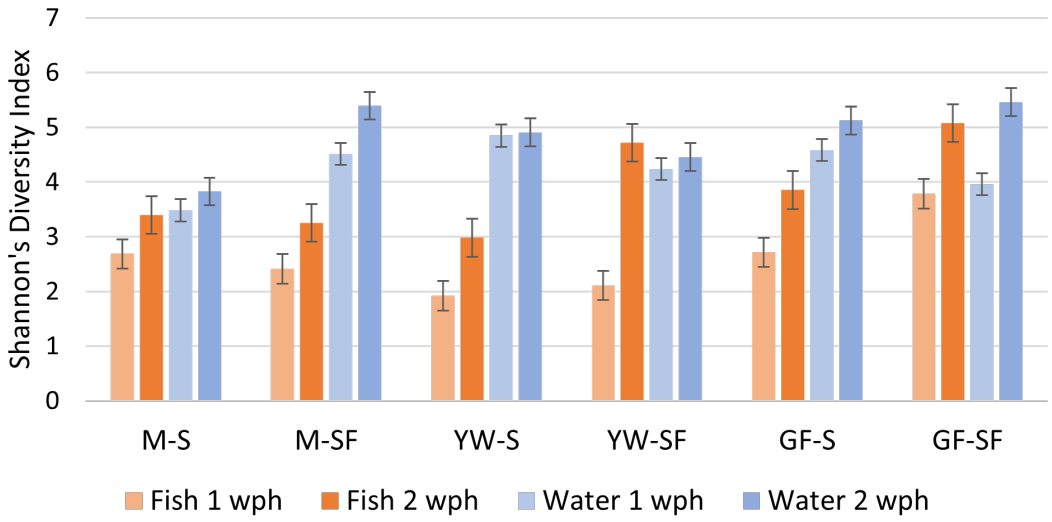
When comparing the treatment groups in terms of their first microbial treatments on the eggs (M, YW, and GF), a significant difference was observed between the YW and the GF groups at 1 wph, in terms of ASV richness and Shannons diversity index (Wilcoxon Rank Sum Test without multiple testing correction, $p = 0.02$ and $p = 0.00015$, respectively). However, these differences were not significant at 2 wph (Wilcoxon Rank Sum Test without multiple testing correction, $p > 0.5$). Furthermore, no significant differences were observed between the M and GF groups or the M and YW groups (Wilcoxon Rank Sum Test without multiple testing correction, $p > 0.05$).

When analyzing the treatment groups in terms of the microbial treatments at 0 dph (S and SF), the SF treatment groups (M-SF, YW-SF, and GF-SF) showed higher observed ASV richness and Shannon's diversity index for both fish and water samples compared to the S groups (M-S, YW-SF, GF-SF), suggesting that the start-feeding water treatment contributed to the highest alpha diversity. Notably, the difference was only significant for the YW group (YW-S vs. YW-SF) at 2 wph (Wilcoxon Rank Sum Test without multiple testing correction, $p = 0.015$).

In summary, the water samples exhibited higher ASV richness and diversity compared to the fish samples. While in terms of the first microbial treatment, YW and GF groups revealed significant differences at 1 wph and not 2 wph, indicating that the richness and diversity converged over time. Lastly, the start-feeding water added at 0 dph contributed to a higher alpha diversity in comparison to the smolt water treatment at 0 dph, however, it was only significant for the YW treatment group.



(a)



(b)

Figure 3.2.2: Average observed ASV richness and Shannon's diversity index for fish and water samples from the different treatment groups in the Atlantic salmon experiment. a) Observed ASV richness. b) Shannon's diversity index. The alpha diversity indices were calculated based on the normalized ASV table. The average values (±SE) are based on twelve fish samples and six water samples for each group. The error bars represent the standard error of the mean.

3.3 Community Composition of the Microbial Communities in the Atlantic Salmon Experiment

To get an overview of the microbial communities in the samples, the composition at the family level was analyzed (Figure 3.3.1). Certain families such as *Oxalobac-*

teraceae and *Flavobacteriaceae* were consistently abundant across all treatment groups and sample types. However, the family *Commomonadaceae* was more prevalent in the treatment groups that were exposed to skin mucus (M), while *Pseudomonadaceae* was more common in the treatment groups that were exposed to yolk-sac fry rearing water (YW) during the egg stage, as well as in the group that did not receive any treatment at the egg phase (GF).

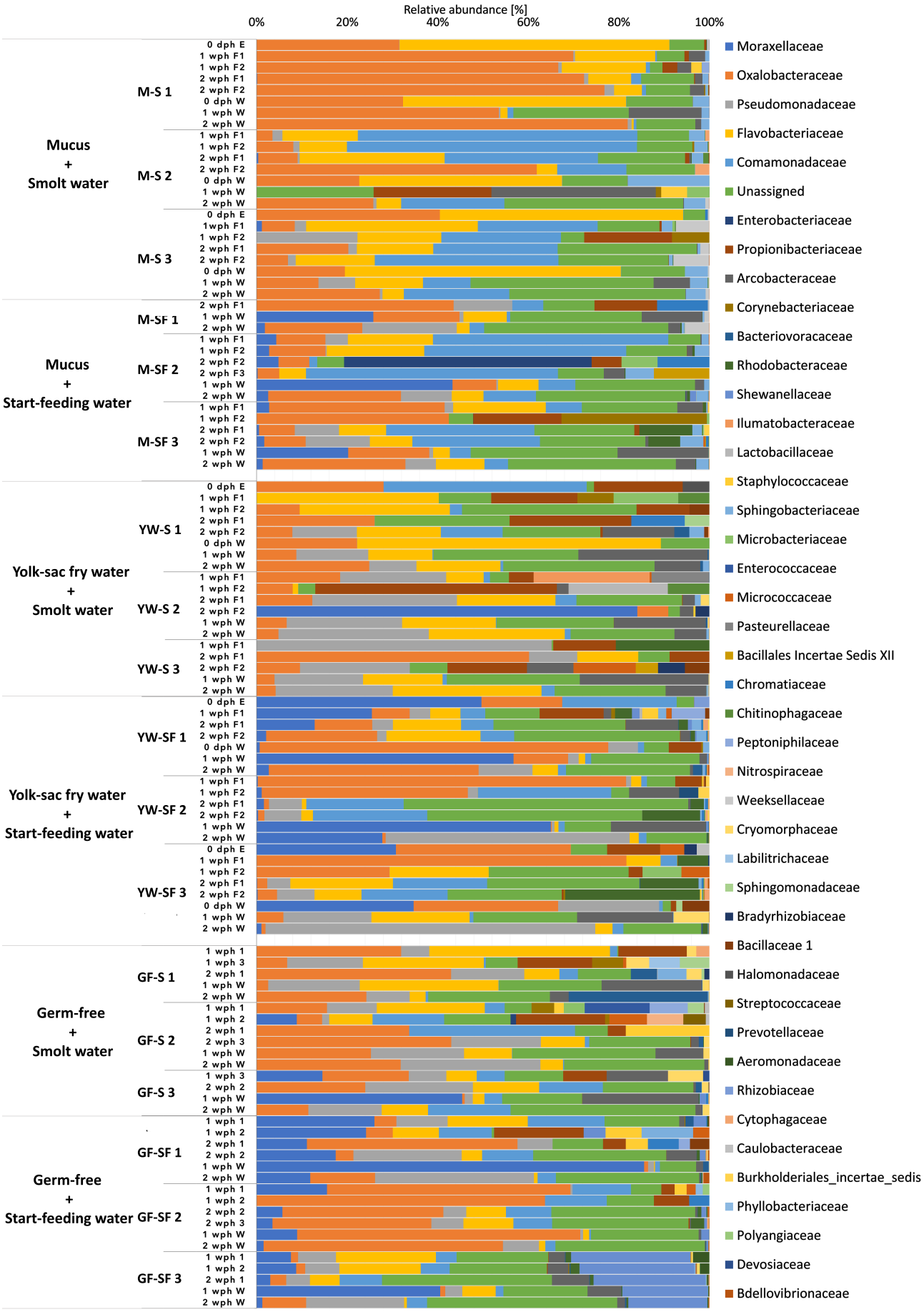

Relative abundance [%]
0%
20%
40%
60%
80%
100%
Mucus + Smolt water
M-S 1
M-S 2
M-S 3
Mucus + Start-feeding water
M-SF 1
M-SF 2
M-SF 3
Yolk-sac fry water + Smolt water
YW-S 1
YW-S 2
YW-S 3
Yolk-sac fry water + Start-feeding water
YW-SF 1
YW-SF 2
YW-SF 3
Germ-free + Smolt water
GF-S 1
GF-S 2
GF-S 3
Germ-free + Start-feeding water
GF-SF 1
GF-SF 2
GF-SF 3
Moraxellaceae
Oxalobacteraceae
Pseudomonadaceae
Flavobacteriaceae
Comamonadaceae
Unassigned
Enterobacteriaceae
Propionibacteriaceae
Arcobacteraceae
Corynebacteriaceae
Bacteriovoracaceae
Rhodobacteraceae
Shewanellaceae
Ilumatobacteraceae
Lactobacillaceae
Staphylococcaceae
Sphingobacteriaceae
Microbacteriaceae
Enterococcaceae
Micrococcaceae
Pasteurellaceae
Bacillales Incertae Sedis XII
Chromatiaceae
Chitinophagaceae
Peptoniphilaceae
Nitrospiraceae
Weeksellaceae
Cryomorphaceae
Labilitrichaceae
Sphingomonadaceae
Bradyrhizobiaceae
Bacillaceae 1
Halomonadaceae
Streptococcaceae
Prevotellaceae
Aeromonadaceae
Rhizobiaceae
Cytophagaceae
Caulobacteraceae
Burkholderiales_incertae_sedis
Phyllobacteriaceae
Polyangiaceae
Devosiaceae
Bdellovibrionaceae

Figure 3.3.1: Composition of the microbial communities at the Family level for the samples collected in the Atlantic salmon experiment. The experiment consisted of six different treatment groups: M-S, M-SF, YW-S, YW-SF, GF-S, and GF-SF, with sampling points at 0 dph, 1 wph, and 2 wph. The sample types are defined as egg (E), fish (F, 1-2 replicates), and water (W). Families with relative abundance below 1% in all samples are not included in the figure.

3.3.1 The Effect of Initial Microbial Treatments on Fish and Rearing Water Microbiota

To investigate the impact of the initial egg treatment with skin mucus and yolk-sac fry rearing water on the microbiota of fish and rearing water, a PCoA based on Bray-Curtis similarities was conducted (Figure 3.3.2). The PCoA revealed differences in the microbial communities between the treatment groups. Specifically, samples from the M treatment groups exhibited a different microbial community compared to the YW and GF treatment groups, while the microbial communities of the YW and the GF treatment groups appeared more similar to each other. A one-way PERMANOA confirmed that there was a significant difference between the fish microbiota of the M treatment group and the combined YW and GF treatment groups for both 1 and 2 wph ($p = 0.0001$). These findings indicate that the treatment with skin mucus on the eggs prior to hatching contributed to a significantly different microbiota on the newly hatched yolk-sac fry in comparison to the other treatments (yolk-sac fry rearing water and germ-free conditions). Furthermore, a separate one-way PERMANOVA comparing samples from the YW and GF treatment groups showed a significant difference in their microbial communities, however at a higher p-value ($p = 0.0211$). These findings indicate that both the skin mucus treatment and the yolk-sac fry rearing water treatment influenced the microbiota of the newly hatched fry, but that the skin mucus treatment influenced it the most.

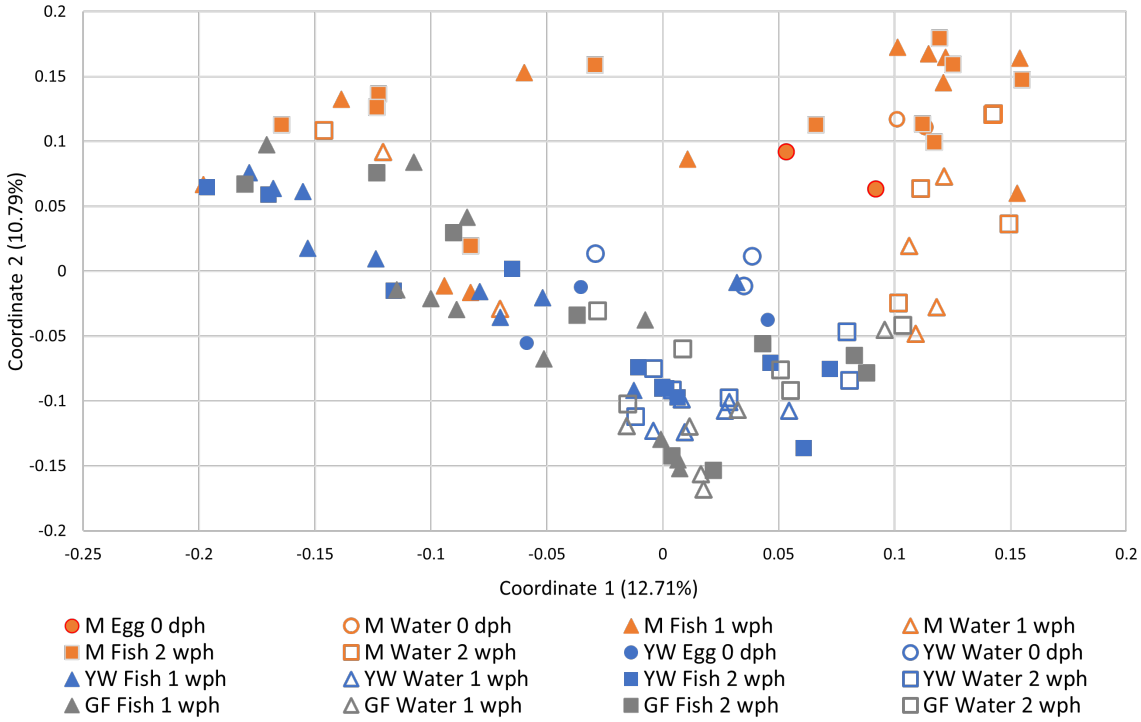


Figure 3.3.2: PCoA plot based on Bray-Curtis similarities for comparison of the microbial community profiles of the fish and rearing water samples from the three initial treatment groups: M, YW, and GF. All sample types and time points from each group are represented, also the ones after the second treatment groups (S and SF). Each point represents a sample, and the distance between points indicates the degree of similarity in bacterial community composition between samples.

To further explore the dissimilarities in the fish and water microbiota among and within the initial treatment groups, the average Bray-Curtis similarity was calculated (Figure 3.3.3). The largest difference was observed between the M and YW treatment groups, however, the differences were not significant (Wilcoxon Rank Sum Test without multiple testing correction, $p = 0.25$).

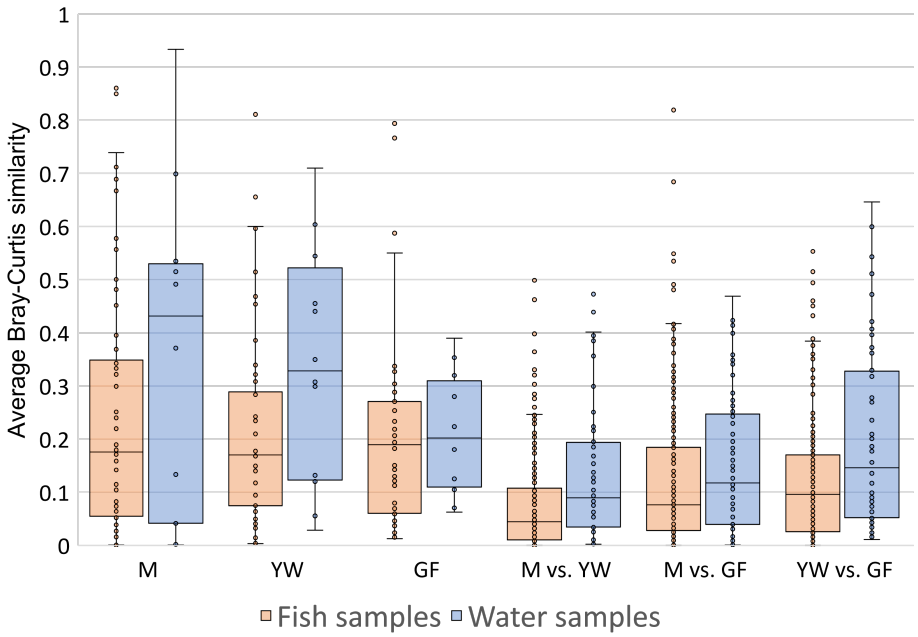


Figure 3.3.3: Average Bray-Curtis similarities (±SE) for comparison of water and fish microbiota within and between the three initial treatment groups: M, YW, and GF. The groups include both water and fish samples collected at 1 and 2 wph. The line across each box represents the average value. The standard error (±SE) is indicated by the error bars.

A SIMPER analysis based on Bray-Curtis similarities was conducted for further comparison of the fish and water samples to identify the ASVs that contributed the most to the differences in the microbiota between the three initial treatment groups (M, YW, and GF). ASV3, (*Polaromonas*) and ASV1, (*Janthinobacterium*) were found to contribute the most to the differences between the M and YW treatment groups (Table 3.3.1). ASV3 was predominantly present in the M samples (average relative abundance of 14.12%), while ASV1 was predominantly present in the YW samples (average relative abundance of 10.12%). This trend is also evident in the variations observed when comparing the M and YW groups to the GF groups (Table 3.3.1b and 3.3.1c). ASV3 accounted for 7.6 % of the variations in the M and GF samples, while ASV1 contributed to 7.06 % of the variation in the YW and GF samples. Furthermore, ASV1 (*Janthinobacterium*) was present in all three groups, but at varying abundances, whereas ASV3 (*Polaromonas*) was predominantly present only in the M samples.

Furthermore, an ASV representing *Flavobacterium* (ASV4), was also among the ASVs contributing to the most differences in the microbial communities between these treatment groups. ASV4 was more abundant in the M samples (4.12% relative abundance) compared to the YW and GF samples (0% and 0.16% relative abundance, respectively).

Table 3.3.1: The ASVs contributing to the most difference between the microbial communities of the M, YW, and GF treatment groups, identified by SIMPER analysis based on Bray-Curtis dissimilarity. a) Comparison of water and fish samples between the M and YW groups. b) Comparison of water and fish samples between the M and GF groups. c) Comparison of water and fish samples between the YW and GF groups. All water and fish samples from 1 and 2 wph were included in the analysis. The ASVs contribution and relative mean abundances are given with the taxonomy specified at the genus level (g) which was the lowest taxonomic level obtained.

(a)

ASV	Taxonomy	Contrib. [%]	Relative abundance M [%]	Relative abundance YW [%]
ASV3	g:*Polaromonas*	7.55	14.12	0.03
ASV1	g:*Janthinobacterium*	7.47	7.81	10.12
ASV5	g:*Cutibacterium*	3.77	3.92	4.58
ASV7	g:*Janthinobacterium*	3.72	6.85	0.28
ASV2	g:*Undibacterium*	3.19	5.95	2.20
ASV4	g:*Flavobacterium*	2.19	4.12	0.00

(b)

ASV	Taxonomy	Contrib. [%]	Relative abundance M [%]	Relative abundance GF [%]
ASV3	g:*Polaromonas*	7.06	14.12	0.00
ASV1	g:*Janthinobacterium*	5.16	6.81	6.58
ASV7	g:*Janthinobacterium*	3.86	6.85	1.72
ASV2	g:*Undibacterium*	3.47	5.92	3.96
ASV5	g:*Cutibacterium*	2.86	3.92	2.99
ASV8	g:*Undibacterium*	2.32	1.22	4.62
ASV4	g:*Flavobacterium*	2.24	4.12	0.16

(c)

ASV	Taxonomy	Contrib. [%]	Relative abundance YW [%]	Relative abundance GF [%]
ASV1	g:*Janthinobacterium*	7.60	10.12	6.58
ASV5	g:*Cutibacterium*	3.42	4.58	2.99
ASV8	g:*Undibacterium*	2.70	1.53	4.62
ASV9	g:*Acinetobacter*	2.69	1.99	3.68
ASV2	g:*Undibacterium*	2.69	2.20	3.96
ASV10	g:*Pseudarcobacter*	2.22	3.20	2.18
ASV6	g:*Flavobacterium*	1.78	0.42	2.98

In summary, both of the initial treatments of the salmon eggs led to significant variations in the microbiota on the yolk-sac fry after hatching. Specifically, the M treatment groups exhibited the most distinct microbial community compared to the other treatment groups (GF and YW), with ASV3 (*Polaromonas*) and ASV4 (*Flavobacterium*) contributing the most to the group's distinct composition.

3.3.2 Impact of the Microbial Treatment on the Day of Hatching

In order to assess the influence of the microbial treatment conducted on the day of hatching (0 dph) on both the fish and water microbiota, all samples (1 wph and 2 wph) from the S group (M-S, YW-S, and GF-S) and the SF group (M-SF, YW-SF, and GF-SF) were compared in a PCoA plot (Figure 3.3.4a). The PCoA plot did not reveal a clear effect of the two water treatments on the fish and water microbiota, as they did not form distinct clusters. The PCoA further indicated that the water microbiota for both treatment groups (S and SF) had similar microbial communities. However, a one-way PERMANOVA test conducted for all S samples and SF samples demonstrated a significant variation between the groups ($p = 0.0001$). Furthermore, when assessing each time point separately, one-way PERMANOVA indicated significant variation between the groups at each time group ($p = 0.0008$ at 1 wph, and $p = 0.0001$ at 2 wph).

To further investigate the dissimilarities within the S and SF treatment groups, PCoA plots were conducted for each of the three initial treatment groups to examine the effect of the second microbial treatment separately (Figure 3.3.4 b, c, and d). The PCoA revealed no distinct pattern between the M-S and M-SF treatment groups (Figure 3.3.4b), and no significant differences were found between the microbiota of the fish and water samples from the groups at 1 wph or 2 wph (PERMANOVA, $p = 0.1384$ and $p = 0.0833$, respectively). However, significant differences were found in the microbiota of fish and water samples between the YW-S and the YW-SF groups at 2 wph (PERMANOVA, $p = 0.0002$), and between the GF-S and GF-SF fish and water samples at 2 pwh (PERMANOVA, $p = 0.0006$). These results indicate that the microbial treatment with smolt and start-feeding water at 0 dph had a significant effect on the microbiota of the YW and GF groups, but not the M groups.

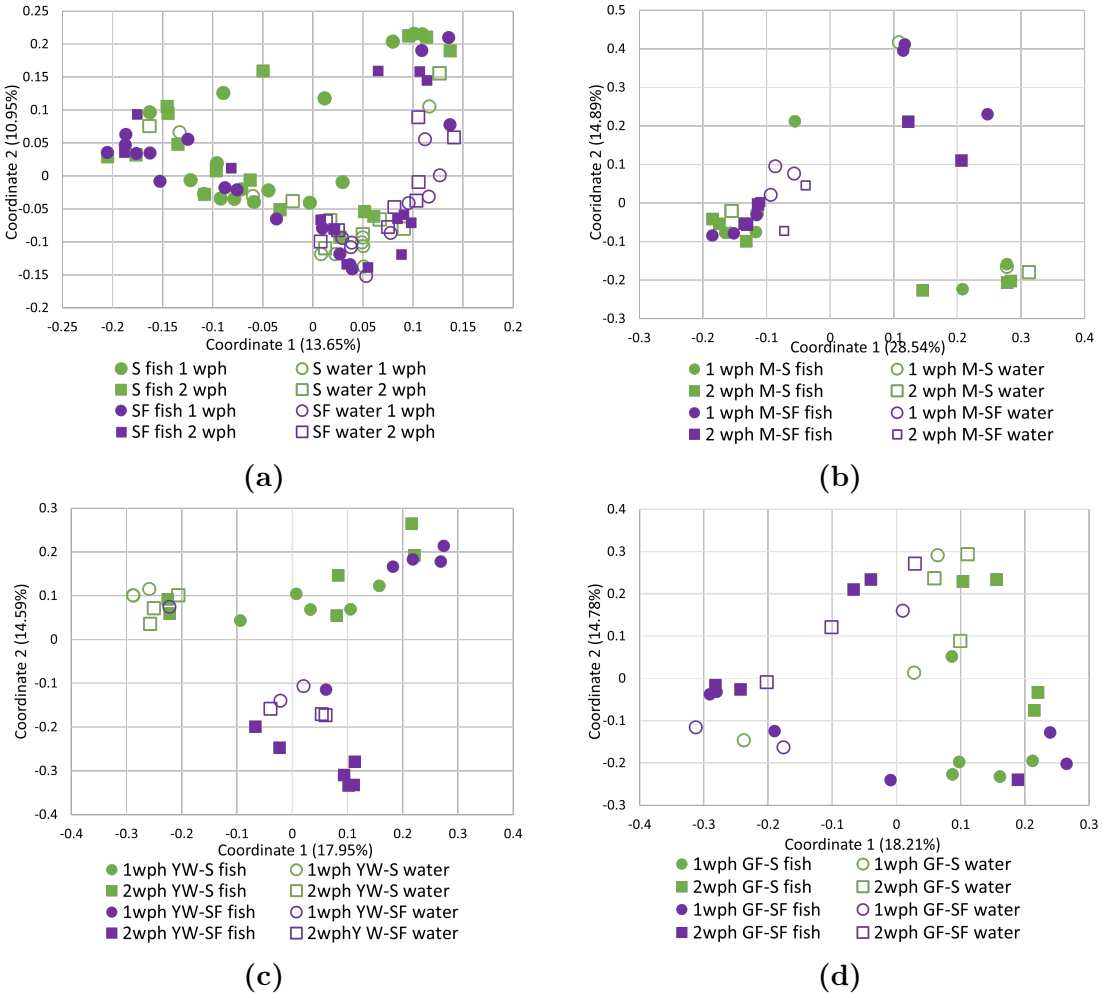


Figure 3.3.4: PCoA plots based on Bray-Curtis similarities for comparison of the microbial community profiles of the fish and rearing water samples in the S and SF treatment groups a) All samples labeled in respect of the S and SF treatments. b) M-S and M-SF treatment groups. c) YW-S and YW-SF treatment groups. d) GF-S and GF-SF treatment groups. All the plots include fish and water samples from 1 and 2 wph. Each point represents a sample, and the distance between points indicates the degree of similarity in bacterial community composition between samples.

To further analyze the impact of the microbial treatment at 0 dph, Bray-Curtis similarities were calculated for comparison of microbial communities within each of the six treatment groups and between the S and SF treatment groups (Figrue 3.3.5).

In general, both the water and fish microbiota varied among samples within the experimental groups, despite exposure to the same microbial treatments. However, the fish samples of the M groups (M-S and M-SF) revealed the most similar microbial communities (PERMANOVA, $p = 0.17$, at 2 wph) while the YW (YW-S vs. YW-SF) and GF (GF-S vs. GF-SF) groups revealed significantly different microbial communities between their S and SF fish samples (PERMANOVA, $p = 0.00021$ and $p = 0.062$, at 2 wph, respectively). These results further confirm that the fish and water microbiota in the YW and GF

groups were significantly affected by the second microbial treatment and that the M groups were not significantly affected. Additionally, the YW groups were more affected by the second microbial treatment than the GF groups were.

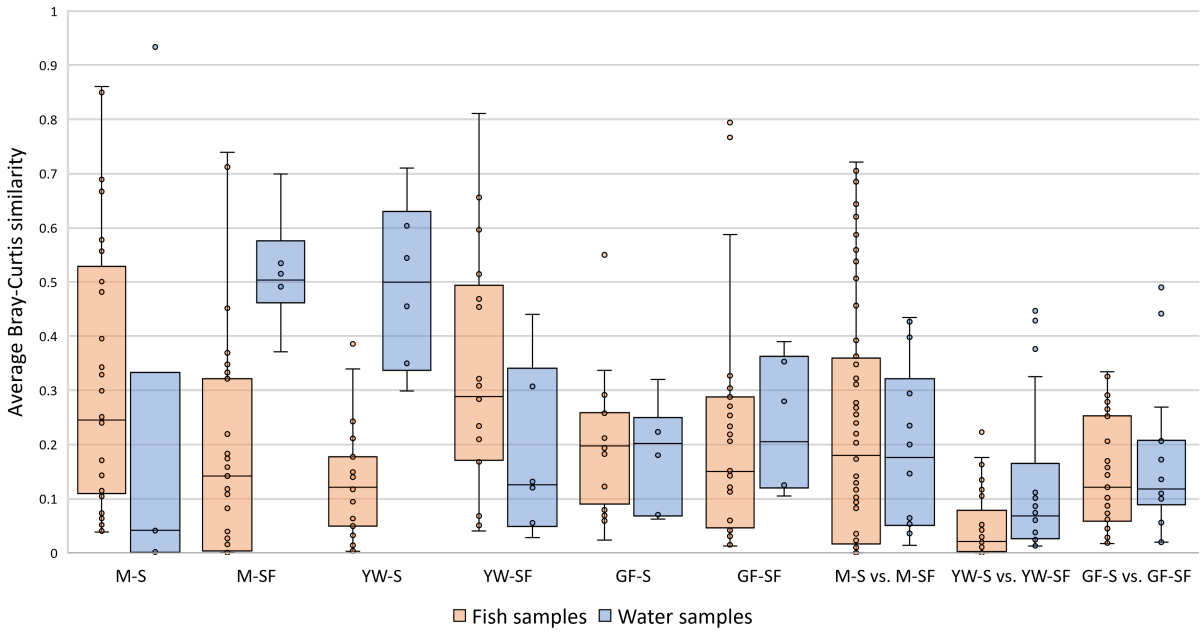


Figure 3.3.5: Average Bray-Curtis similarities (±SE) within the six treatment groups, and between the S and SF treatment groups. The groups represent water and fish samples collected at 1 and 2 wph. The line across each box represents the average value. The standard error (±SE) is indicated by the error bars.

SIMPER analysis based on Bray-Curtis dissimilarity was conducted for further comparison of the two microbial treatment groups (S and SF) from 0 dph (Table 3.3.2). ASV3 (*Polaromonas*) contributed the most to the difference between the M-s and M-SF groups (11.28%), while ASV1 (*Janthinobacterium*) contributed the most to the difference between the S and SF treatments in the YW and GF groups (9.18% and 5.79%, respectively). Notably, none of these ASVs were exclusively present in either of the groups.

ASV14 (*Bacteroidetes*) was exclusively present in the YW-S group (5.65% relative abundance) and absent in the YW-SF group (Table 3.3.2b). This suggests that ASV14 was introduced by the smolt water treatment. Similarly, ASV9 (*Acinctobacter*) was present in the samples of the YW-SF group (3.96% relative abundance) but not in the YW-S group. However, this ASV also contributed to differences between the GF-S and GF-SF groups and was present in the GF-S group at a relative abundance of 1.83%. Consequently, this observation makes it difficult to determine if ASV9 was introduced solely by the start-feeding water.

Table 3.3.2: The ASVs contributing to the most difference between the microbial communities of the S and SF treatment groups, identified by SIMPER analysis based on Bray-Curtis dissimilarity. a) Comparison of water and fish samples between the M-S and M-SF groups. b) Comparison of water and fish samples between the YW-S and YW-SF groups. c) Comparison of water and fish samples between the GF-S and GF-SF groups. All samples from 1 and 2 wph were included in the analysis. The ASVs contribution and relative mean abundances are given with the taxonomy specified at the lowest taxonomic level obtained, either at phylum (p), family (f), or genus (g) level

(a)

ASV	Taxonomy	Contrib. [%]	Relative abundance M-S [%]	Relative abundance M-SF [%]
ASV3	g:*Polaromonas*	11.28	15.85	12.31
ASV7	g:*Janthinobacterium*	19.20	12.96	0.32
ASV1	g:*Janthinobacterium*	25.82	8.35	5.19
ASV2	g:*Undibacterium*	30.23	4.54	7.42
ASV5	g:*Cutibacterium*	34.30	2.63	5.27
ASV4	g:*Flavobacterium*	37.32	5.04	3.12

(b)

ASV	Taxonomy	Contrib. [%]	Relative abundance YW-S [%]	Relative abundance YW-SF [%]
ASV1	g:*Janthinobacterium*	9.18	6.19	14.00
ASV5	g:*Cutibacterium*	4.32	7.96	1.24
ASV14	p:*Bacteroidetes*	3.01	0.00	5.65
ASV10	g:*Pseudarcobacter*	2.63	4.85	1.57
ASV13	g:*Pseudomonas*	2.36	4.38	0.51
ASV9	g:*Acinetobacter*	2.11	0.00	3.96

(c)

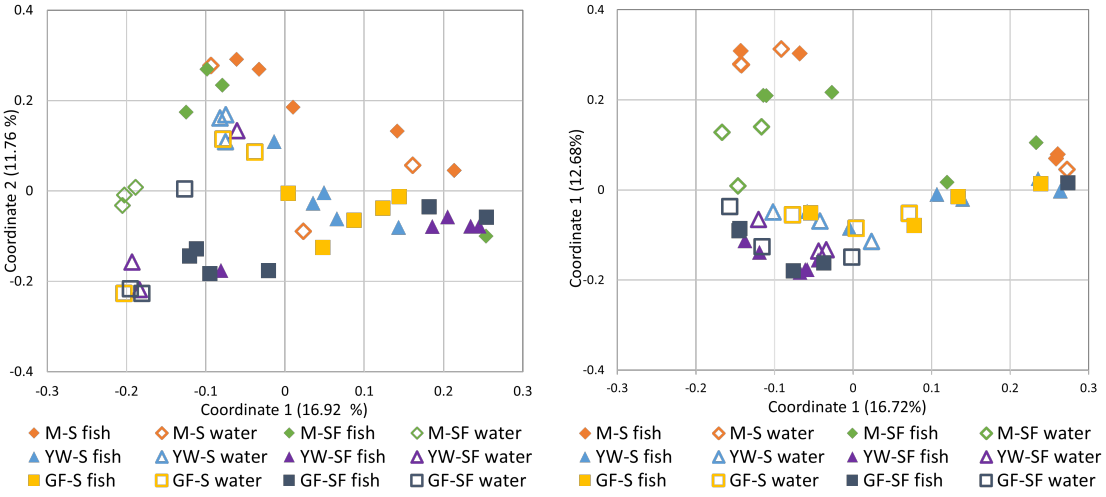
ASV	Taxonomy	Contrib. [%]	Relative abundance GF-S [%]	Relative abundance GF-SF [%]
ASV1	g:*Janthinobacterium*	5.79	5.65	7.38
ASV8	g:*Undibacterium*	3.60	5.04	4.23
ASV2	g:*Undibacterium*	3.47	2.17	5.54
ASV9	g:*Acinetobacter*	3.24	1.83	5.31
ASV6	g:*Flavobacterium*	2.90	4.38	1.72
ASV5	g:*Cutibacterium*	2.84	4.12	2.01
ASV34	f:*Comamonadaceae*	2.10	3.30	0.63

3.3.3 Comparison of the Microbial Community Profiles of the Water and Fish Microbiota

To assess the differences between water and fish microbiota observed in the previous sections, PCoA based on Bray-Curtis similarities were conducted for fish and rearing water samples collected at 1 and 2 wph (Figure 3.3.6 a and b).

At 1 wph, the microbial communities of the fish and water samples differed significantly between treatment groups (PERMANOVA, p = 0.0016) (Figure 3.3.6a). However, after 2 wph the fish and water microbiota differed less, but still significantly, between the treatment groups (PERMANOVA, p = 0.024)(Figure 3.3.6b). Furthermore, Bray-Curtis similarities revealed that the M-S group displayed the most similar fish and water microbiota at 2 wph (PERMANOVA, p = 0.4081), while the M-SF and the YW-SF group revealed significant differences between the fish and water microbiota (PERMANOVA , p = 0.037 and p = 0.0238, respectively)(Figure 3.3.7).

Overall, differences in the microbiota of fish and water samples were observed. Additionally, the differences observed between sampling times indicated that the microbiota of fish and water became more similar over time.



(a) (b)

Figure 3.3.6: PCoA plots based on Bray-Curtis similarities for comparison of the microbial community profiles of the fish and rearing water samples at a) 1 and b) 2 wph. All six treatment groups are displayed in the plot, M-S, M-SF, YW-S, YW-SF, GF-S, and GF-SF. Each point represents a sample, and the distance between points indicates the degree of similarity in bacterial community composition between samples.

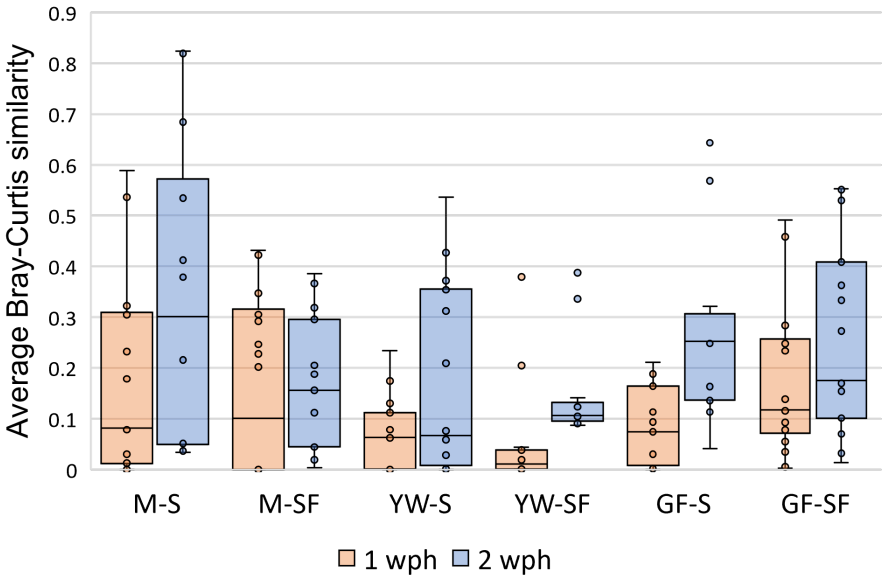


Figure 3.3.7: Average Bray-Curtis similarities (±SE) for comparison of the microbiota of fish and water samples within the six treatment groups. Each time group represents water and fish samples. The line across each box represents the average value. The standard error (±SE) is indicated by the error bars.

3.3.4 Exploring Variations in the Microbial Communities among Replicate Rearing Flasks and within Treatment Groups

To assess the differences in microbial communities of water and fish between the replicate flasks, as observed in Figure 3.3.1, PCoA was conducted for each of the six treatment groups (Figure 3.3.8).

The M-S treatment group appeared to exhibit the most different microbiota between its replicate flasks (Figure 3.3.8a). However, within the replicate flasks, the fish and water microbiota appeared to have similar microbial communities, with some exceptions observed in rearing flask 2. Furthermore, the M-SF, YW-S, and YW-SF treatment groups, also had variations between the fish samples of the replicate flasks, however, the microbial communities in the water samples appeared more similar between the replicate flasks (Figure 3.3.8b and 3.3.8c).

Among the six groups, the group that was germ-free until 0 dph, GF-S and GF-SF, revealed the most differences in the microbial communities both between replicate flasks and between the samples within each replicate flasks (Figure 3.3.8e and Figure 3.3.8f).

Overall, the microbiota of the replicate fish samples appeared to become more similar after 2 wph. Additionally, the fish microbiota appears to become more similar to the water microbiota over time.

In summary, all groups displayed differences between the fish and water microbiota in the replicate flasks and between replicate samples within each flask. Furthermore, many of the replicate samples exhibited a tendency to develop more similar microbial communities over time. Due to a limited number of samples, further statistical analyses were not possible. However, it is important to note the observed differences within individuals and replicate flasks.

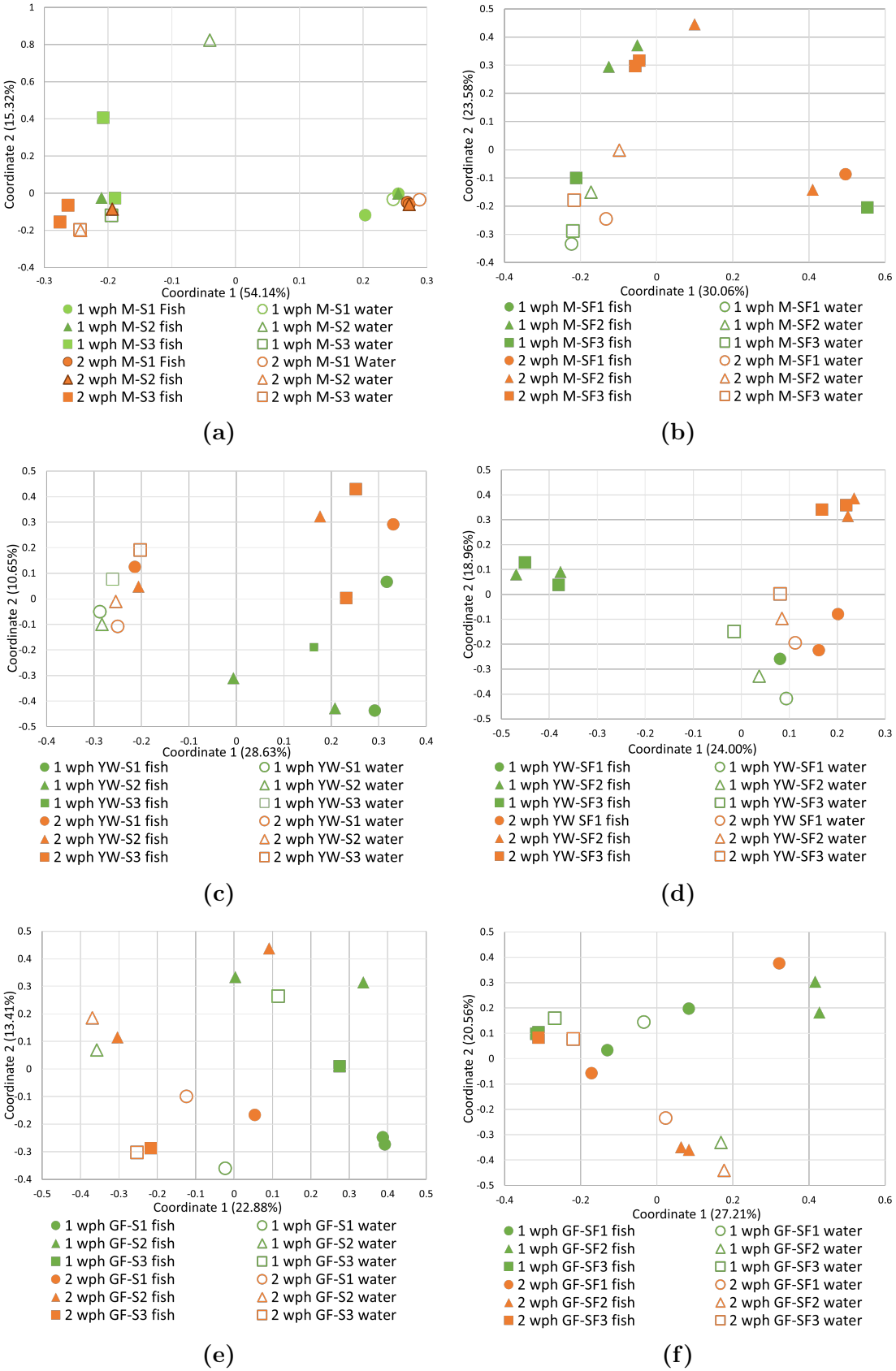


Figure 3.3.8: PCoA plots based on Bray-Curtis similarities for comparison of the microbial community profiles between replicate flasks. a) M-S, b) M-SF, c) YW-S, d) YW-SF, e) GF-S, and f) GF-SF treatment groups. Each treatment group represents three replicate rearing flasks with fish and water samples from 1 and 2 wph.

CHAPTER

FOUR

DISCUSSION

4.1 Evaluation of Survival Rates and Microbial Contamination

The gnotobiotic model used for the Atlantic salmon experiment demonstrated a high survival rate, with only one egg found deceased during the rearing period. Additionally, only three yolk-sac fry were found deceased. The high survival rate of 98.6% indicates that the experimental conditions provided a suitable environment for the development and survival of the Atlantic salmon yolk-sac fry, suggesting that treatments and rearing conditions exerted a minimal impact on fish survival.

Conversely, sterility and contamination analyses revealed the presence of bacterial contamination in some of the GF-rearing flasks. Despite the first sterility controls indicating a 100% successful disinfection procedure, bacterial growth was detected in three GF-rearing flasks (GF-S1, GF-SF1, GF-SF2) during the second sterility control at 0 dph. The contaminants identified were *Undibacterium* and *Flavobacterium*, with the latter also observed as a contaminant in a similar experiment described in the connected specialization project[40].

Among the two ASVs classified as *Flavobacterium*, ASV6 was detected in the community profile of the start-feeding water which was used to colonize the SF treatment groups at 0 dph. However, ASV29 was not observed in any of the community profiles of the microbial treatment samples. Interestingly, both ASVs were found in the community profiles of all the GF-S and GF-SF rearing flasks as well as in two other rearing flaks, YW-S1 and M-S1. The ASV6 in the start-feeding treatment could potentially explain its occurrence in the GF-SF rearing flasks. However, it does not account for its presence in the GF-S rearing flasks or the M-S1 and YW-S1 rearing flasks, which did not receive SF treatment.

Flavobacterium strains are known to be resistant to several types of antibiotics and are often recurring issues in the aquaculture industry[51, 52, 53]. Interestingly previous studies at ACMS tested *Flavobacterium*'s resistance following the same disinfection protocol and found that among the in the antibiotics used, only

Oxalinic Acid could inhibit its growth[54]. In response to these findings, the Oxalinic Acid concentration was increased in the disinfection protocol.

Considering the limited presence of the relevant ASVs (ASV6 and ASV29) in other rearing flasks and the known resistance of *Flavobacterium*, it is less likely that the cross-contamination occurred. Instead, the contamination likely resulted from an unsuccessful disinfection procedure that went unnoticed in the first sterility control. Notably, all GF flasks and only two other rearing flasks (M-S1 and YW-S1) from the other treatment groups indicated unsuccessful disinfection. While it may be a coincidence that only these flasks were unsuccessfully disinfected, it is intriguing to speculate whether more flasks were unsuccessfully disinfected, with microbial colonization from the treatments outcompeting the contamination and thus making its detection difficult. Regardless, the absence of relevant ASVs at 2 wph suggests the limited impact by these *Flavobacterium* populations on rearing flasks

Furhtermore, the GF-SF2 rearing flask was found to be contaminated by *Undibacterium*. The associated ASVs (ASV2 and ASV8) were also identified in several community profiles of other samples across the experiment. Importantly the ASVs were found in the skin mucus treatment, particularly in the M1 replicate, which exhibited a distinct community profile compared to its other two replicates (M2 and M3). The presence of the ASVs in this microbial treatment would explain the presence of the ASVs in the associated M-treatment groups. However, several other rearing flasks exhibited ASV2 and ASV8 in their community profiles despite not receiving the skin mucus treatment. Unlike *Flavobacteirum*, *Undibacterium* has no known history of surviving the disinfection procedure, and considering that the ASVs were present in several other rearing flasks, it is more likely that *Undibacterium* contamination in the GF flasks resulted from cross-contamination from the other treatment groups during the experiment, rather than from an unsuccessful disinfection procedure. It is difficult to ascertain whether the high abundance of ASV2 and ASV8 in the samples throughout the experiment originated from the microbial treatments or from cross-contamination. None of these ASVs contributed to large differences between the treatment groups. However, it is worth noting that these ASVs were present in high abundance across many of the samples.

In summary, the Atlantic salmon experiment demonstrated a high survival rate of eggs and yolk-sac fry, highlighting favorable rearing conditions. *Flavobactterium* contamination likely resulted from resistance to the disinfection procedure, while *Undibacterium* contamination probably originated from cross-contamination from other rearing flasks during sampling and water exchanges. Interestingly, no ASVs linked to these contaminations were found to significantly contribute to differences between the groups.

4.2 Interactions and Variations in Water and Yolk-Sac Fry Microbiota

From the community profiles of the water and fish samples across the experiment, several taxa that are known to be associated with the early development of yolk-sac fry were identified. Notably, the families *Pseudomonadaceae*, *Oxalobacteraceae*, *Comamonadaceae* and *Moraxellaceae*, belonging to the *Proteobacteria* phylum were consistently observed across the treatment groups in the experiment. This phylum has previously been reported as stage-specific to the egg stage of Atlantic salmon development[15].

In addition, families within the *Actinobacteria* phylum, including *Propionibacteriaceae*, *Ilumatobacteraceae* and *Corynebacteriaceae*, as well as families within the *Firmicutis* phylum, such as *Lactobacillaceae* and *Staphylococcaceae*, were also abundant in some of the samples across the treatment groups, although at a lower degree compared to the *Proteobacteria* phylum. These phyla (*Firmicutis* and *Actinobacteria*) have previously been reported to be stage-specific of newly hatched yolk-sac fry [15].

Differences between the water and fish microbiota were also observed, as well as variations between individuals within the same treatment groups. At 1 wph differences in the microbial communities between the water and fish samples were observed (Figure 3.3.6a). This finding is consistent with previous studies that have reported differences in the microbial communities associated with fish and their surrounding water[55].

Furthermore, alpha diversity measures supported these observations, as the water samples exhibited higher observed and estimated ASV richness and Shannon's diversity compared to the corresponding fish samples (Figure 3.2.2). This indicates that the microbial communities in the rearing water were more diverse and contained a greater number of ASVs compared to the microbial communities in the fish microbiota, despite being subjected to the same microbial treatment. These results align with the general trend observed in aquaculture systems, where the alpha diversity of water microbiota is typically significantly higher than that of fish [56, 57, 23].

As the rearing period progressed to 2 wph, the fish and water samples exhibited more similar microbial communities (Figure 3.3.6b). Quantitative analysis using Bray-Curtis similarities further supported the convergence of microbial community composition between fish and water samples over time (Figure 3.3.7). The increasing similarity suggests the potential transfer of microorganisms from water to fish, as well as the fish themselves shaping the water microbiota through the release or shedding of skin or egg-associated microbiota.

In addition to the differences between water and fish microbiota, Differences in the microbiota between rearing flasks within the same treatment groups and among individuals within the same flasks were observed (Figure 3.3.8). This

variation within the treatment groups is intriguing, considering that the replicates were exposed to the same microbial treatments. However, similar variability within replicate samples has been observed in other fish studies, particularly in the microbiota of cod larvae[58]. These variations may be attributed to stochastic factors influencing the colonization of bacteria in larvae, suggesting that specific events may significantly influence the complexity and diversity of individual microbial communities, even under individual environmental conditions. Furthermore, host genetics have also been suggested to contribute to inter-individual variations in fish microbiota[59].

In summary, stage-specific phyla were observed in the community profiles of the newly hatched yolk-sac fry. Differences between water and fish microbiota were evident at 1 wph, but convergence was observed at 2 wph, suggesting an ongoing interaction between fish and their surrounding water. These findings deviate from previous studies that reported a more distinct relationship between water and fish microbiota over time [55], however, it is likely that the dynamics of the water and fish microbiota would become different again over time. Additionally, variations within replicate flasks and among individuals within the same flasks were observed, emphasizing the dynamic nature of microbial communities and the potential of both environmental and host-associated factors.

4.3 Impact of the Microbial Treatment of Salmon Egg Microbiota

Fish egg microbiota is an intriguing area of research that holds broader significance, as the complex interactions between eggs and microbes during early development can have profound effects on subsequent stages of fish development[60]. Primarily the surrounding water is assumed to be the main source of initial colonization of yolk-sac fry. The understanding of microbial colonization of fish eggs and its impact on newly hatched fry remains limited. However, a few studies have shed light on the potential for broodstock interaction to lead to vertical transmission of microbial symbionts and pathogens to the eggs and their progeny.

In one study[61], the role of vertical and horizontal transmission of microbial symbionts in discus fish was investigated. It found that the gut microbiota of the fry was initially influenced by vertical transmission from the parents, with the fry acquiring microbial symbionts from the skin mucus of their parents during early development. Similarly, another study focused on Rainbow trout and coho salmon[62] and provided evidence of vertical transmission of *Flavobacterium psychrophilum* from infected broodstock to their eggs and progeny. The prevalence of this pathogen was significantly higher in eggs obtained from infected broodstocks compared to those from uninfected ones, and the progeny also had a higher abundance of *Flavobacterium psychrophilum* compared to progeny from uninfected broodstock.

Based on the limited understanding of microbial colonization of fish eggs, the primary aim of this study was to investigate the role of microbial colonization of salmon eggs and its influence on the resulting microbiota of yolk-sac fry. To assess this, microbial diversities and colonization patterns across different treatment groups were evaluated.

In this study, notable differences were found in the fish and water microbiota among three distinct treatment groups. The yolk-sac fry that received skin mucus treatment in the rearing water pre-hatching, the yolk-sac fry that received yolk-sac fry rearing water pre-hatching, and the yolk-sac fry that did not receive any microbial treatment at the egg phase exhibited significantly different microbiotas. Particularly, the treatment group that received skin mucus (M) displayed the largest difference in the microbial communities compared to the other two groups.

Among the key differences between the groups, ASV3 (*Polaromonas*) and ASV4 (*Flavobacterium*) contributed the most to the distinct microbiota of the M-treatment group. Notably both of these species, *Polaromonas* and *Flavobacterium*, are commonly found on the skin of Atlantic salmon[63, 64], indicating that the microbiota from the skin mucus had colonized the yolk-sac fry through egg colonization.

In contrast, the YW and GF treatment groups exhibited more similar community profiles and did not exhibit any ASVs that were exclusively dominant in either group. However, these observations suggest that the yolk-sac fry water treatment, which consisted of rearing water from a tank of young Atlantic salmon fry, probably introduced a microbial community that was less adapted to egg and fish colonization. While in contrast, the skin mucus treatment, derived from the skin of Atlantic salmon fry, probably introduced a microbiota that was ideally suited for egg and fish colonization. This discrepancy likely explains why the YW treatment groups exhibited more similarity to the GF groups that only received microbial treatment after hatching.

To summarize, both the treatments exposed to the salmon eggs before hatching resulted in significantly different microbiota of the yolk-sac fry post-hatching. Yolk-sac fry that were colonized with the skin mucus of older Atlantic salmon had a microbial community with skin mucus-associated taxa, indicating that the microbiota was successfully transferred from the egg to the yolk-sac fry. Furthermore, the yolk-sac fry that were colonized with the yolk-sac fry rearing water had a less distinct microbial community when compared to the group that did not receive microbial treatment at the egg phase.

Though it is clear that the skin mucus treatment significantly influenced the fry colonization at both 1 and 2 wph, it is not determined whether this colonization was more effective when introduced at the egg stage, as opposed to the water stage. What it does, however, is to confirm that the microbiota on eggs successfully transfers to the fish and that water microbiota is not the sole source of microbial colonization.

4.4 Impact of the Microbial Treatment added after Hatching

On the day of hatching, the rearing water was exchanged with new water sources from one of two different RAS systems (one for smolt production and one for start-feeding of salmon). Exploring the influence of water microbiota on fish microbiota is essential for shaping microbial communities in aquaculture systems. Previous studies have demonstrated that changes in rearing water microbiomes can influence the gut microbiota of fish, indicating a strong correlation between water and fish microbiota dynamics[65, 66].

Upon evaluating the observed and estimated ASV richness, alongside Shannon's diversity index, the groups that received start-feeding water exhibited the greatest ASV richness and Shannon's diversity, with the GF-SF group exhibiting the highest values. This implies that start-feeding water contributed more to the ASV richness in all groups, with the most substantial impact on the group without treatment on the eggs prior to hatching. These findings align with the previous studies highlighting the influence of water sources on the microbial diversity of fish[65, 66].

However, no significant differences were observed between S and SF treatments in the M group, indicating that the microbiota of the M groups, which were colonized with skin mucus at the egg stage, did not undergo significant changes after the introduction of new water sources after hatching. This suggests that the pre-existing colonization with skin mucus microbiota provided a protective effect against major shifts in microbial communities caused by the water sources.

In contrast, a significant difference was found between the S and SF treatments of the YW and the GF groups. The presence of *Janthinobacterium* (ASV1), which has previously been isolated from Atlantic salmon fry during previous work at ACMS[67], contributed the most to the microbial community differences but was present in the fish microbiota of both treatment groups (S and SF). However, this ASV was not observed in either of the RAS water sources in the normalized ASV table, it was however detected in both of the initial treatments, skin mucus and yolk-sac fry rearing water (3.1.2).

In the aquaculture industry, it is common practice to disinfect eggs prior to hatching. Interestingly, these results reveal that the yolk-sac fry that hatches from disinfected, non-colonized eggs are more susceptible to the influence of the surrounding water compared to the yolk-sac fry that hatches from colonized eggs. By allowing colonization of eggs after disinfection, or by not disinfecting the eggs, the yolk-sac fry may possess a stronger defense upon exposure to the rearing water after hatching. Therefore, introducing microbial treatments at the egg stage may potentially enhance the health and survival rate of farmed salmon.

4.5 Complexitiy of the Experiment and the Statistical Analyses

Complexities and sources of variability in the experimental results are important aspects to consider in any study, including the Atlantic salmon experiment conducted here. Replicate samples play a crucial role in accounting for the variability and ensuring the reliability of the obtained results. However, the analysis of the replicate samples in this study revealed significant differences, particularly among the samples of the added skin mucus and yolk-sac fry rearing water treatments, where one of the skin mucus samples exhibited an unusually different community profile compared to its replicates, while the two other replicates were similar to the yolk-sac water replicates (Figure 3.1.2). These unexpected variations raise questions about the factors contributing to such discrepancies.

One possible explanation for the observed variations in the replicate samples could be the viscosity of the mucus sample. The viscous nature of the mucus may have resulted in an uneven distribution of microbial species within the replicate, leading to the differences between them. However, this explanation alone does not account for the uneven distribution observed in the replicate samples of the yolk-sac fry rearing water treatment.

Accidental mixing of samples or contamination during the Illumina library preparation process is another potential factor that could contribute to the observed variations. Strict control over experimental procedures is crucial to minimize such issues. Interestingly, the replicate samples of the smolt water and start-feeding water treatments did not exhibit substantial variations between replicates. These samples underwent the same library preparation procedure and were prepared on the same normalization plates, suggesting that the variations seen in the mucus and yolk-sac fry water treatment replicates may be specific to those treatments or other factors associated with them.

Additionally, the small number of replicates used in the Atlantic salmon experiment limits the ability to evaluate statistically significant differences between rearing flasks and individuals within the same flasks. Research has indicated that the pooling of individuals may obscure inter-individual variations[58]. This study was limited in terms of the number of replicates used, as only one Illumina MiSeq-run was conducted, however, a larger sample size would provide a more comprehensive understanding of the microbial communities and their variations, while allowing a more robust statistical evaluation of inter-individual differences.

Another important aspect of these results is the limited knowledge regarding the quantity of bacterial cells of the microbial treatment applied. Although the community composition of microbial treatments was characterized by amplicon sequencing the actual number of microbes added to the rearing flasks was not quantified. Consequently, the disparities in the amount of microbiota introduced to the different rearing groups remained unknown. This raises the important question: Could the stronger colonization of yolk-sac fry observed in the M treatment groups be attributed to a higher microbial load being added? In other

words, if the yolk-sac fry rearing water treatment had been administrated in equivalent quantity, would the outcomes have been comparable? Either way, the important result of skin mucus microbiota significantly contributing to the colonization of the newly hatched yolk-sac fry would remain the same. Yet having a consistent quantity of microbial treatment could have helped with understanding if the microbial colonization of yolk-sac fry was due to the fact that the communities were more adapted to colonizing the eggs and fish or that it was due to a larger population.

In summary, the complexities and sources of variability observed in the Atlantic salmon experiment points to the importance of replicating samples for ensuring reliable results. The variations seen in the replicate samples of the skin mucus and yolk-sac fry rearing water could be influenced by factors such as the viscosity of the mucus sample or accidental mixing during library preparation. Additionally, the variations observed within replicate samples of fish and rearing water samples emphasize the dynamic nature of microbial communities in aquatic environments and the need for larger sample sizes to achieve statistically robust conclusions. Furthermore, the absence of information concerning the quantity of added microbial treatment represents a knowledge gap in the study.

4.6 Future work

While this study focused on investigating the influence of egg microbiota on the colonization of yolk-sac fry, the specific impact of the microbiota on host health and development was not thoroughly explored. Therefore, future research should aim to investigate the long-term effects of microbial colonization on eggs. This will provide a deeper understanding of the implications and potential benefits associated with egg microbiota. Additionally, the interplay between the host's genetics and the microbiota should be further investigated, as it could significantly impact the composition of the microbial communities.

To enhance the survival rate, health, and performance of farmed Atlantic salmon, as well as to mitigate the negative effects of egg disinfection, targeted microbial treatment at the egg stage should be further explored. This could involve the application of specific beneficial microbial taxa or probiotics that have demonstrated their efficiency in promoting growth, immune function, and disease resistance in fish. By exploring the potential of such treatments, strategies to optimize rearing conditions for Atlantic salmon and improve their overall well-being can be developed.

In terms of the experimental design, future studies should incorporate quantitative measures to determine and standardize the amount of microbial treatment added to each experimental group. This will enable a more accurate assessment of the influence of microbial dosage on colonization dynamics and facilitate meaningful comparisons between treatments. Additionally, an increasing number of replicate flasks and taking more replicate samples would improve the statistical robustness

and reliability of the results.

Finally, it is essential to conduct a thorough evaluation of the sterility procedures employed in similar experiments. This evaluation should encompass a review of the disinfection protocols to identify areas for potential improvement. Additionally, measures to minimize the risk of cross-contamination should be carefully implemented to ensure the integrity of the experimental setup.

CHAPTER

FIVE

CONCLUSION

This study found that the fish and water microbiota exhibited variations between the treatment groups. However, the families *Oxalobacteraceae* and *Flavobacteriaceae* were consistently abundant across all treatment groups. The family *Commomonadaceae* was more prevalent in the treatment groups that were exposed to skin mucus, while *Pseudomonadaceae* was more common in the treatment groups that were exposed to yolk-sac fry rearing water during the egg stage, as well as in the group that did not receive any treatment at the egg phase.

Moreover, a high survival rate of eggs and yolk-sac fry demonstrates favorable rearing conditions and indicates no observable impact of the treatments on their survival. However, the sterility and contamination analyses raised concerns regarding bacterial contaminants in germ-free rearing flasks, particularly *Flavobacterium* and *Undibacterium*, in some of the rearing flasks. The presence of *Flavobacterium* strains highlighted a potential weakness in the disinfection procedure employed, suggesting the need for re-evaluation.

Furthermore, the study revealed that the microbial treatments applied to germ-free salmon eggs were found to significantly impact the colonization of newly hatched yolk-sac fry, suggesting that water microbiota is not the sole source of colonization of newly hatched fry. Both yolk-sac fry that were exposed to skin mucus and yolk-sac fry that were exposed to yolk-sac fry rearing water at the egg stage had significantly different microbiotas in comparison to yolk-sac fry that did not receive any microbial treatments at the egg stage. Moreover, the yolk-sac fry that were exposed to skin mucus at the egg stage were not significantly impacted by microbial treatments added after hatching, while the yolk-sac fry that were colonized by the yolk-sac fry rearing water at the egg stage were significantly affected by microbial treatments added after hatching. This implies that depending on the microbial treatment exposed to the eggs, the yolk-sac fry can be more or less influenced by the exposure to new microbial environments after hatching.

In conclusion, this study highlights the significance of egg microbiota in influencing the microbiota of newly hatched yolk-sac fry. This finding holds particular relevance as the aquaculture industry often employs disinfection procedures on salmon

eggs, which eliminates both beneficial and pathogenic bacteria. The results underscore the vulnerability of disinfected eggs to colonization by water microbiota upon hatching when lacking colonization during the egg stage. Therefore, introducing microbial treatments at the egg stage may potentially enhance the farmed salmon's survival rate and overall health.

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APPENDICES

A - ANTIBIOTIC SOLUTION

Table A.1: Components of the antibiotic solution used in disinfection procedure of Atlantic salmon eggs.

Antibiotic	**Working concentration [mg/L]**	**Volume of stock added to 1L SGM [µL]**
Rifampicin	10	200
Erythromycin	10	200
Kanamycin	10	200
Ampicillin	100	1000
Amphotericin B	0.25	1000
Penicillin	150	1500
Oxalinic acid	37.5	3000

B - ASV PLOT

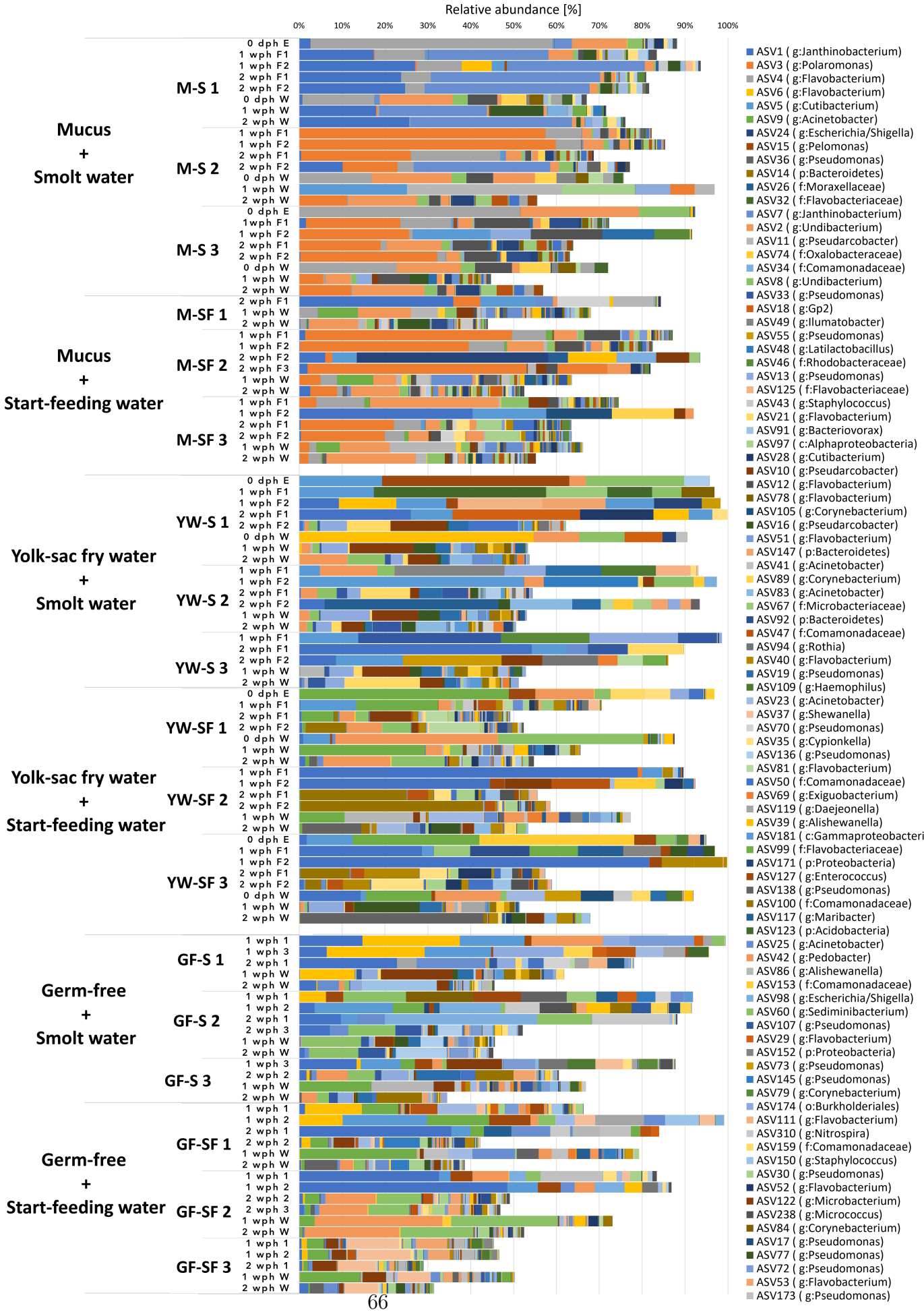
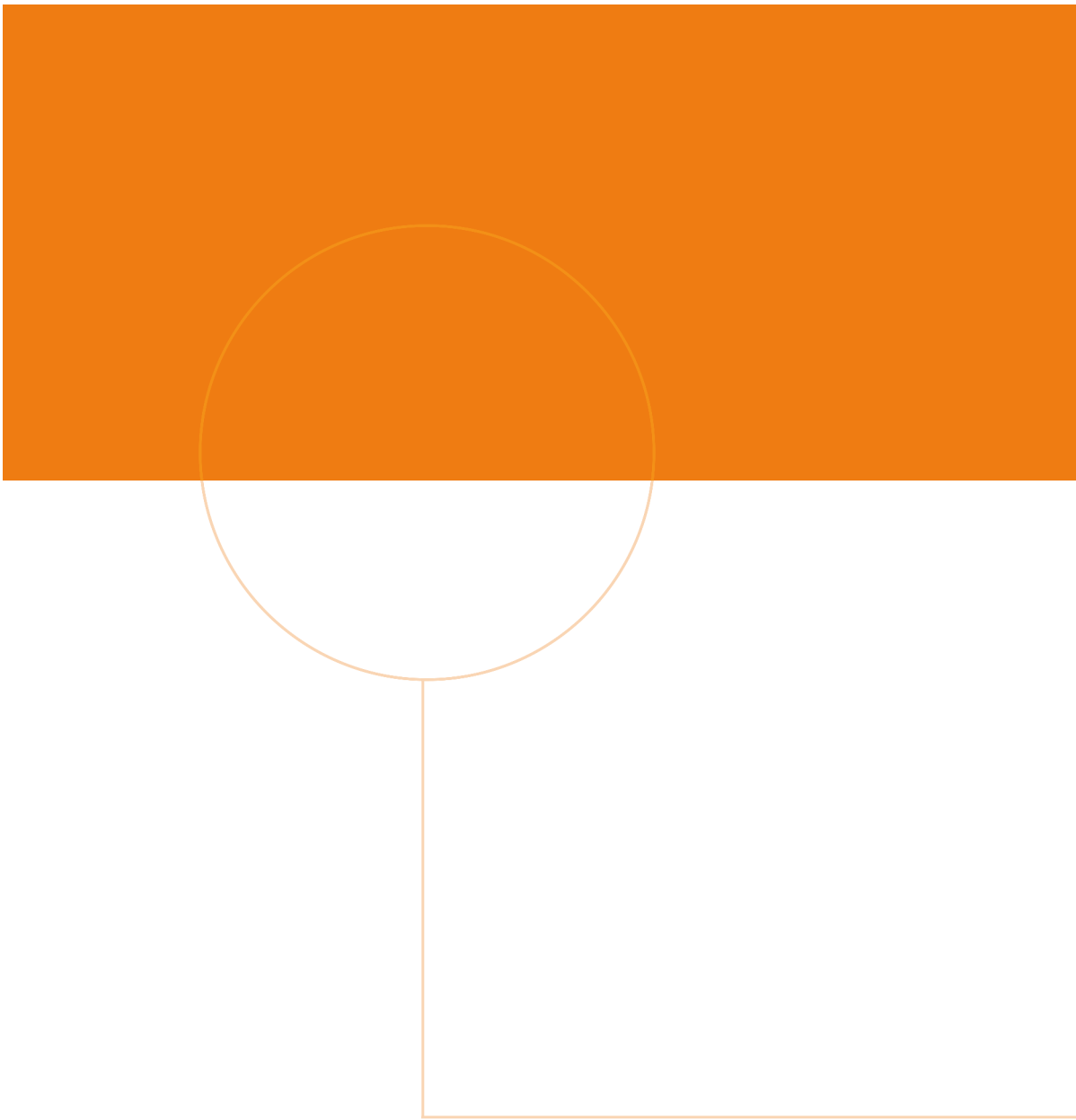

Relative abundance [%]
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Mucus + Smolt water
M-S 1
M-S 2
M-S 3
Mucus + Start-feeding water
M-SF 1
M-SF 2
M-SF 3
Yolk-sac fry water + Smolt water
YW-S 1
YW-S 2
YW-S 3
Yolk-sac fry water + Start-feeding water
YW-SF 1
YW-SF 2
YW-SF 3
Germ-free + Smolt water
GF-S 1
GF-S 2
GF-S 3
Germ-free + Start-feeding water
GF-SF 1
GF-SF 2
GF-SF 3
ASV1 (g:Janthinobacterium)
ASV3 (g:Polaromonas)
ASV4 (g:Flavobacterium)
ASV6 (g:Flavobacterium)
ASV5 (g:Cutibacterium)
ASV9 (g:Acinetobacter)
ASV24 (g:Escherichia/Shigella)
ASV15 (g:Pelomonas)
ASV36 (g:Pseudomonas)
ASV14 (p:Bacteroidetes)
ASV26 (f:Moraxellaceae)
ASV32 (f:Flavobacteriaceae)
ASV7 (g:Janthinobacterium)
ASV2 (g:Undibacterium)
ASV11 (g:Pseudarcobacter)
ASV74 (f:Oxalobacteraceae)
ASV34 (f:Comamonadaceae)
ASV8 (g:Undibacterium)
ASV33 (g:Pseudomonas)
ASV18 (g:Gp2)
ASV49 (g:Ilumatobacter)
ASV55 (g:Pseudomonas)
ASV48 (g:Latilactobacillus)
ASV46 (f:Rhodobacteraceae)
ASV13 (g:Pseudomonas)
ASV125 (f:Flavobacteriaceae)
ASV43 (g:Staphylococcus)
ASV21 (g:Flavobacterium)
ASV91 (g:Bacteriovorax)
ASV97 (c:Alphaproteobacteria)
ASV28 (g:Cutibacterium)
ASV10 (g:Pseudarcobacter)
ASV12 (g:Flavobacterium)
ASV78 (g:Flavobacterium)
ASV105 (g:Corynebacterium)
ASV16 (g:Pseudarcobacter)
ASV51 (g:Flavobacterium)
ASV147 (p:Bacteroidetes)
ASV41 (g:Acinetobacter)
ASV89 (g:Corynebacterium)
ASV83 (g:Acinetobacter)
ASV67 (f:Microbacteriaceae)
ASV92 (p:Bacteroidetes)
ASV47 (f:Comamonadaceae)
ASV94 (g:Rothia)
ASV40 (g:Flavobacterium)
ASV19 (g:Pseudomonas)
ASV109 (g:Haemophilus)
ASV23 (g:Acinetobacter)
ASV37 (g:Shewanella)
ASV70 (g:Pseudomonas)
ASV35 (g:Cypionkella)
ASV136 (g:Pseudomonas)
ASV81 (g:Flavobacterium)
ASV50 (f:Comamonadaceae)
ASV69 (g:Exiguobacterium)
ASV119 (g:Daejeonella)
ASV39 (g:Alishewanella)
ASV181 (c:Gammaproteobacteria)
ASV99 (f:Flavobacteriaceae)
ASV171 (p:Proteobacteria)
ASV127 (g:Enterococcus)
ASV138 (g:Pseudomonas)
ASV100 (f:Comamonadaceae)
ASV117 (g:Maribacter)
ASV123 (p:Acidobacteria)
ASV25 (g:Acinetobacter)
ASV42 (g:Pedobacter)
ASV86 (g:Alishewanella)
ASV153 (f:Comamonadaceae)
ASV98 (g:Escherichia/Shigella)
ASV60 (g:Sediminibacterium)
ASV107 (g:Pseudomonas)
ASV29 (g:Flavobacterium)
ASV152 (p:Proteobacteria)
ASV73 (g:Pseudomonas)
ASV145 (g:Pseudomonas)
ASV79 (g:Corynebacterium)
ASV174 (o:Burkholderiales)
ASV111 (g:Flavobacterium)
ASV310 (g:Nitrospira)
ASV159 (f:Comamonadaceae)
ASV150 (g:Staphylococcus)
ASV30 (g:Pseudomonas)
ASV52 (g:Flavobacterium)
ASV122 (g:Microbacterium)
ASV238 (g:Micrococcus)
ASV84 (g:Corynebacterium)
ASV17 (g:Pseudomonas)
ASV77 (g:Pseudomonas)
ASV72 (g:Pseudomonas)
ASV53 (g:Flavobacterium)
ASV173 (g:Pseudomonas)

Figure F.1: Microbial community composition at ASV level for the samples taken in the Atlantic salmon experiment. The experiment consisted of six different rearing groups: M-S, M-SF, YW-S, YW-SF, GF-S, and GF-SF, with sampling points at 0 dph, 1 wph, and 2 wph. The sample types are defined as eggs (E), Fish (F, 1-2 replicates), and water (W). Families with relative abundance below 1% in all samples are not included in the figure.



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