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The microbiome of rainbow trout (Oncorhynchus mykiss)

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The microbiome of rainbow trout (Oncorhynchus mykiss)

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ABSTRACT

Commensal organisms are an integral part of all vertebrates, contributing heavily to development, pathogen defense, and metabolism. Commensals reside at different body sites within vertebrate animals creating unique and distinct communities that vary between locations. The human microbiome project has revealed distinct bacterial community compositions at the diverse range of body sites that have been sampled, providing evidence for different functional purposes of each microbiome. Rainbow trout, *Oncorhynchus mykiss*, serves as a model organism for the study of mucosal physiology and immunology. Teleost fish are evolutionarily important as one of the first jawed vertebrates with a dedicated adaptive mucosal immune system, as well as being vital to aquaculture practices. Studies of the microbiome of rainbow trout have the potential to 1) reveal important mucosal evolutionary processes 2) discover particular symbtiotic bacteria that can be used in aquaculture to improve fish health. The hypothesis of the present study is that different body sites of rainbow trout possess distinct commensal bacterial communities. Using 454 pyrosequencing of the 16S bacterial rRNA, we present the first topographical map of the microbiome of rainbow trout. Body site is a strong predictor of bacterial community composition in trout. Both ANOSIM and Adonis

statistical analysis revealed p values below 0.001 when using body site as a variable to describe diversity. The most diverse mucosal sites are the skin and the olfactory organ with 17 and 18 different phyla, respectively. We also discovered a novel and high diversity of bacteria present within the skin epithelium of rainbow trout, dominated by *Propionibacterium* sp. and *Staphyloccus* sp. This may represent a unique adaptation in salmonids to avoid swimming drag forces that bacteria attached to the external mucus may cause.

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Chapter 1

A comprehensive overview of the microbial associations at mucosal surfaces in the teleost fish rainbow trout, *Oncorhynchus mykiss*, and the importance of these associations for the immune system.

1.1 Commensalism and symbiosis

Symbiosis is a conserved feature within all organisms, both prokaryotes and eukaryotes. Symbiosis can be defined as an interaction between two or more biologically different organisms. Symbiosis is typically centered on metabolic capabilities that allow either or both species to utilize an unavailable or relatively un-accessible nutrient source (Perret et al, 2000). These symbiotic interactions can last for differing amounts of time. There are three types of symbiosis: mutualism, commensalism, and parasitism. Mutualism is a symbiotic relationship in which both organisms involved gain a benefit from their interaction. Parasitism is a symbiotic relationship in which one of the organisms involved is harmed, while the other gains a benefit. Finally, commensalism describes a symbiotic relationship in which one organism gains a benefit while the other remains neutral. Many instances of commensalism are often composed of a single dominant eukaryotic cell genome and an array of microbial genomes, both eukaryotic and prokaryotic (McFall-Ngai 2002).

Microorganisms started forming complex communities and interacting with each other almost 3.4 billion years ago (BYA), as evidenced by those trapped within the fossil record (Allwood et al, 2006). In many cases, microbes have evolved to directly coexist with a host (Hill & Artis 2010; McFall-Ngai 1999; Wilkinson 2001). This evolutionary

process is a result of directly trying to increase the microorganism's fitness, which also happens to often increase the fitness of the host (Dethlefsen et al, 2007). Microbe-host relationships are various and complex and the many beneficial commensal associations found in nature can have multiple functions. Such symbiotic relationships have evolved to represent a conserved feature of multicellular life, important for normal development and physiology in plants, insects, nematodes, fish, birds, and mammals (Hill & Artis 2010). Specifically, it becomes important to recognize the framework in which these symbiotic relationships occur, and the benefits conferred to the host. Amongst the benefits conferred to hosts are digestion of carbohydrates, fat metabolism, and resistance to infection by mechanisms such as competition for nutrients and niches or production of anti-microbial peptides. It has also been shown that commensal bacterial interactions are required for normal immune system development, homeostasis and regulation (Abt et al, 2012; Hill & Artis 2010, Kosiewicz et al, 2013).

1.2 Evolution of the association between commensals and eukaryotes

The association of commensals and eukaryotes has evolved to respond to needs dictated by the environment of the host. The majority of metabolic pathways evolved in prokaryotes long before the arrival of multicellular organisms (Gottschalk 1986). Prokaryotes were able to use both organic and inorganic sources for energy before the evolution of eukaryotes (Madigan et al, 1997). Eukaryotes, however, had difficulty metabolizing nutrients from otherwise scarce resources. Thus, partnerships with prokaryotic bacteria were likely created to take advantage of the metabolic opportunities afforded by colonizing communities of prokaryotes (McFall-Ngai 1998). The ability to

garner resources in otherwise nutrient deficient environments represents a significant fitness benefit, and one that has been conserved in all domains of life. Together, the host and microbial population have a combined ability to gather nutrients from different energy sources. The sum of the metabolic processes of both the host and the microbial population residing within the host can then be defined as the metabolome. The microbiome's highly adaptive metabolic engine substantially increases the host's ability to harvest nutrients from food. It can be deduced that as cellular mass and metabolic needs increase in complex metazoans, the associated commensal communities increase in complexity. This is illustrated by the human lower intestine, which appears to be the most densely populated microbial ecosystem that has been studied (Andersson et al, 2008). Moreover, humans have complex microbial communities in other points of contact with the environment such as the skin, nasopharyngeal cavity, respiratory tract and reproductive tract.

In evolutionary history of *Homo sapiens*, diet shifts have had their impact upon the genome, and reveal the importance of bacterial interactions (Ley et al, 2008). As these diet shifts occurred, it became necessary to adjust to, and process different sources of energy. Studies of the human gut microbiota have revealed complex flora consistent with distinct diet types in world regions (Ley et al, 2008). This being said, it has been shown that vertebrates other than humans have evolved the ability to harbor large populations of microbial communities throughout their life cycles (Rawls et al, 2004). It is also important to recognize that studies on the gut microbial communities of invertebrate animals reveal different microbial complexities based upon diet (Colman et al. 2012). This leads to a potential hypothesis that diet, not host complexity, is a driver for

symbiotic bacterial community diversity. Overall, we can say that multiple interactions between the host, environment, and microbiome occur to determine microbial community structure and symbiotic relationships within eukaryotes.

As mentioned earlier, the commensal-host interaction has also driven the evolution and development of the immune system. In particular, some researchers have proposed that complex commensal communities were in fact the driving factor for the emergence of the adaptive immunity. Without the constant interactions between multicellular organisms and commensals for so many millions of years, it is possible that adaptive immunity would never have arisen. The evolution of vertebrates was nearly simultaneous with the emergence of adaptive immunity, around 500 MYA (Maynard et al, 2012). Adaptive immunity is characterized by an antigen specific response to a pathogen or danger signal. Adaptive immune receptors are created by recombination of genes and addition of diversity through molecular mechanisms. There is a tremendous amount of antigen binding site diversity that can be created in immunoglobulins and T cell receptors using VDJ recombination. This is a hallmark of the adaptive immune system of vertebrates. The great diversity of B and T cell repertoires that can be generated provides the ability to recognize almost any antigen specifically. The first exposure during an adaptive immune response leads to a slow expansion of lymphocytes and antigen specific machinery to eliminate the microorganism. Adaptive immunity establishes a state of immunological memory, in which the adaptive immune system can remember the pathogen from the first invasion. After recognition in a secondary exposure, the adaptive immune system can quickly expand and clear the invading microbe at a much faster rate than the first exposure.

Despite the many benefits that commensal microorganisms provide to the immune system, commensalism also poses important challenges to the vertebrate immune system. Many different strains of bacteria, both commensals and pathogens, share common antigen on their cell surface and within their flagella. These antigens are known as microbe-associated molecular patterns (MAMPs) (Mackey & McFall 2006). These molecular elements that are on the surface of microbes are not present in the host, and thus are recognized as foreign (Mackey & McFall 2006). For example, the immune system cannot distinguish if the lipid A which is a core binding motif of LPS that interacts with TLR4, in fact originates from a commensal or a pathogen. With increasingly complex microbial communities present at different sites within vertebrates, it becomes critical to distinguish between pathogens and beneficial commensals. Whereas innate immunity aims to eliminate all microorganisms, adaptive immunity can "remember" commensals and pathogens, and it is capable of tolerating the first and fighting the second. Ultimately, the association with commensals requires determining "self" versus "non-self" and mounting an appropriate immune response. The commensal microbial communities that reside within the host can be viewed as the "extended self" (Maynard et al, 2012). They are a vital ecosystem within the host that confers multiple benefits. The commensals must be recognized as "self" in order to be protected against the hosts own immune system.

Another of the challenges that commensals pose to the vertebrate immune system is illustrated by the instances when the homeostasis of the epithelial barrier is disrupted. In these instances, even in the absence of pathogens, commensal microorganisms are not always safe for the host (Drake 2008). In the event of dysbiosis, or disregulation of

microbial communities, shifts can occur in community compositions that result in the proliferation of opportunistic species or pathobionts. This can, in turn, result in the adaptive immune response against antigens present in the normally beneficial microbial communities present (Maynard et al, 2012). Adaptive immune responses to commensals have been linked to be one of the causes of diseases such as irritable bowel syndrome, rheumatoid arthritis, and diabetes, as well as others (Tlaskalova-Hogenova 2004; Strober et al, 2007; Erridge 2011). With the adaptive immune response able to create specific responses towards any microbe, the delineation between commensal and pathogenic microbiota can become blurred as community disruption occurs. This has been coined by some authors as the "frustrated commensal model", in which prolonged inflammation responses are the result of an immune response mounted against shared antigens between pathogens and commensals (Nussbaum & Locksley 2012).

1.3 Immune Responses at Mucosa-Associated Lymphoid Tissues (MALT) in Vertebrates

Complex commensal relationships can be found at mucosal surfaces of vertebrate animals, which are the first interface between the environment and the animal host. Apart from providing niches for commensal microorganisms, the physical and chemical barriers that are formed by mucosal tissues are important for preventing invasion from pathogens. They also represent a major site for interaction between the host, symbiotic microbial communities, and the environment. For example, we know that bacterial communities are important for the development of the immune system, particularly in the gut (Olszak et al, 2012; Hansen et al, 2012; Szeri et al, 1976; Ostman et al, 2006). The interactions between

the mucosal immune system in vertebrates and bacterial communities have long lasting effects, and contribute to the overall health of the organism.

Mucosal surfaces act as barriers to the environment, but they also have immunological protection in the form of cellular and humoral immunity (Salinas et al, 2011). The barriers at mucosal surfaces are made out of epithelial cells, mucus producing cells, neuroendocrine cells and a local immune system, the mucosa-associated lymphoid system (MALT). The mucosal immune system is very complex, even more so than the systemic immune system (Brandtzaeg 2009). The mucosal immune system can be divided into two separate sites according to the Society of Mucosal Immunology. First there are inductive sites where T and B leukocytes are stimulated with antigen gathered from the mucosal surface, and secondly the effector sites where effector cells arrive to carry out their functions (Brandtzaeg & Pabst 2004). In terms of structure, inductive sites usually have organized MALT as well as lymph nodes that drain the surrounding area of lymphatic fluid containing potential antigens (Brandtzaeg & Pabst 2004). Effector sites are known to have distinct localizations, including the lamina propria (LP), the epithelial cells, as well as the supportive tissue of exocrine glands (Brandtzaeg & Pabst 2004). MALT prevents the entry of foreign bodies or potential pathogens, and is intimately linked with the lymphatic system (Garcia-Garcia et al, 2013). MALT is present at different anatomical sites including the gut, respiratory tract, and nasal tissues in mammals (MacDonald 2003) but varies amongst the different vertebrate groups. For instance, in aquatic vertebrates such as fish, the skin is also considered part of the MALT (Salinas et al, 2011). MALT contains both innate and adaptive immune components that are essential for prevention of disease and regulation of commensals.

The innate immune response is a non-specific reaction to pathogens by the host immune system in an effort to eliminate any microbial invaders. At mucosal sites, secretion of mucus is one of the most important non-specific defense mechanisms and it is often the first line of defense against pathogens (Woof & Mestecky 2005). Mucus lines mucosal surfaces and prevents binding of pathogens to cells, as well as protecting mucosal sites from injury by enzymes or mechanical damage (Esteban 2012; Linden 2008). Mucus is a complex, viscous and adhesive fluid containing many macromolecules. One of the primary molecules that are important for the physical properties of mucus is mucins. Mucins are the major macromolecules that are responsible for the fluid and solid-like properties of mucus (Linden 2008). Mucins are a group of glycosylated proteins that can either be antimicrobial by themselves, or carry additional molecules that can damage microbes (Linden 2008). Mucus also contains immune molecules that are important for protection against foreign microbes. Many important innate defense proteins and enzymes such as lysozyme, lectins, C-reactive protein, proteolytic enzymes, transferrins, alkaline phosphatase and other antibacterial proteins and peptides have been found in mucus (Shoemaker et al, 2006). Mucus also contains adaptive immune molecules in the form of secretory Igs (sIgs), which can activate complement in classical pathways, as well as neutralize and opsonize potential pathogens and coat commensals. Thus, mucus is an important aspect of all mucosal epithelia, and ultimately determines which bacterial species (commensals and pathogens) adhere and how abundant they are.

Vertebrate MALT also contains a network of B cells, plasma cells and immunoglobulins (Igs) that are essential for mucosal homeostasis. Igs function is to very specifically target and neutralize any foreign cognate antigen present within the

organism. After binding to a pathogen or the cognate antigen Igs mediate a number of different actions, including complement activation, phagocytosis by opsonization, neutralization, antibody dependent cellular cytotoxicity, and agglutination. Secretory Igs are mainly produced by plasmablasts and plasma cells. The majority of Igs produced at mucosal surfaces of mammals are IgA (Brandtzaeg 2009). IgA is also found in birds and crocodilians (Orlans & Rose 1972; Magadan-Mompo et al, 2013). This Ig isotype is almost exclusively associated with mucosal immunity, although other forms of Igs are present at mucosal surfaces, such as IgM and IgG. IgA is normally in a dimeric form, while IgM can form pentamers. The dimeric IgA configuration allows this Ig to bind to additional antigen sites, promoting cross linking. Due to the need for Igs to be secreted to the external mucus layer to provide additional pathogen neutralization, Igs must be exported across the epithelium. Both IgA and IgM can be secreted across the epithelium to areas of pathogen invasion and inflammation through the mechanism of the pIgR/SC receptor (Brandtzaeg 2009).

sIgs also play an important role in commensal regulation. Igs coat the surface of commensal bacteria, preventing translocation, a process known as immune exclusion (Stokes et al, 1975). The Igs that bind to the surface of the bacteria can be recognized by the host immune system. This recognition prevents lymphocytes from inducing an immune response against those bacteria. Immune exclusion allows the host immune system to determine “self” and “non-self”, and amount a proper immune response against pathogenic bacteria while beneficial commensals are left unharmed. Without regulation in an immune response, all bacteria containing a singular antigen will be eliminated by the host immune system. It is vital to maintain homeostasis and determine which bacteria

are commensals to prevent destruction of essential bacterial microflora. It has been shown that even after an inflammation event, memory against commensals is maintained for long periods afterward (Hand et al, 2012). Low-affinity IgA has enough ability to protect the host from the immune activation induced by commensals, but affinity maturation of IgA is required to protect the host from more dangerous microorganisms (Slack et al, 2012).

T cells are also important in the adaptive mucosal immune response, as they induce switching of B cells to secretory plasma cells among other functions. At mucosal surfaces, T regulatory cells (Tregs) are particularly important for microbial communities since they induce tolerance to commensal bacteria by expressing TGF-β (Giacinto et al, 2005). Commensals can also induce Tregs in germ free animals, reducing inflammation (Round & Mazmanian 2010). Furthermore, another subset of mucosal T cells is the cytotoxic T cell. Cytotoxic T cells have processes which allow them to destroy other cells or to induce apoptosis. Cytotoxic T cells kill cells infected by pathogens or containing foreign antigens presented by the major histocompatibility complex I. In the case of mucosal surfaces, cytotoxic T cells are often responsible for eliminating epithelial cells that become infected with virus or bacteria.

1.4 Teleost Mucosa-Associated Lymphoid Tissues (MAL) and Mucosal Immune Responses

Teleostei is one of three infraclasses in class Actinopterygii, the ray-finned fishes. The Teleostei, with an estimated 23,600 extant species (Nelson 1994), is the most diverse

group of actinopterygians. It also has the most species among vertebrate animals. The first jawed fishes emerged 360-450 MYA during the Devonian period (Volff 2005).

The importance of studying the mucosal immune system of teleosts is two-fold. First, it is important from an evolutionary perspective. Due to their important phylogenetic position, comparative studies have the potential to reveal how certain mucosal immune molecules and pathways possibly evolved from a very early stage during vertebrate evolution. Secondly, the importance of fish health to aquaculture cannot be overstated. Global aquaculture production has more than quadrupled since 1985 (FAO 2007). In the last decade it has more than doubled, reaching 62.9 million tons in 2005 (FAO 2007). Also according to the FAO, as of currently 52% of the 600 wild fish species with economic value are heavily depleted, 17% are overfished, and 7% have been fully exploited. With the extreme amount of food that aquaculture produces, we must better understand how make this food industry sustainable. Fish farming sustainability relies on minimizing economic losses due to aquatic infectious diseases. The study of fish immunity has generated solutions to many problems faced by this industry. This also, in turn, has a large impact on human health since millions of tons of fish produced by aquaculture are consumed worldwide each year.

Teleosts, like other vertebrates, contain both an adaptive and innate immune system, which is thought to have evolved independently of that of jawless fish (Boehm et al, 2012). Teleost fish, like the rest of jawed vertebrates, have an adaptive immune system based on B and T cells. Adaptive immunity is present systemically and also in the periphery, at mucosal sites. The mucosal barriers of teleost fish, specifically the gut, skin, gills, and nose, form the interface between the host and their environment. These barriers

prevent pathogens from entering the teleost host through mucosal sites. There are distinct differences between these mucosal sites, both in terms of the tissue structure and the distribution of immune cells. Teleost are known to have three major mucosal sites with associated lymphoid tissue. First, there is the gut-associated lymphoid tissue (GALT), which contains both the lamina propria (LP) and intraepithelial (IEL) compartments (Salinas et al. 2011). Secondly there is the skin-associated lymphoid tissue (SALT), and lastly the gill-associated lymphoid tissue (GIALT) (Salinas et al. 2011). These tissues harbor resident commensals at multiple levels, and each tissue has distinct immunological properties. The different tissue types are illustrated in Figure 1. Since fish are in constant contact with the water environment, their immune system needs to regulate both symbiotic and pathogenic microbes.

Many important fish pathogens affect mucosal surfaces. Of all of the fish mucosal surfaces, the gut and its associated GALT is the most intensively studied (Esteban 2012). The GI tract of fish varies much between different species and feeding strategies (Evans 1998). The variation in structure of the GI tract is due to a number of factors, including the presence or absence of a stomach, differences between the length of the intestine, whether the pyloric caeca are present as well as how many exist, and the loops and valves within the intestine (Evans 1998). Regardless of its morphology, the main role of the GI tract is nutrient processing and absorption. The first evidence that fish have a local immune system associated with their gastrointestinal (GI) tract was reported by Fletcher and White in 1973 (Fletcher & White 1973). We know there is also a difference in the ability to uptake particles between the anterior and posterior gut of fish (Rombout et al, 2010). Immunologically speaking, the posterior gut is arguably the most important as the

majority of antigen uptake occurs within this region of the GI tract (Rombout et al, 2010). In carnivorous fish, the first portion of the GI tract is generally associated with break down and absorbance of proteins and lipids, as shown by varying levels of protease and lipase activity along the intestine and stomach (Xiong et al, 2011). In addition, the pH differences present along the GI tract have been well described. As seen in catfish (*Ictalurus punctatus*) the pH can range between 2 and 4 in the stomach, then shifts to an alkaline environment below the pylorus, with a pH between 7 and 9 (Pillay & Kutty 2005). A pH of 8.3 is present in the foregut and it is near neutral in the hindgut (Pillay & Kutty 2005). These pH gradient differences are another instance in which the anatomy and physiology of the gut differs widely in teleost, creating distinct microenvironments along the GI tract.

Unlike endotherms, fish do not have an organized MALT, such as Peyer's Patches or mesenteric lymph nodes (Rombout et al, 2010). For instance, teleost GALT is diffuse, with leukocyte populations scattered along the GI tract forming the LP and IEL populations. The LP of teleost gut is known to harbor different types of immune cells, such as macrophages, granulocytes, lymphocytes and plasma cells (Salinas et al, 2011). The intraepithelial lymphocytes (IEL), generally are T and B cell populations, with T cells being the most abundant (Salinas et al. 2011). The sIgs present in the mucus layer are primarily tetrameric IgM and multimeric IgT. IgT has similar function to that of IgA within mammals, and may play a role in immune exclusion within teleost fish (Zhang et al. 2010). Gut associated bacteria are mostly coated by IgT and to a lesser extent by IgM. As seen in mammals, pIgR is expressed within the gut and the skin (Zhang et al. 2010; Xu et al. 2013). As explained earlier, pIgR is responsible for transporting sIgs across the

epithelial barriers. However, even though there are similarities between teleost and mammal gut mucosal tissues, little is known about the distribution of B cell subsets and Igs along the GI tract of teleosts (Salinas et al, 2011).

SALT was first coined in mammals by Dr. Streilein in 1985 in the Journal of Investigative Dermatology (Streilein 1985). It is important to note that teleost SALT is significantly different from mammalian SALT. The skin layer, which covers the outer surface of a fish's body and fins, also known as the integument, is a multifunctional organ. The skin has different morphological features at these sites that are specially adapted to help carry out these functions. The integument consists of two layers. The outer layer, the epidermis, is essentially cellular in structure, comprised of a multilayered epithelium that usually includes specialized cells. The inner layer of the skin, known as the dermis, has very few resident cells (Elliot 2011). However, it is known that the dermis can contain a wide variety of cell types, including pigment cells, scales, blood vessels, and nerves (Elliot 2011). The first indication of the presence of cutaneous immunity in fish dates back to the 1970's, where Fletcher described antibodies in the skin mucosal secretion of the plaice (Fletcher & White 1973). In 1985, the presence of lymphocytes in the skin of salmonid fish was first reported (Peleteiro and Richards 1985). This was done using light and electron microscopy. As a tissue layer, teleost SALT is a living skin layer that can replicate and divide, and does not have an exterior keratinized layer (Salinas et al, 2011). In the dermis of fish, bony scales replace what would normally be the connective tissue found in tetrapods (Esteban 2012). Scales are different among the many groups of teleost. The types of scales that are present include the scales of chondrichthyans (placoid scales), the scales of basal actinopterygians (ganoid scales), the

bony scales of some actinopterygian taxa (dermal bony scales and scutes) and the scales of basal sarcopterygian taxa and most actinopterygian species (elasmoid scales) (Sire & Akimenko 2004). Most of the scales in teleost belong to the elasmoid type (Sire & Akimenko 2004). As water flows past the scales, it comes in contact with all surfaces. However, the environments above and below the scales are very different, due to the amount of flow of current. As water passes past the anterior scales, the oxygen content may increasingly decrease as it moves towards the posterior. Thus, anterior scales may have more access to oxygen microenvironments than posterior scales. This great diversity of niches within fish skin anatomy allows for the opportunity of extremely diverse microbial communities.

In mammals, SALT is not generally considered a MALT. However, for consistency purposes, the same term is used for fish skin-associated lymphoid tissue, which forms diffuse MALT. Four types of secretory cells can be found in different fish epidermis. These four secretory cells are: malpighian cells, goblet cells, sacciform cells and club cells (Salinas et al, 2011). These tissues and cells have morphological and biochemical differences, and change with different species of teleost (Salinas et al, 2011). In addition to secretory cells, immune cells are present within teleost SALT. These include macrophages, granulocytes, mast cells, dendritic cells and plasma cells (Iger et al, 1988; Peleteiro & Richards 1990; Davidson et al, 1993; Herbomel et al, 2001). Though these leukocytes have been described in different species of teleosts, we are not entirely sure of the different functions they have during an immune response in teleost fish (Salinas et al, 2011).

Teleost gills are mucosal surfaces with the main purpose of uptaking oxygen from the water. Gills have an associated local immune system (GIALT) that has a diverse system of leukocytes and immune molecules. Anatomically, teleost gills usually consist of four gill arches which are supported by cartilage or bone tissue and are covered by an operculum (Wilson & Laurent 2002). Gill arches contain gill filaments, also known as primary lamellae, which are then further subdivided into gill lamellae (Wilson & Laurent 2002). These are also known as secondary lamellae (Wilson & Laurent 2002). Secondary lamellae are formed by a very thin epithelium that is supported by pillar cells, which allows for capillary flow of erythrocytes to these sections (Salinas et al, 2011). Water flow through the gills is countercurrent to blood within the secondary lamellae (Randall 1982). The external gill arch is the one most directly exposed to the environment (Wilson & Laurent 2002), and will be in the most contact with water, and thus oxygen exchange. Differences in oxygen exchange and environmental exposure create different niches for microbes within the gill. Gill epithelium is generally thicker than that of the epithelium lining the lungs of mammals (Randall 1982). Within the gills, mucus production occurs at a very high rate. It has been shown that mucus production at the gill cover area is higher than the production at any other skin site (Shephard 1994). These mucus secretions contain all the innate immune molecules previously described. Adaptive immune cells have also been discovered within gills including IgM and IgT B cells. Clusters of leukocytes were identified at the base of gill filaments, containing T cell receptors (Haugarvoll et al, 2008).

While the anatomy and immune responses at these teleost mucosal sites have been well characterized, the microbial communities present in fish MALT remain largely

uncharacterized. These microbial communities likely play a crucial role in maintaining homeostasis of the mucosal immune system and also modulating the local immune responses against pathogens.

1.5 Study of the Microbiome

The study of the physiological and immune system interactions that occur between the host and resident microbial populations has led to the study of multicellular microbiomes. Each of the resident communities that form symbiotic interactions with the multicellular host has a specific set of microbiota. This is the complete set of microbial lineages that live in a particular environment (Ley et al, 2008). A microbiome can be described as the complete collection of genes in the genomes of microorganisms that live in a particular environment (that is, the set of genes contained in a microbiota) (Ley et al, 2008). Each microbe possibly plays a functional role in the mucosal immune response, and the studies of these interactions have led to a greater understanding of these collaborations.

There have been four main methods used to study the microbial diversity in specific microbiomes. The last three methods are molecular based, whereas the first one is not. First, scientists used a culture and microscope based system. This system had specific problems, as many of the bacteria collected from tissue samples were very hard to culture, or would not grow (Morgan et al, 2013). As a consequence, this method of study leads to a very incomplete analysis and understanding of microbial communities in different organisms. Moreover, there was very minimal study of those bacteria that could not be cultured, until the advent of DNA sequencing techniques (Morgan et al, 2013).

Secondly, fingerprinting has been used to determine bacterial community diversity. Fingerprinting methods include terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), and temperature gradient gel electrophoresis (TGGE), (Anderson & Cairney 2004). Simply, fingerprinting involves amplification of a bacterial gene, not necessarily 16S, which is then applied to gel electrophoresis to separate community diversity (Hamady & Knight 2009). The bands will be separated based upon size, as each individual bacterial species will have some variability in gene length. The separation of bands then creates a banding pattern on the gel, which can be compared to a reference to determine community structure. Fingerprinting can be very cheap, and relatively effective. However, it does not divulge the full range of microbiota and microbiome data that next generation sequencing can provide. Though bands of separation can be detected and applied generally to bacteria, it becomes very difficult to assign this information to the species or genus level, as is possible in next generation sequencing. Also, it is difficult to relate small changes in banding patterns to species, or to compile all data into a single study (Hamady & Knight 2009). Third, Sanger sequencing allows for longer reads of bacterial genes to be sequenced. This sequencing technology adopts the whole genome shotgunning method of sequencing, which is very useful from a metagenomics perspective in which multiple genes can be analyzed for species richness. In the Sanger sequencing method, Sanger dideoxy chain terminator methodology is used to sequence DNA clone libraries which can have sequence lengths of 600 to 900 base pairs (bp) (Sanger et al, 1977). This process has close to 99.97% accuracy (Wommack et al, 2008). The downfalls of Sanger sequencing are the costs of sequencing, as well as the difficulty and inherent bias of

creating DNA libraries for sequencing (Wommack et al, 2008). More recently, the advent of 16S rRNA pyrosequencing has resulted in high throughput production of data for multiple samples, as opposed to singular sample processing in the Sanger method (Tringe & Hugenholtz 2008). 16S rRNA sequencing has become a mainstay of microbial community study over the past quarter century (Tringe & Hugenholtz 2008). With the advent of cheaper sequencing costs, as well as additional sequencing techniques, 16S rRNA data collection continues at a rapid pace (Tringe & Hugenholtz 2008). Particularly, sequencing using the 454/Roche pyrosequencing platform has virtually replaced the Sanger-based 16S rRNA sequencing method (Tamaki et al, 2011). Though it does not provide complete sampling of microbiome diversity and abundance, it however does offer an analysis for novel composition (Tamaki et al, 2011). The 454 GS FLX Titanium platform usually generates sequences of 400–500 bp. It can sometimes be very difficult to taxonomically assign a sequence length of less than 500 bp to the species or genus level (Engelbrektson et al, 2010). Thus, it becomes very important to pick primers for a variable region of the 16S rRNA that can yield enough information for taxonomical assignment (Engelbrekston et al, 2010). Pyrosequencing is significantly cheaper than Sanger sequencing, though it uses much shorter reads. This makes it unsuitable for metagenomic analysis, as amplification of only a single gene is required for pyrosequencing.

The Human Microbiome Project has sought to increase understanding of the microbial communities, and their contribution to normal physiology and disease (Turnbaugh et al, 2007). While studies have been conducted on numerous other organisms, the Human Microbiome Project is by far the largest in scale. Studies of the

human oral cavity, vaginal cavity, as well as the gut have revealed remarkable levels of bacterial diversity and the presence of distinct microbial communities (Dewhirst et al, 2010; Gill et al, 2006; Turnbaugh et al, 2007). Diverse skin samples have revealed that there are skin sites with more phylotypes than the oral cavity and the gut, as well as more phylogenetic diversity (Costello et al, 2009). However it appears that oral and stool samples have been seen to be particularly diverse, expanding upon previous knowledge (Huttenhower et al, 2012). Recently, it has been shown that 247 species level phylotypes are present within the human mouth (Bik et al, 2010). Most of the bacteria sampled for the Human Microbiome Project for the gut and oral cavity derive from fecal samples or oral swabs, respectively. These bacteria most likely come from the mucus and excretions within gut and oral mucosal epithelia, although some commensal microbiota can be intracellular. For example, it has been show that several different bacteria can adhere and invade the colonic epithelial cells within mice (Ohkusa et al, 2010) or that mucosal dendritic cells maintain live commensal bacteria in their cytoplasm for a few days (MacPherson & Uhr 2004). Understanding the composition of microbial communities at different locations of an organism can lead to understanding of potential functions by bacterial groups or species. The Human Microbiome Project provides a model for the study of microbiomes in other organisms.

1.6 The Microbiome of Teleost Fish

The first compilation of knowledge about teleost microbial flora was published by R.W. Horsley in 1977 (Horsley 1977). This knowledge has been expanded with work in

recent years on the microbiome of the model species the zebrafish (*Danio rerio*). Gnotobiotic zebrafish have been used to further understand the effects of a core microbiota on a large spectrum of biological processes, such as nutrient processing and development of the mucosal immune system (Rawls et al, 2004). Gnotobiotic zebrafish have also been used to detect selective pressures exhibited within the gut upon bacterial communities through reciprocal microbiota transplants with germ free mice (Rawls et al, 2006). A core gut microbiome appears to be present in zebrafish, consisting of the phyla Proteobacteria, Fusobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Chloroflexi, and Planctomycetes (Roeselers et al, 2011). Though there have been studies on the intestinal microbiomes of teleosts, (Desai et al, 2010; Sanchez et al, 2012; Navarrete et al, 2012; Rawls et al, 2004) the whole body microbiome of rainbow trout (*Oncorhynchus mykiss*) remains largely unidentified. In addition, no core microbiome in the gut of rainbow trout has been established, since different studies have reported different gut bacterial communities.

The gut of rainbow trout is by far the best characterized of all the rainbow trout mucosal sites in terms of its microbiota, as there have been comprehensive studies on the effect of diets on the gut microbial communities (Desai et al, 2012; Kim et al, 2007). Kim et al. reported a retrieval of 41 culturable phylotypes (Kim et al, 2007), which is significantly less than what has been seen in human microbiome studies. It was also noted that the mucus of the rainbow trout gut seemed to have a different population than the gut tissue, as evidenced by the absence of key genera seen in the gut tissue (Kim et al., 2007). The current dogma amongst fish biologists is that microbial communities associated with fish are similar to the microbial community present in the water

environment (Hansen & Olafsen 1999). However, 16S rRNA pyrosequencing of environmental water is not often included in the studies so far published, making this statement a speculation. Moreover, 16S rRNA bacterial sequences have largely not been processed for every fish mucosal site such as the skin, olfactory, and gills. In summary, a geographical map of the commensal microbial communities of fish using pyrosequencing techniques is not available.

Rainbow trout is a great model organism for microbiome research due to their extremely transient microbial environment. The mammalian microbiome was initially thought to be stable. However, recent studies continue to unravel how dynamic and temporary mammalian microbiomes can be (Martin et al, 2007; Nicholson et al, 2005; Blaser et al, 2013). In other words, fish and mammalian microbiomes may have more in common than originally acknowledged. Fish mucosal surfaces, unlike their mammalian counterparts, are constantly in contact with the outside environment (water circulates through them). Importantly, water sustains microbial growth better than air, thus fish live in microorganism-rich environments compared to terrestrial vertebrates. A complex relationship with both beneficial and pathogenic microbes exists between the fish host and the aquatic environment. Fish species such as rainbow trout or zebrafish are commonly used in the field of comparative immunology and, for this reason, reagents and immune assays are available to study the immune system of these species. Fish models can therefore help advance our understanding on how mucosal immunity maintains homeostasis with commensals in health and disease.

1.7 Figures and Figure Legends

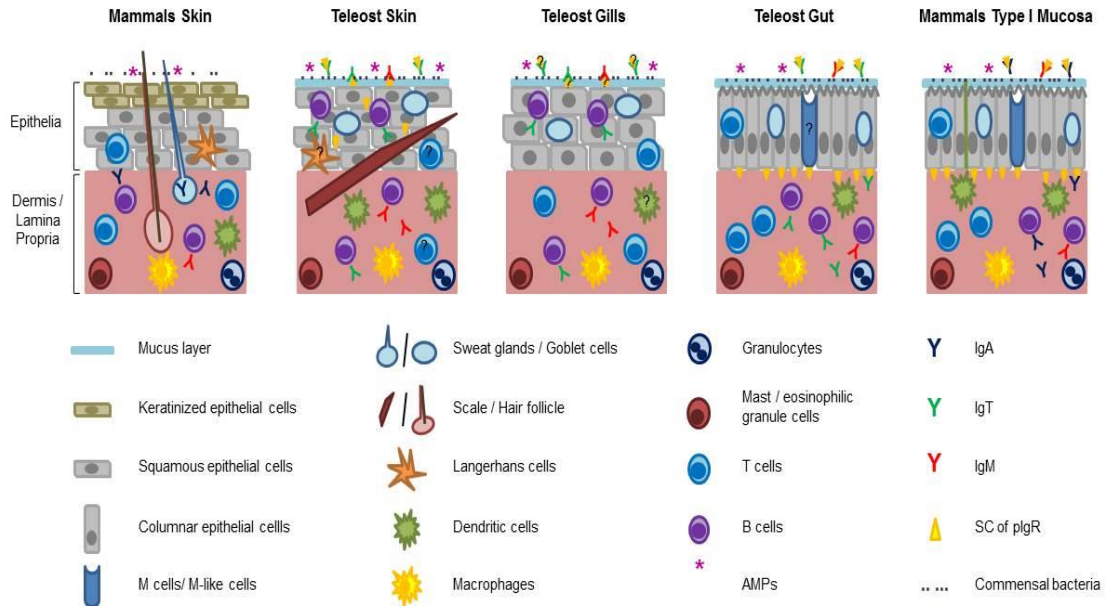

Figure 1: Esquematic representation of the similarities and differences between teleost fish skin, gills and gut, and mammalian skin and type I mucosal surfaces. Structural differences in the type and number of layers of epithelial cells, as well as the presence of keratin or mucus (and mucus producing cells) are displayed in the upper half of each diagram. In the bottom the connective tissue, named dermis in skin and lamina propria in gut and gills, is shown. Similarities in the cellular components of the innate immune system (Langerhans cells, dendritic cells, macrophages, granulocytes and mast cells) are also displayed. Differences in the localization of B and T cells, the isotype of immunoglobulins and the presence of the secretory component (SC) of the polymeric immunoglobulin receptor (pIgR) are represented as well. Finally, the presence of commensal bacteria and antimicrobial peptides (AMPs) is shown in the outer mucosal surface or over the keratin layer. Elements that are suspected to be present in a tissue, but have not been studied so far are marked as unknown (?) (Gomez et al, 2013).

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Chapter 2

Topographical mapping the rainbow trout (*Oncorhynchus mykiss*) microbiome reveals a highly diverse and potentially antifungal resident bacterial community living within the skin epithelium

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Keywords: microbiome, teleosts, rainbow trout, skin epithelium, antifungal properties

2.1 Abstract

The mucosal surfaces of wild and farmed aquatic vertebrates face the threat of many aquatic pathogens including fungi. These surfaces are colonized by diverse symbiotic bacterial communities that may contribute to fight infection. Whereas the gut microbiome of teleosts has been extensively studied, the composition of the bacterial communities present at other mucosal surfaces of teleosts remains uncharacterized. Here we provide for the first time a complete map of the mucosal microbiome of an aquatic vertebrate, the rainbow trout (*Oncorhynchus mykiss*). Using 16S rRNA pyrosequencing, we reveal novel bacterial diversity at each of the five body sites sampled and show that body site is a strong predictor of the community composition. The skin has the highest diversity followed by the olfactory organ, gills and gut. *Flectobacillus* sp. is highly represented within skin and gill communities. PCoA analysis and plots revealed clustering of external sites apart from internal sites. A highly diverse community is present within the epithelium as demonstrated by confocal microscopy and pyrosequencing. This intraepithelial resident microbiota in trout highlights the presence specific adaptations in teleost fish, which may allow aquatic vertebrates to better exploit microbiota-derived benefits without adding swimming drag forces. This intraepithelial bacterial community, as well as the skin, olfactory organ and gill bacterial communities, contain high numbers OTUs with potentially antifungal properties. This indicates the potential contribution of these communities against aquatic fungal pathogens such as Saprolegnia sp. These results underscore the importance of symbiotic bacterial communities of fish in the control of emerging aquatic fungal diseases.

2.2 Introduction

The mucosal surfaces of vertebrate animals are at the interface between the environment and the animal host. Mucosal epithelia form important mechanical, chemical and microbiological barriers that prevent pathogen invasion but permit colonization by beneficial symbiotic microorganisms, the microbiota. The microbiota is crucial for the development, homeostais and immune function of animal's mucosal epithelia (Cebra 1999; Sellon et al, 1998; Lee and Mazmanian 2010).

The association between metazoans and commensal microorganisms is one of the most ancient and successful associations found in nature (McFall-Ngai et al, 2013; Fierer et al, 2013). The microbial communities of different organisms such as plants, corals, annelids, gastropods, insects and many vertebrates are being characterized. In the particular case of vertebrates, mucosal surfaces have undergone drastic changes over the course of evolution due to the transition of vertebrate animals from water to land. These evolutionary pressures especially affected some mucosal barriers such as the skin. While the skin of fish is a living cell layer that secretes a mucous layer and has imbricated scales for protection (Schempp et al, 2009), amphibians have a cornified layer of skin that has developed into a more uniform epidermis (Schempp et al, 2009). Finally, in birds and mammals, the presence of feathers, scales, hair, sweat glands, coats or the leather-like thickening of the dermis represent unique adaptations to terrestrial environments. All these structures and appendages, in turn, provide unique niches within the skin for microbial colonization (Schemp et al, 2009; Belkaid & Naik 2013).

The vertebrate transition from water to land likely affected the relationships between the host and the microbiota. Water is a microbial-rich environment that promotes bacterial growth compared to air. In other words, aquatic vertebrates have evolved mechanisms to benefit from symbiotic bacteria in an external environment where these microorganisms thrive. These symbiotic bacteria help the aquatic hosts to fight against mucosal pathogens. For example, the mucosal microbiota of aquatic vertebrates can function to protect against fungal pathogens such as the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) affecting amphibians (Harris et al, 2009), or *Saprolegnia sp.* affecting fish and amphibians (Liu et al, 2014; Petrisko et al, 2008). It is clear that the mucosal surfaces of wild and farmed aquatic vertebrates along with their associated microbiota play a critical role in the control of emerging aquatic diseases.

The Human Microbiome Project has offered revolutionary insights into the different microbial communities present at different mucosal surfaces (Erb-Downward et al, 2011; Turnbaugh et al, 2007; Grice et al, 2009). While the gut is by far the best characterized site, it is now clear that distinct microbial communities inhabit different anatomical sites such as the gut, mouth, skin, and vaginal cavity, and each site contains different ratios of major groups of bacteria (Koren et al, 2013). Thus, body site is a strong determinant factor for the composition of the microbiota in terrestrial vertebrates. However, detailed topographical maps of these communities in other animal species are currently missing. The main mucosal barriers of teleost fish are the gut, skin and gills. As in mammals, they form the interface between the host and its environment. Teleost fish gut, skin and gills are known to harbor complex microbial communities (Salinas et al, 2011; Xu et al, 2013). Though there have been a number of studies on the intestinal

microbiomes of teleosts (Desai et al, 2012; Sanchez et al, 2012; Rawls et al, 2004; Navarrete et al, 2012), the diversity present at other mucosal sites remains largely unexplored. A core gut microbiome appears to be present in zebrafish (*Danio rerio*), consisting of the phyla Proteobacteria, Fusobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Chloroflexi, and Planctomycetes (Roeselers et al, 2011).

The purpose of this study is to fingerprint the microbial communities present at each of the mucosal surfaces of healthy hatchery-reared rainbow trout (*Oncorhynchus mykiss*) using high throughput sequencing. We provide the first topographical map of the microbiome of a teleost species, and describe the relative contribution of potentially antifungal bacteria found at different body sites. Our results contribute to our understanding of the evolution between commensals and mucosal surfaces in vertebrates.

2.3 Materials and Methods

Animals and tissue samples

Six hatchery-reared adult female triploid rainbow trout (*O. mykiss*) were obtained from the Lisboa Springs Hatchery in Pecos, New Mexico. The average length of the fish was 11.25 inches from head to tail and mean weight was approximately 220g. Fish were maintained in the hatchery raceways in an open water circulation system from the Pecos River. Sampling was conducted in October 2012, when water temperatures are approximately 13°C. Fish were starved for 48h prior to sampling. Rainbow trout were first euthanized using an overdose of MS-222. Skin, gills, olfactory rosettes, anterior gut and posterior gut tissue samples were collected. The sampling scheme was selected based

on the main physiological and physicochemical properties of these sites, which are likely to generate different habitats for bacteria (Table 1). Skin samples were 1cm^2 in size and were obtained above the lateral line on the left side of the fish. Gill samples were taken from the second left gill arch for consistency purposes. Both olfactory rosettes were removed from the olfactory cavity after removing the skin covering. Anterior gut samples (1cm long) were collected immediately after the stomach whereas posterior gut samples (1cm long) were obtained 1 cm before the anus. Figure 1 shows the sampling scheme used in the present study. Samples were placed in sterile sucrose lysis buffer and stored at -80°C until processing.

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Mexico, protocol number 12-100854-MCC.

DNA isolation, bacterial 16S rRNA PCR amplification and pyrosequencing

Sterile tungsten beads were used to lyse the tissue samples. Total genomic DNA was extracted from whole tissue samples, including both fish and bacterial DNA. 200µL of homogenized sample was added with 400µL of CTAB buffer and 3µL of 3% proteinase K solution to centrifuge tube. This solution was incubated at 60ºC for 1h, with inversion of samples occurring every 15min. Incubation was followed by addition of 120µL of 10% SDS detergent. Incubation occurred for one hour with inversion at regular periods of 15min. Samples were then incubated overnight at -20ºC for precipitation. Addition of 1 volume of 25:24:1 phenol isoamyl-alcohol chloroform to sample tubes then occurred. Tubes were inverted and mixed, and centrifuged at 13000 rpm for 5min at 4ºC.

Supernatant was removed and an equal volume of chloroform was added. Tubes were inverted and mixed, and centrifuged at 13000 rpm for 5min at 4ºC. Chloroform separation was repeated twice, until samples were clean. Supernatant was then removed, and 0.1 volumes of 3M NaOAc as well as 2 volumes of 95% ethanol were added. Samples were then incubated overnight at -20ºC for precipitation. Supernatant was removed and DNA pellets were first air dried to remove excess ethanol. Pellets were then re-suspended in 30µL of DNase/RNase free molecular water. Samples DNA concentration and purity was measured in a Thermo Scientific Nanodrop 2000c.

Bacterial community composition was determined using barcoded pyrosequencing.. Fourteen twelve-bp barcodes were used to provide high throughput analysis. Total genomic DNA was amplified using barcoded V1-V3 16S rRNA gene primers (A17F 5' GTTTGATCCTGGCTCAG 3', 519R 5' GTATTACCGCGGCAGCTGGCAC 3') (Kumar et al, 2011), with initial activation of the enzyme at 94°C for 2min, followed by 33 cycles of 94°C for 30s, 55°C annealing for 30s and 72°C for 1min and 30s. Amplification finished with a 10min extension cycle of 72°C. Melting temperature of the primers was determined by primer design, as well as optimal gradient trial runs in a thermocycler. PCR products were run in a 2.0% agarose gel to confirm amplification. In the event that amplification did not occur using the original A17F and 519R primers, a semi-nested PCR was used, with the first round consisting of the original forward, A17F, and the reverse primer P934R, 5' ACCGCTTGTGCGGGYC 3' (with Y being C or T). After amplification of this larger band, a semi nested PCR with the original primers, A17F and 519R, was run. This process occurred for only 2 samples, a skin and a gill sample (fish 5 and 2 respectively).

We still were unable to amplify the 16S rRNA from three samples. A single band of approximately 500bp was extracted using the Invitrogen E-Gel® SizeSelect™ system. Each sample was run in triplicate. After amplification, samples were pooled into libraries and sequenced on a Roche 454 GS FLX Platform with Titanium reagents at the Molecular Biology Facility at the University of New Mexico.

Sequence Analysis

We obtained an average of 6,490 sequences per sample, with a minimum and maximum of 1,665 and 14,135 sequences respectively. In order to account for 454 sequencing base errors, as well as errors due to PCR amplification, and low quality products, the final sequences from the Roche 454 GS FLX Titanium platform were processed with Ampliconnoise (Quince et al, 2011). This included chimera checking with Perseus (Quince et al, 2011) to ensure proper diversity estimates. All sequence analyses were performed in Quantitative Insights Into Microbial Ecology (Qiime version 1.8) pipeline (Caporaso et al, 2010) with default settings. Operational taxonomic units (OTUs) were aligned to the Greengenes August 2013 (DeSantis et al, 2006) database at 97% identity, and those that did not match were subsampled at 10% of the failed aligned reads and clustered to determine new reference OTUs. Taxonomic summaries were produced to compare bacteria occurring at the five body sites sampled and the epithelial layer sampled by laser capture microdissection described below.

To determine level of sequencing depth, rarefaction curve analysis was conducted using Qiime. 1,600 sequences was the lowest number of reads for all of our samples, so for consistency purposes we rarefied all samples to this depth. Alpha diversity metrics

included Shannon diversity index, chao1, PD, Good's coverage and number of OTUs, as well as number of phyla and genera were compared among body sites. Microbial diversity among samples (beta-diversity) was evaluated with Qiime using weighted and unweighted UniFrac, a phylogenetic distance metric that measures community similarity based on the degree to which pairs of communities share branch length in a common phylogenetic tree (Lozupone et al. 2011). Principle coordinate analysis, core microbiota analysis and unique OTUs analysis were also performed in Qiime.

Proportion of inhibitory OTUs with antifungal properties

We generated a database of 1,255 16S rRNA gene sequences from cultured bacteria isolated from amphibian skin using published data sets. A number of studies have tested these isolates for bioactivity against fungal pathogens including *Batrachochytrium dendrobatidis*, *Mariannaea elegans* and *Rhizomucor variabilis* in co-culture challenge assays (Belden & Harris 2007; Harris et al, 2009; Lauer et al, 2007; Lauer et al, 2008; Woodhams et al, 2008; Lam et al, 2010; Walker et al, 2010; Stevenson et al, 2013; Flechas et al, 2012; Becker unpublished, Davis unpublished, Holden unpublished, Woodhams unpublished). Because freshwater fish have similar mucosal defenses and fungal pathogens to amphibians, we used this database to generate a list of OTUs by clustering sequences at 97% similarity using the Greengenes August 2013 reference. The full Sanger sequences (mean 1074.8 bp) of antifungal isolates included 819 isolates clustered into 291 OTUs. This list was expanded to include potentially inhibitory neighboring OTUs within 0.1 Jukes-Cantor distance on the Greengenes phylogenetic tree (7,266 OTUs). We then filtered our OTU table in this study to retain only the potentially antifungal OTUs (180 OTUs found). We compared the relative abundance of antifungal

sequences among the five trout body sites sampled and tested for differences among site by Kruskal-Wallis test.

Fluorescent in situ hybridization, microscopy and laser capture microdissection

Skin (n=6) was snap frozen in OCT (TissueTek) and 5 μm-thick cryosections were obtained and stained with 5' end labelled indodicarbocyanine-labeled EUB338 and indodicarbocyanine-labeled NONEUB (control probe complementary to EUB338) oligonucleotide probes (Eurofins MWG Operon). EUB targets the 16S rRNA of ~90% of all eubacteria. Details on oligonucleotide probes are available at probeBase. Hybridizations were performed at 37°C for 14h with hybridization buffer (2x SSC/50% formamide) containing 1μg/ml of the labeled probe. Slides were then washed with hybridization buffer without probes followed by two more washes is washing buffer (0.1x SSC) and two washes in PBS at 37°C. Nuclei were stained with DAPI (5ng/ml) solution for 25min at 37°C. Slides were mounted with fluorescent mounting media and images were acquired and analyzed with a Nikon Ti fluorescence microscope and the Elements Advanced Research Software (version 4.0) and with a Zeiss LSM 780 confocal microscope and the Zen software.

Additionally, skin cryosections from two different rainbow trout specimens were used for laser capture microdissection (LCM) using an ArcturusXT LCM microscope (Applied Biosystems). The epithelium from six 5 μm-thick sections from each fish was captured and pooled into one sample for DNA purification. As a negative control, muscle underlying the dermis was also dissected. Total DNA was extracted from LCM the epithelial layer (including epithelial cells and goblet cells, see Supplementary Fig. 1) or

muscle cells using Arcturus PicoPure DNA Isolation Kit (Applied Biosystems) following manufacturer's instructions. DNA was subject to the same PCR amplification and pryosequencing protocols as those explained for the rest of the samples in this study. Muscle dissected samples failed to amplify by PCR (not shown).

Statistical analysis

Differences in alpha-diversity among body sites were tested by Kruskal-Wallis tests in IBM SPSS Statistics v.22. To test for significant differences in community composition among body sites we used non-parametric multivariate analysis of variance (Adonis) and analysis of similarity (ANOSIM) in Qiime.

2.4 Results

General aspects of rainbow trout bacterial communities characterized by 454 pyrosequencing

Out of the six fish samples for each mucosal site, one anterior gut, one olfactory organ, and one skin failed to amplify the 16S rRNA. Those samples were therefore not included in our analyses.

The number of reads obtained for each individual sample ranged between 14,135 and 1,665 reads except for one skin sample that only produced 600 sequences. Thus, in order to normalize inter-sample variability all analyses were performed using 1,600 sequences. This excluded the skin sample with 600 sequences. Shannon-diversity differed significantly among body sites (Kruskal-Wallis test, $P = 0.006$) (Fig. 2a). The anterior gut

had a significantly lower diversity index than the rest of the body sites, the highest being the skin. Phyla richness analysis (number of unique phyla per individual sampling site at 1,600 sequences) revealed that the skin was the most diverse site followed by the olfactory organ, gills, and both gut sites (Fig. 2b). Total numbers of unique phyla came from addition of all unique phyla discovered at the respective body site. Total numbers include all replicates. For the skin, up to 17 total different phyla were present (mean of 10.5). The olfactory organ, gills, anterior gut and posterior gut had a total of 18, 14, 13, and 13 phyla, respectively (with a mean phyla richness of 10, 8.5, 6.8, and 9; respectively). Analysis at the genus level showed a higher number of total genera present within the skin than at any other site (total 199). The total number of genera found in each sample is presented in Fig. 2c. After the skin, the most diverse sites at the genus levels were the olfactory organ, gills, posterior gut and anterior gut (total 187, 140, 118, 104; respectively). We report Good's coverage values ranging from 93.9% to 99.9%. The mean values for good's coverage at the anterior gut, posterior gut, gills, olfactory organ, and skin were 98.4, 98.2, 97, 97.6, and 97.3% respectively. Faith's PD mean values for anterior gut, posterior gut, gills, olfactory organ, and skin were 4.4, 6.0, 7.9, 10, and 10.4 respectively.

The composition of the skin microbiome of rainbow trout

The skin microbiome contained the highest diversity at the genera level of bacterial communities of all sampled sites in the present study. A total number of 17 phyla were observed with Proteobacteria, Actinobacteria, Bacteriodetes and Firmicutes being the most dominant phyla (Fig. 3a-c). While the skin had one less phylum than the olfactory organ, the number of genera represented was the highest among all body sites with 199

different genera. Skin had a maximum of 288 OTUs observed, with a minimum of 46. The mean value of OTUs observed was 152. Overall, all fish sampled showed a similar bacterial community composition indicating that these phyla represent the core microbiome of the skin of rainbow trout. At the genus level, the bacterial community was consistently composed by *Flectobacillus* sp. in the family *Flexibacteriaceae* which accounted for 3.4 - 10.6% of the total bacterial community (Supplementary Fig. 2). *Flavobacteriaceae, Propionibacteriaceae,* and *Streptococcaceae* accounted for 3.0 – 24.0%; 5.0 - 5.6%; and 2.8 – 16.0% of the sequences, respectively.

The composition of the gill microbiome of rainbow trout

The gill microbiome was the third most diverse of the sites sampled in this study with a total number of 14 different phyla. Gills had a maximum of 180 OTUs observed, with a minimum of 39. The mean value of OTUs observed was 95. The gills showed the highest level of inter-individual variability, as shown in Supplementary Fig. 3 and Table 1. The dominant phyla were Bacteriodetes and Proteobacteria (Fig. 3a-c). At the genus level, the diversity of the gill bacteria community was lower than that of the skin (Fig. 3c and Supplementary Fig. 2). The dominant genera included *Flectobacillus* sp., *Flavobacterium* sp. and the *Commamonadaceae* family (Supplementary Fig. 2). *Flectobacillus* sp. was present in all gill samples although in one fish it only accounted for 1.8% of all reads. In the rest of the samples *Flectobacillus* sp. contributed to 0.1 - 35.3% of all sequences. *Flavobacterium* sp., on the other hand, comprised between 7.7 - 61.7% of the bacterial community of the gills.

The composition of the olfactory organ microbiome of rainbow trout

The bacterial community of the olfactory organ contained 18 total phyla (Fig. 3a), with the highest number of phyla present among all body sites. The olfactory organ had a maximum of 186 OTUs observed, with a minimum of 95. The mean value of OTUs observed was 133. The community was dominated by Proteobacteria, Actinobacteria, Bacteriodetes and Firmicutes (Fig. 3a-c). At the genus level, inter-individual variability was present (Supplementary Fig. 2). The class Betaproteobacteria (undetermined genus) accounted for 15.1 - 53.6% of all sequences. The genus *Staphylococcus* sp. comprised 0.1 -6.6% of the bacterial community (Supplementary Fig. 2). The family *Streptococcaceae*, in turn, was present at 0.1 - 7.8%.

The composition of the anterior and posterior gut microbiome of rainbow trout

The anterior and posterior gut bacterial communities were similar to each other. In terms of total numbers of phyla, 13 phyla were observed in the anterior gut and 13 in the posterior gut (Fig. 1a). The anterior gut had a maximum of 136 OTUs observed, with a minimum of 3. The mean value of OTUs observed was 45. The posterior gut had a maximum of 160 OTUs observed, with a minimum of 20 and a mean value of 63 OTUs represented. The anterior and posterior guts showed the lowest level of inter-individual variability, as shown in the distance plot analysis (Supplementary Fig. 3 and Supplementary Table 1). These sites were the only to contain sequences which were not classified to bacteria. Considerable variability amongst individuals was present. Both gut sample sites were dominated by Tenericutes whereas Proteobacteria, Firmicutes, Cyanobacteria, Bacteriodetes and Actinobacteria were also present. At the genus level, *Mycoplasma* sp. dominated both the anterior and posterior gut samples (Supplementary Fig. 2).

Core microbiome analysis and comparisons across body sites

The core microbiome analysis (Fig. 3b) revealed that the core microbiota that accounts for 50% of the total diversity is very distinct in each of the body sites sampled. Generally speaking, the core microbiota among external sites (skin, olfactory organ and gills) was more similar and distinct from the anterior and posterior gut samples.

Principal coordinate analysis using the weighted Unifrac distance matrix (Fig. 4) indicates a clear separation between the microbial communities present at external and internal body sites. Internal sites were tightly clustered while external sites were more loosely grouped, indicating some commonalities in community structure, while still revealing unique groups present at each site. Anosim and Adonis analyses confirmed that body site is a significant predictor of variability in bacterial communities of rainbow trout, with both P values being less than 0.005 (0.001).

The rainbow trout skin possesses a rich intraepithelial microbiome

16S FISH experiments revealed that bacteria reside within the epithelial layer of rainbow trout. Confocal microscopy studies show that bacteria were associated with both epithelial cells and goblet cells (Fig. 5a and Supplementary Fig. 4). Further experiments using LCM successfully amplified the 16S rRNA genes of the intraepithelial bacterial community. The composition of this community at the phyla level is shown in Fig. 5b and compared to the total skin microbial associated community. Strikingly, a total of 10 different phyla and 53 different genera were present inside the skin epithelium of two rainbow trout specimens (pooled sampled). The intraepithelial community was enriched in two major groups: *Propionibacterium* sp. and *Staphylococcus* sp. which accounted for

22.5% and 14.5% of the total intraepithelial diversity respectively, compared to 5.6 - 6.8% and 3.0 - 3.5% in the total skin microbiota (mucus and epidermis combined).

Inhibitory OTUs with antifungal properties analysis

Trout samples contained up to 28.6% antifungal bacterial sequences matching those from a database developed from amphibian cultures. Proportions differed significantly among body sites (Kruskal-Wallis test, $p = 0.015$). The gills, skin, and olfactory organ had higher proportions of potentially antifungal bacteria than either the posterior or anterior gut (Fig. 6a).

Compared to other body sites, gills host abundant *Flavobacterium sp.* and various Comamonadaceae and Oxalobacteraceae (Fig. 6b). The skin and olfactory organ both contain members of 10 out of 12 taxonomic orders of antifungal bacteria found, while members of these orders are much less abundant in the gut (Fig. 6b). The intraepithelial bacterial community also contained potentially antifungal OTU's. Antifungal total skin communities were dominated by Flavobacteriales whereas Bacillales dominated skin intraepithelial community (Fig. 6c). The range of antifungal OTUs was 10-35 in the skin and 14 by LCM. The proportion of antifungal sequences in total skin ranged from 16.2-23.7% compared to 21.5% in the pooled LCM epithelium sample.

2.5 Discussion

Aquatic vertebrates are completely covered by mucosal epithelia that are at the interface between the host and the environment. Teleost fish mucosal surfaces are highly specialized to provide critical physiological functions, such as nutrient uptake in the gut or gas exchange in the gills. Importantly, the skin of teleost fish is significantly different

to that of terrestrial vertebrates. Teleost skin produces a mucus layer, it is composed of living cells, it lacks keratin, and it possesses defense components such as scales.

All mucosal epithelia have an associated symbiotic bacterial community. The composition of this community is determined by the specific habitat that each of the body sites offers to symbiotic microorganisms as well as those permitted by the local immune system. In the case of fish, there have been extensive studies on the gut microbiome of rainbow trout, as well as how the gut microbiome is affected by dietary changes (Desai et al, 2010; Sanchez & Wong 2012; Navarrete et al, 2012; Rawls et al, 2004; Kim et al, 2007). These studies represent a thorough analysis of the interior mucosal microbiome of rainbow trout, however the microbial communities present at exterior body sites have not been previously characterized. The present study represents the first analysis of a teleost fish microbiome across its main mucosal body sites.

Using 16S rRNA pyrosequencing we reveal here the presence of distinct bacterial communities across several teleost body sites. Body site is a key determinant of microbiota composition in other vertebrate species. Our results therefore indicate that the specialization of symbiotic bacteria with particular body sites emerged early during vertebrate evolution. Importantly, the core bacterium of internal sites (anterior and posterior gut) was markedly different from that of the external sites sampled in this study. Differing tissue architecture and chemical properties can lead to differences in potential niche space for microbial communities to establish. Whereas fish gut epithelial cells are ciliated and the gut contains intestinal folds to increase surface area, fish skin harbors unique niches with scales increasing potential surface area for colonization. The gills and olfactory organ also have unique architecture and functional differences, which can affect

communities present at these sites. Moreover, chemical properties such as pH and oxygen saturation at tissue surfaces can impact aerobic and anaerobic species, selecting for groups that are more tolerant to a specific microenvironment. The higher diversity observed in external sites may be a reflection of niche and environmental diversity, whereas the gut may offer more stable habitats that shape specialized microbial communities.

Out of all five sites sampled, the skin showed the highest bacterial diversity in rainbow trout. We obtained a total of 17 different phyla present within the skin tissue samples, a comparable number to that found in both amphibian and human skin communities (McKenzie et al, 2011). While this number of phyla was not as high as the diversity found in the olfactory organ (18), there were more genera of bacteria present within the skin compared to the olfactory organ (199 to 187 respectively). Interestingly, the most represented phyla were Proteobacteria followed by Bacteriodetes. This is very different to the skin microbiota of terrestrial or semi-terrestrial vertebrates. For instance, in humans the skin microbiota is mostly composed by Firmicutes and Actinobacteria, in dogs is composed by Proteobacteria, Actinobacteria and Firmicutes whereas in amphibians is dominated by Betaproteobacteria (McKenzie et al, 2011; Hoffmann et al, 2014; Huttenhower et al, 2012). In aquatic larval amphibians and interestingly the humpback whale, a marine mammal, Proteobacteria and Bacteriodetes dominate the skin microbiome (Apprill et al, 2014; Kueneman et al, 2014).

Previous studies had concluded that teleosts have low numbers (10^2-10^4/cm^2) of bacteria associated with the skin and in some cases bacteria were not even observable using microscopy methods (Crouse-Eisnor et al, 1985; Austin 2006). Whilst the latter may be

true with regards to numbers of culturable bacteria, our results indicate that there is a very diverse microbiota living in association with the skin of rainbow trout. Interestingly, both the gill and skin microbiota was highly represented by one genus, *Flectobacillus* sp., which accounted for 14.2-35.3% of the diversity in the gills and 3.4-10.6% in the skin. It is likely that the association with *Flectobacillus* sp. brings particular benefits to rainbow trout at the gills and skin surfaces. Future studies will address which beneficial interactions are involved.

Our initial pyrosequencing results and the finding that the skin is the most diverse site in trout led us to hypothesize that some of the diversity may be associated with bacterial colonization of the trout skin epidermis. FISH 16S staining revealed that bacteria in fact live within the skin epithelium of trout. Bacteria were observed within the epithelium and next to goblet cells. The different appendages and structures present in the skin of mammals provide unique habitats for the colonization of particular microbial species (Grice et al, 2009; Rosenthal et al, 2011). In the case of rainbow trout, we found a strikingly diverse bacterial community living inside the epithelium. This community was characterized by the phyla Firmicutes and Actinobacteria, particularly *Propionibacterium* sp. and *Staphylococcus* sp. that were represented at higher proportions than in the total skin microbiome (mucus and epithelium). These phyla were also highly dominant in skin communities of the human microbiome (Huttenhower et al, 2012). Interestingly, *Propionibacterium* sp. colonizes various niches of the human body; particularly the sebaceous follicles of the skin and *Staphylococcus* sp. can live within human keratinocytes (Kintarak et al, 2004) and in deeper skin layers (Nakatsuji & Gallo 2014). It has also been discovered that *Staphylococcus warneri* inhabits the skin epidermis of

rainbow trout and has potential pathogenic characteristics (Musharrafieh et al, 2013). Thus, these two groups of bacteria are well known to exploit specific niches within the skin of vertebrates likely due to the fact that they are facultative anaerobes.

We propose two possible explanations for the "permissive" properties of trout skin towards bacterial colonization. First, teleost skin consists of living epithelial cells instead of keratinized dead cells present in terrestrial vertebrates. Thus, living cells may provide a more beneficial environment for microorganism colonization. Second, the continuous swimming forces make skin mucus a difficult environment for bacterial attachment. This means that symbiotic bacteria may have evolved to preferentially colonize the interior of the epidermis rather than the external mucus layer. This adaptation is also beneficial to the fish host because high number of mucus-associated bacteria would increase the drag forces and decrease swimming performance. Thus, internal colonization of the skin microbiota is advantageous to both the bacteria and the fish host.

One of the benefits that vertebrates draw from establishing symbiosis with bacteria is the production of antimicrobial compounds that help them fight pathogens. In the case of aquatic vertebrates, the skin, gills and olfactory organ can all be main portal of entry for disease agents. Particularly, fungal pathogens such as *Saprolegnia sp*. are a threat for fish and amphibians worldwide (Liu et al, 2014). One of the dominant antifungal members of the fish epithelium is *Staphylococcus epidermidis* (Fig. 6c), a species thought to interact with host immune functioning (Nakatsuji & Gallo 2014) and involved in protection against pathogen infection in mice (Naik et al, 2012). Our studies, and others, support the idea that the bacterial communities associated with external fish surfaces (i.e skin, gills and olfactory organ) are a key mechanism for the host to fight fungal disease agents. Our

findings will help mitigate the impact of fungal pathogens in farmed and wild fish species, particularly salmonids.

As part of the topographical mapping effort of this study we sampled anterior and posterior gut tissue from adult hatchery reared rainbow trout. Our results show a lack of strong differences between anterior and posterior gut. The main phylum present was Tenericutes, with *Mycoplasma* sp. being the predominant genus. This bacterium was ubiquitous within all gut samples, comprising the majority of reads. The presence of large numbers of Tenericutes present in the gut microbiome is in agreement with multiple studies in vertebrate animals, including the porcine gut (Leser et al, 2002) and oyster gut (King 2012). The distal gut microbiome of farmed and wild salmon is dominated by *Mycoplasma* sp. as well (Holben et al, 2002). However, our results are in disagreement with previous studies in rainbow trout (Wong et al, 2013; Sanchez & Wong 2012; Navarrete et al, 2012; Desai et al, 2010; Kim et al, 2007), in which Proteobacteria, Firmicutes, and Actinobacteria represented the majority of phyla. However these studies utilized primarly DGGE analysis and/or did not target the V1-V3 region of the 16S rRNA through pyrosequencing. Furthermore, differences in gut microbial composition may be due to primer design, region of amplification, as well as differences between laboratory and hatchery raised fish. In addition to these factors, absence of fecal contents in our gut samples could contribute to certain bacteria being overrepresented.

In conclusion, this study provides the first detailed topographical map of the microbial communities that are present at the different mucosal sites of a non-tetrapod aquatic vertebrate species, the rainbow trout. Importantly, we have untapped the great diversity associated with the skin of teleosts, previously thought to be an almost sterile site.

Furthermore, we demonstrate here that almost 50% of the skin microbial diversity is found within the epithelium itself. This may be a specific adaptation in fish species living in high current waters so that the skin can benefit from symbiotic bacteria without adding external drag forces. Finally, the bacterial communities associated with trout external mucosal surfaces contain species that are known to produce inhibitory substances against fungal pathogen. Thus, our results not only have important implications from an evolutionary point of view but also for the control of emerging fungal diseases in wild and farmed fish.

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2.7 Figures and Figure Legends

Table 1: Physiological and physicochemical properties of rainbow trout mucosal body sites.

Figure 1: Rainbow trout tissue sampling strategy.

Figure 2: Comparison of bacterial diversity present at rainbow trout mucosal body sites. a) Shannon-diversity index for each body site. Curves calculated as a total from all individuals at each body site. b) Total phyla present at each individual sampling site. Each dot represents an individual sample, horizontal lines represent average values. c) Total genera present at each individual sampling site. Each dot represents an individual sample, horizontal lines represent average values.

Figure 3: Composition of the bacterial microbiome of rainbow trout at different body sites. a) Bar chart of abundance of phyla present at each site and in each individual fish sampled. Total length of bar is equivalent to 100% of the OTUs found. OTUs matched at 97% identity to Greengenes August 2013 database. b) Area chart of core microbiome analysis. Represents bacteria present in 50% of samples at each body site. OTUs matched at 97% identity to Greengenes August 2013 database. c) Map of the bacterial microbiome of rainbow trout at each body site at the phyla level.

Figure 4: Three dimensional principal coordinate analysis plot (PCoA), obtained with the weighted UniFrac distance matrix, comparing the bacterial communities present at each of the sampled body sites. Each dot represents an individual fish. Principal coordinate 1 (P1) vs principal coordinate 2 (P2) vs principal coordinate 3 (P3) are represented.

Figure 5: Rainbow trout skin has a diverse intraepithelial bacterial community. a) Confocal microscopy image of a rainbow trout skin cryosection stained with Cy5-EUB338 oligoprobe by FISH and scanned from above. Bacteria are shown in green. Nuclei were stained with DAPI (blue). b) Bar chart of abundance of phyla present within the LCM sample and all skin samples. Skin 3 is included in this analysis with 600 sequences, and all skin samples as well as the LCM are rarefied to this measure. Total length of bar is equivalent to 100%. OTUs matched at 97% identity to Greengenes August 2013 database.

Figure 6. Potentially antifungal bacteria found on rainbow trout *Oncorhynchus mykiss*. a) Antifungal bacteria differ in abundance among body sites (Kruskal-Wallis test, P = 0.015). Mean number of sequences and standard error displayed. b) Heatmap showing mean number of sequences in each taxonomic order of antifungal bacteria found at each body site. Yellow indicates most abundant and blue least abundant. Red is intermediate. c) A comparison of potentially antifungal bacteria at the order level found in skin samples (N=5) and by LCM of the epithelium (N=1 pooled sample). The Bacillales group in the LCM sample is composed of 92% *Staphylococcus epidermidis*.

Supplementary Figure 1: Laser microdissection of the rainbow trout skin epithelium from unfixed skin cryosections. a) Light micrograph of a rainbow trout skin cryosection. b) Light micrograph of a rainbow trout skin cryosection where the area that was about to be captured in marked with red spheres and lines. c) Light micrograph of the skin epithelium captured by LCM. Ep: Epithelium. Magnification x10.

Supplementary Figure 2: Composition of the bacterial microbiome of rainbow trout at different body sites. Bar chart of abundance of genera present at each site and in each individual fish sampled. Total length of bar is equivalent to 100% of the OTUs found. OTUs matched at 97% identity to Greengenes August 2013 database.

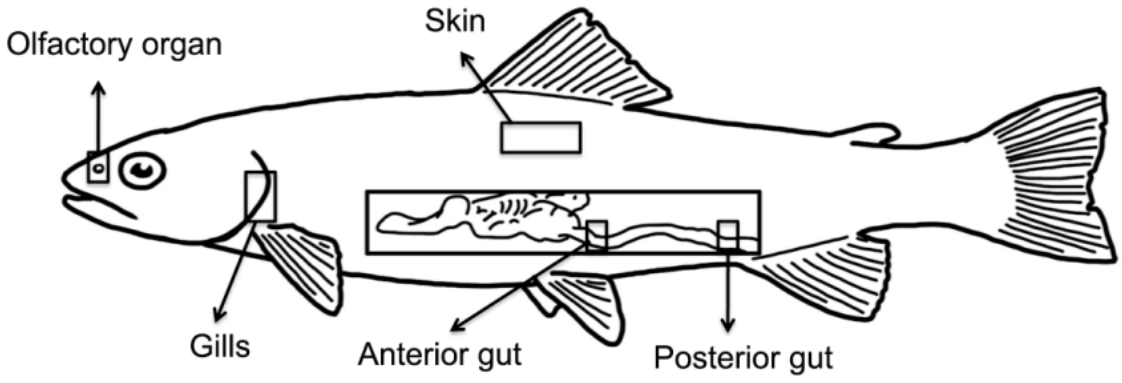
Supplementary Figure 3: Distance plot of XXX

Supplementary Figure 4: 16s rRNA fluoresecent in situ hybridization (FISH) of rainbow trout skin epithelium reveals the presence of bacteria intraepithelially. a) Rainbow trout skin cryosection labeled with Cy5-NON-EUB probe (control). b) Rainbow trout skin cryosection labeled with Cy5-EUB338 probe (magenta). Nuclei (blue) were stained with DAPI. Ep: epithelium. GC: goblet cell. White arrows point to resident bacterial cells.

TABLE 1:

Anatomical Site	Primary Physiological Function	pH	Level of Oxygenation	Habitat Complexity
Gills	Oxygen Uptake/Osmoregulation	Variable, usually close to neutral	High	Medium
Olfactory Organ	Olfaction	Variable, usually close to neutral	High	Low
Skin	Physical Barrier to Environment/Osmoregulation	Variable, usually close to neutral	Medium, Decreasing laterally from the head of the fish	High
Anterior Gut	Nutrient Absorbtion	~8	Low	Medium
Posterior Gut	Nutrient Absorbtion	~7	Low	Medium

FIGURE 1:



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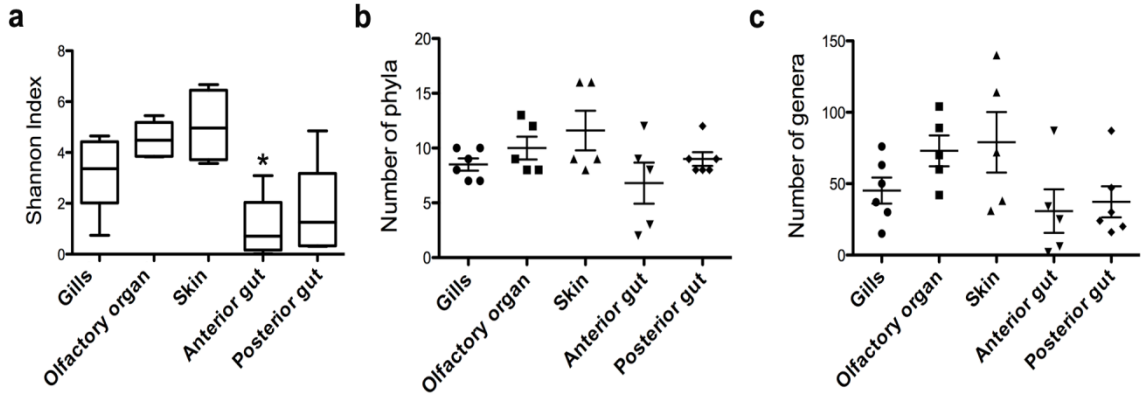

a
Shannon Index
0
2
4
6
8
*
Gills
Olfactory organ
Skin
Anterior gut
Posterior gut
b
Number of phyla
0
5
10
15
20
Gills
Olfactory organ
Skin
Anterior gut
Posterior gut
c
Number of genera
0
50
100
150
Gills
Olfactory organ
Skin
Anterior gut
Posterior gut

FIGURE 3:

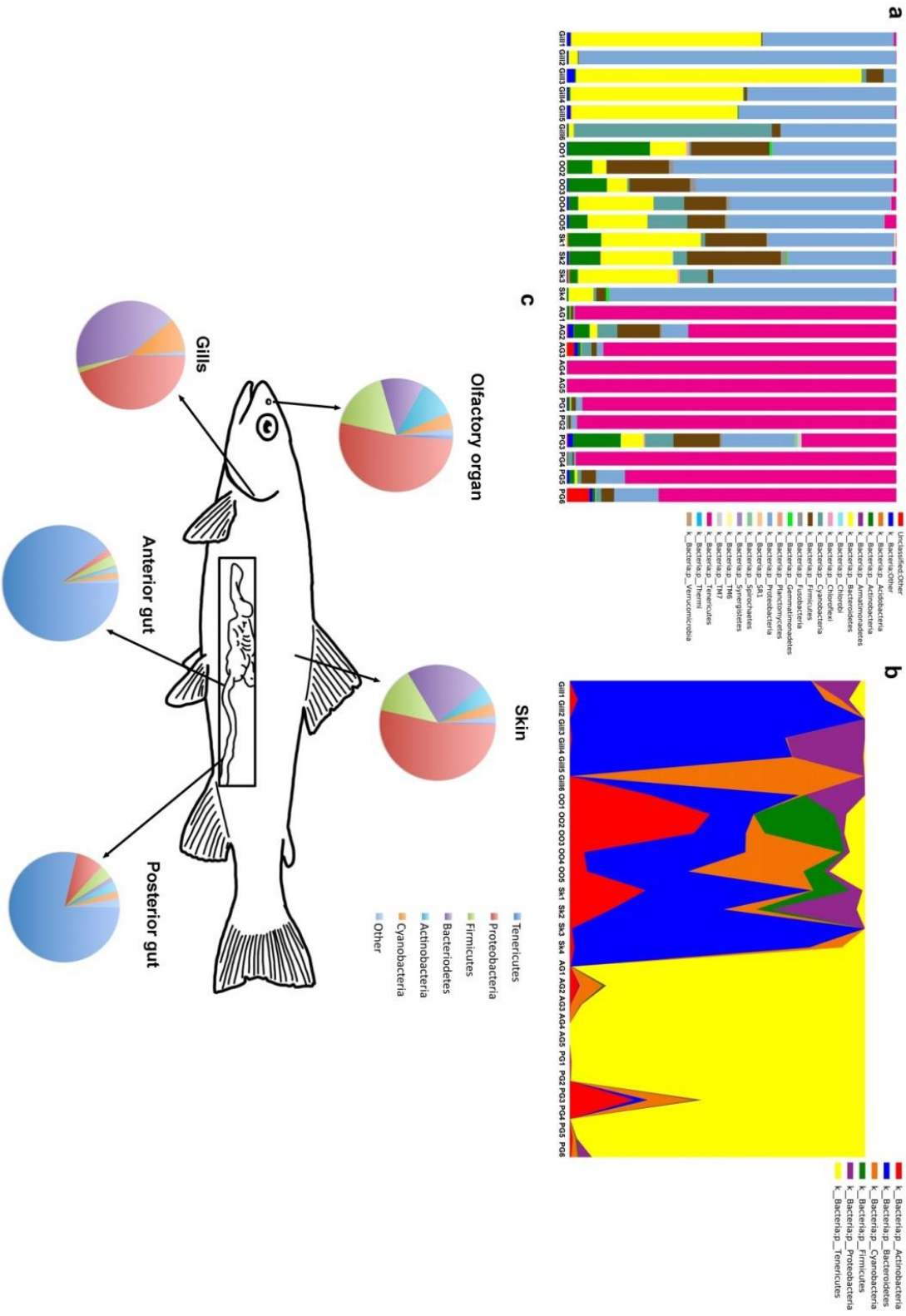

a
b
c
Gills
Olfactory organ
Anterior gut
Posterior gut
Skin
Tenericutes
Proteobacteria
Firmicutes
Bacteriodetes
Actinobacteria
Cyanobacteria
Other

FIGURE 4:

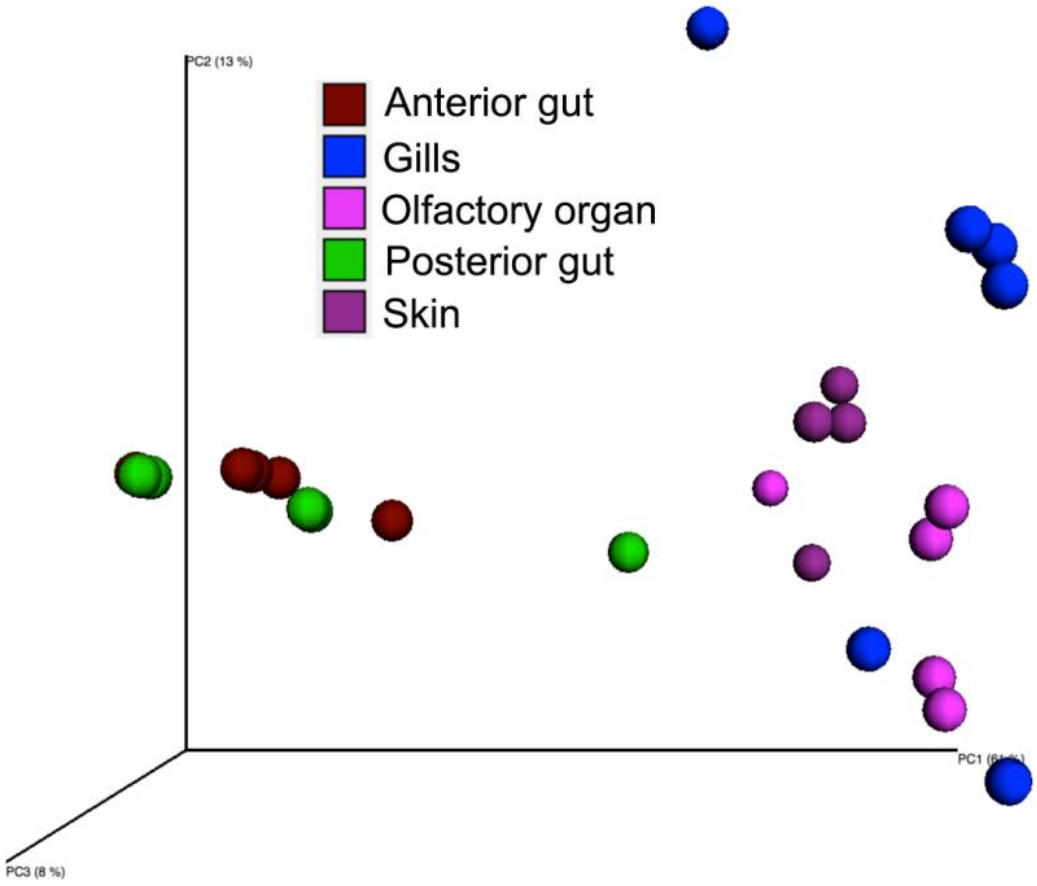

PC2 (13 %)
Anterior gut
Gills
Olfactory organ
Posterior gut
Skin
PC3 (8 %)

FIGURE 5:

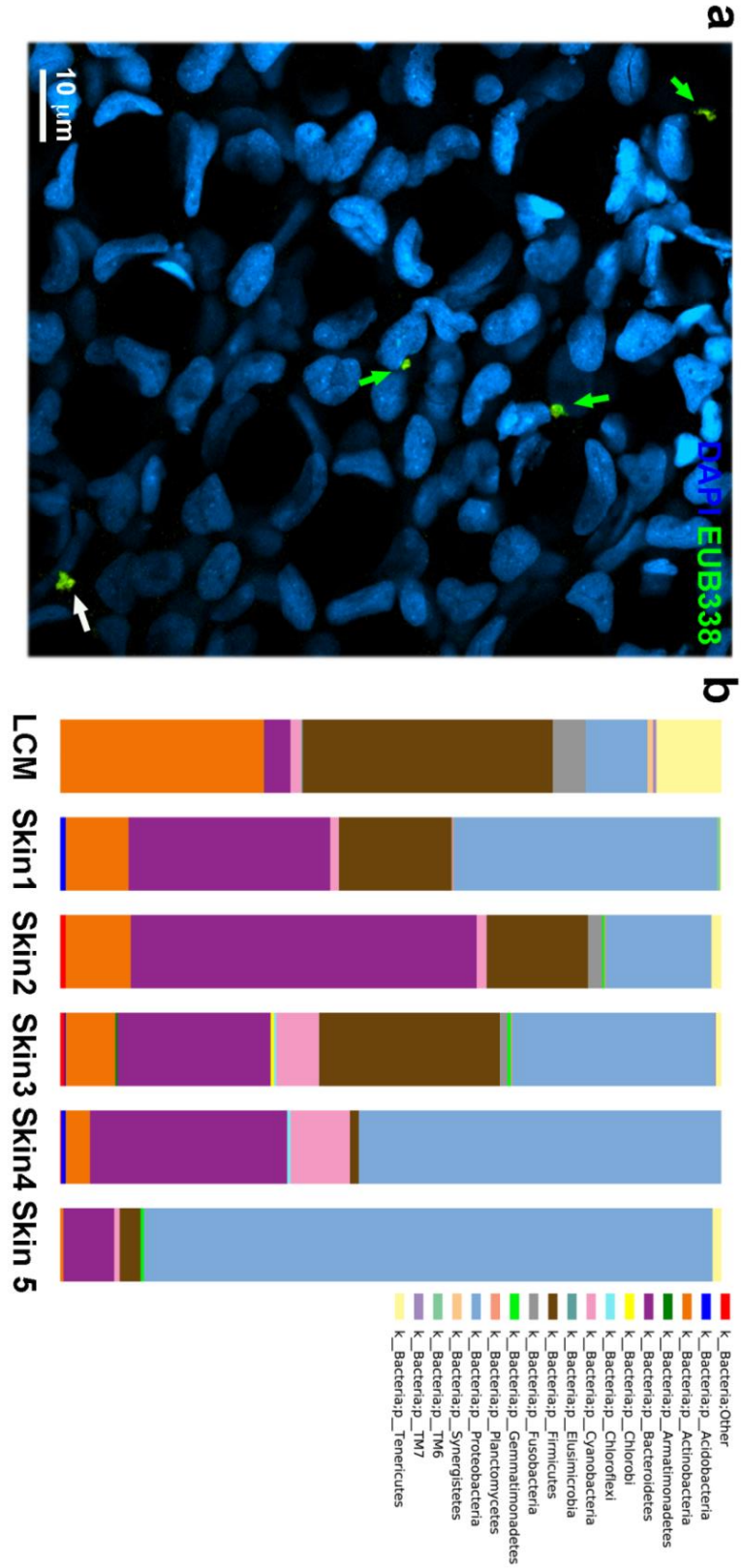
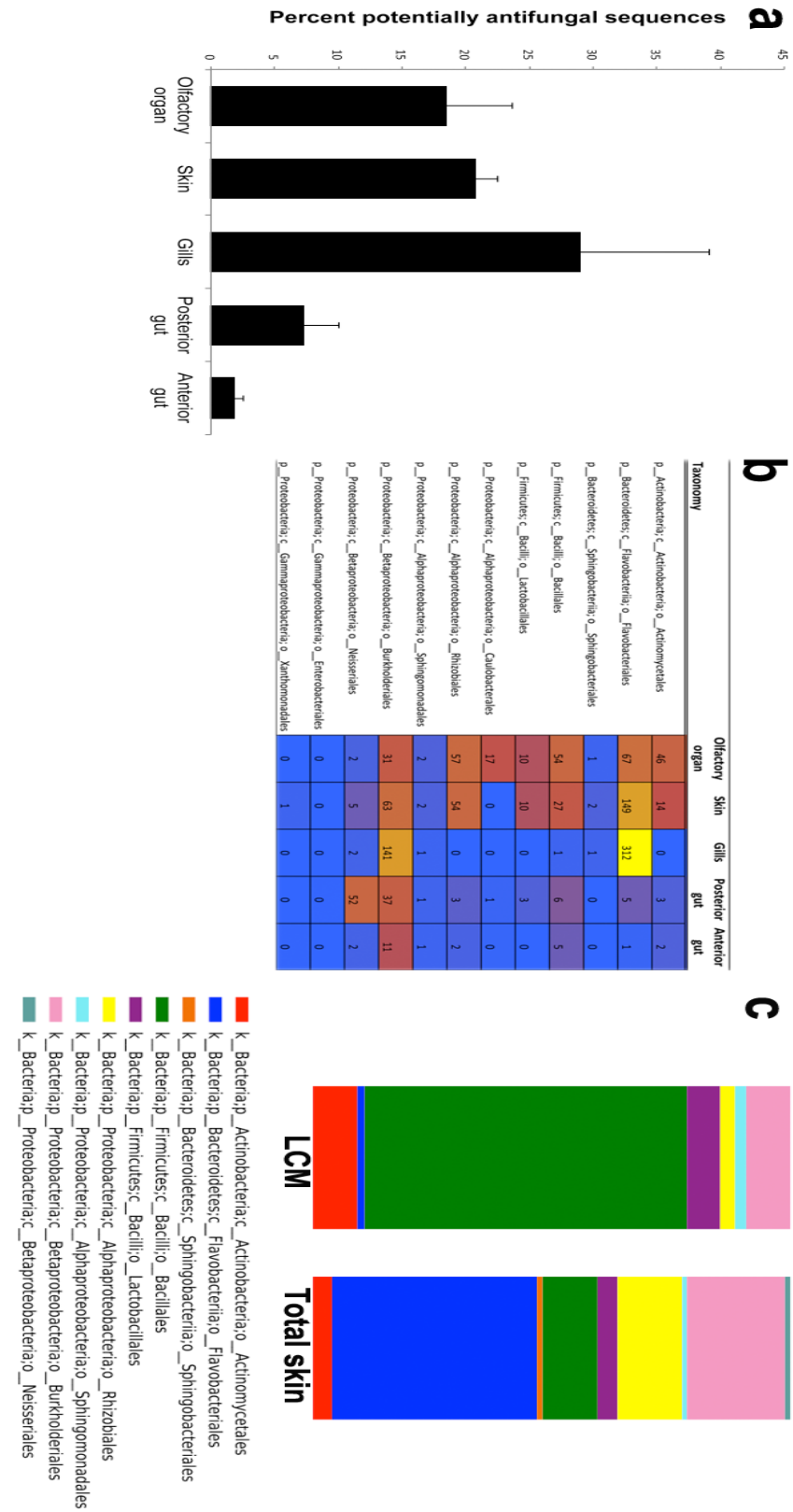
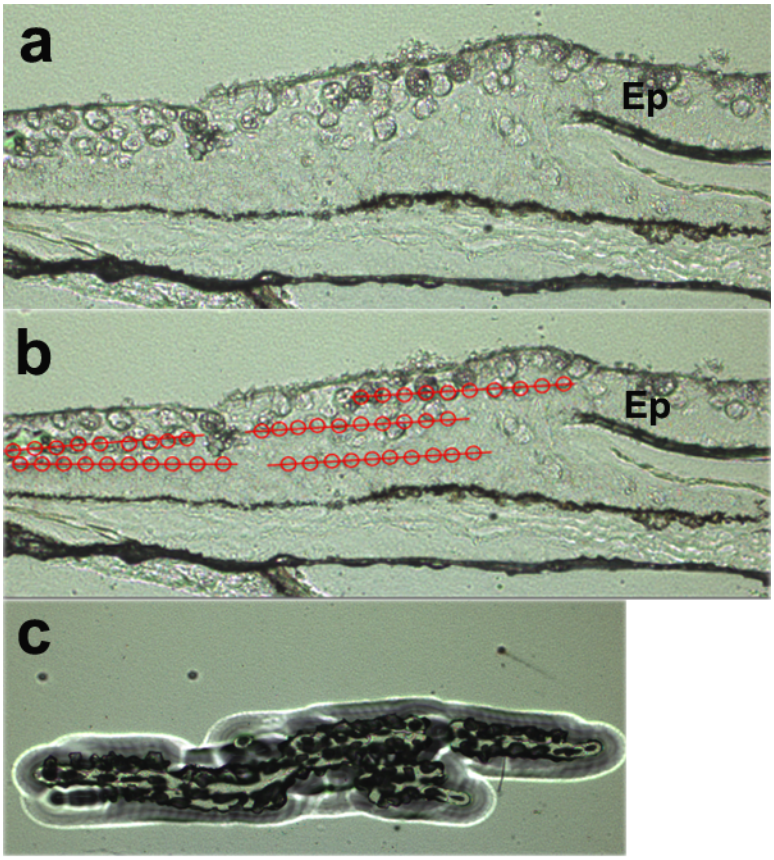

a
10 µm
DAPI EUB338
b
LCM
Skin1
Skin2
Skin3
Skin4
Skin 5
k__Bacteria;Other
k__Bacteria;p__Acidobacteria
k__Bacteria;p__Actinobacteria
k__Bacteria;p__Armatimonadetes
k__Bacteria;p__Bacteroidetes
k__Bacteria;p__Chlorobi
k__Bacteria;p__Chloroflexi
k__Bacteria;p__Cyanobacteria
k__Bacteria;p__Elusimicrobia
k__Bacteria;p__Firmicutes
k__Bacteria;p__Fusobacteria
k__Bacteria;p__Gemmatimonadetes
k__Bacteria;p__Planctomycetes
k__Bacteria;p__Proteobacteria
k__Bacteria;p__Synergistetes
k__Bacteria;p__TM6
k__Bacteria;p__TM7
k__Bacteria;p__Tenericutes

FIGURE 6:

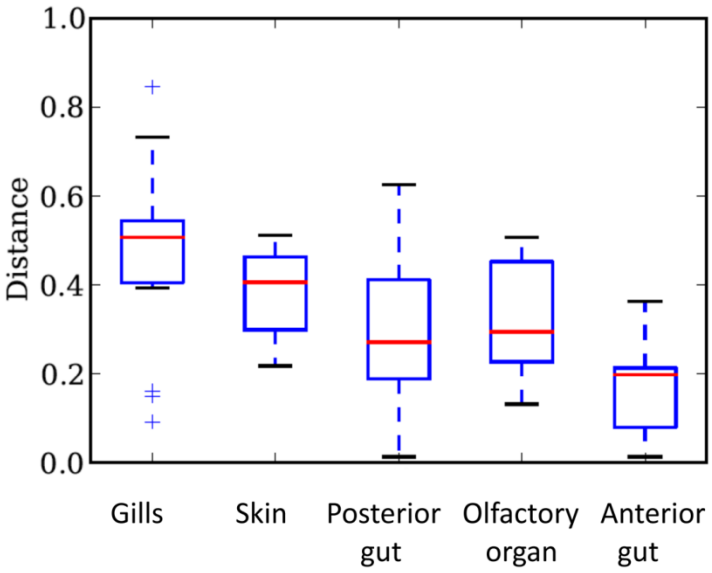

a
Percent potentially antifungal sequences
0
5
10
15
20
25
30
35
40
45
Olfactory organ
Skin
Gills
Posterior gut
Anterior gut
b
Taxonomy
Olfactory organ
Skin
Gills
Posterior gut
Anterior gut
p__Actinobacteria; c__Actinobacteria; o__Actinomycetales 46 14 0 3 2
p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales 67 149 312 5 1
p__Bacteroidetes; c__Sphingobacteriia; o__Sphingobacteriales 1 2 1 0 0
p__Firmicutes; c__Bacilli; o__Bacillales 54 27 1 6 5
p__Firmicutes; c__Bacilli; o__Lactobacillales 10 10 0 3 0
p__Proteobacteria; c__Alphaproteobacteria; o__Caulobacterales 17 0 0 1 0
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales 57 54 0 3 2
p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales 2 2 1 1 1
p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales 31 63 141 37 11
p__Proteobacteria; c__Betaproteobacteria; o__Neisseriales 2 5 2 52 2
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales 0 0 0 0 0
p__Proteobacteria; c__Gammaproteobacteria; o__Xanthomonadales 0 1 0 0 0
c
LCM
Total skin
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales

SUPPLEMENTARY FIGURE 1:

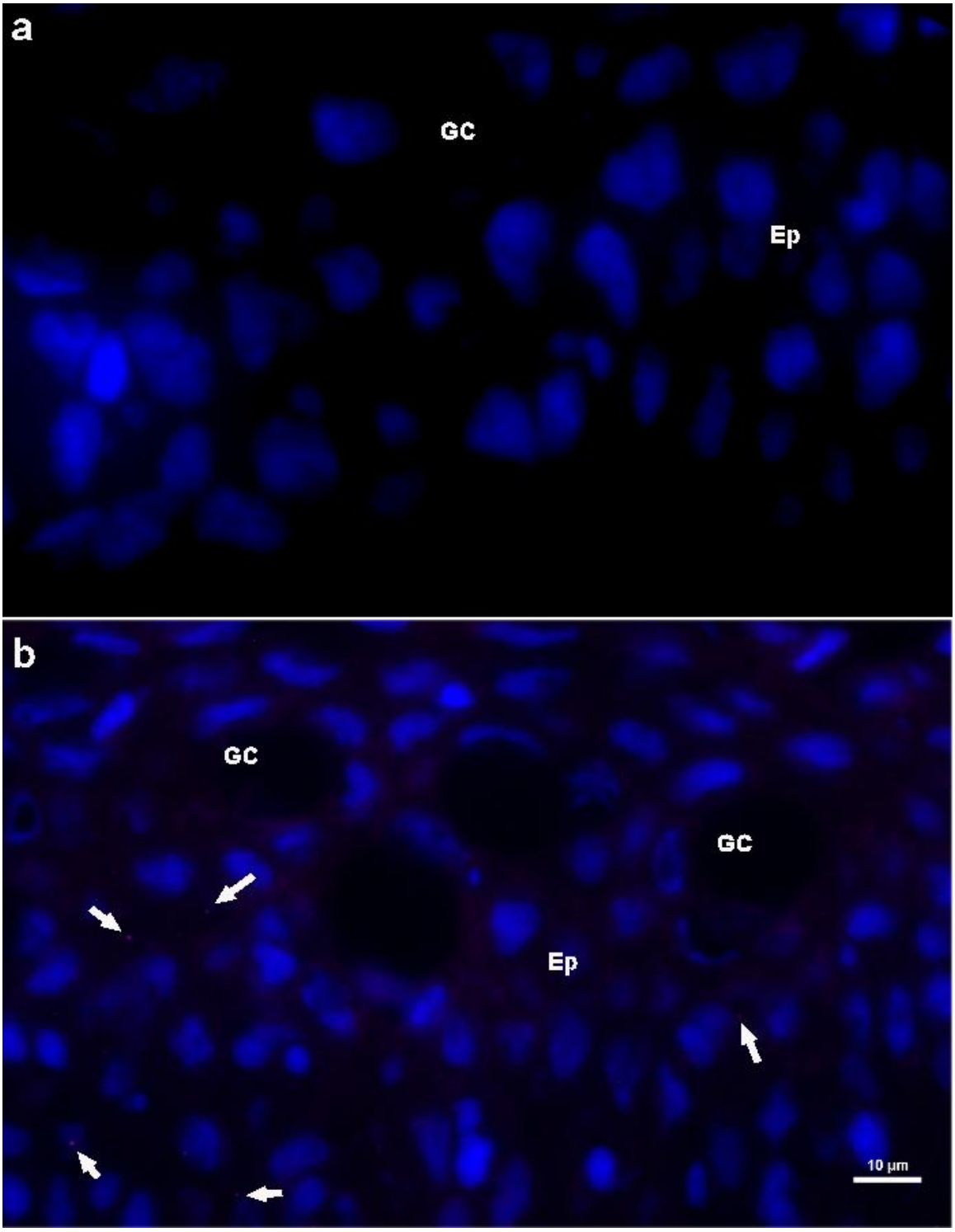


SUPPLEMENTARY FIGURE 2:

SUPPLEMENTARY FIGURE 3:



SUPPLEMENTARY FIGURE 4:



Chapter 3

3.1 Conclusions

This study has discovered, for the first time, the bacterial diversity that is present at the different body sites of rainbow trout. Until this point, only the gut bacterial communities had been described. Though our findings are not identical to those found in other studies on the gut, there are multiple factors that can change the bacterial diversity discovered. Also, fish body site appears as a strong predictor of the bacterial community composition. This is similar to how it has been shown in mammals, particularly humans. While exterior and interior sites cluster differently in a principle coordinate analysis, there are differences in bacterial communities between all body sites.

The skin, gills and gut of fish were known to contain commensal bacteria prior to our study. However, this is the first study to reveal that the olfactory organ of teleost also has a very complex bacterial community, and that it is nearly as diverse as the skin.

We also discovered a highly represent commensal in the gills and skin of rainbow trout. *Flectobacillus* sp., which had never been reported as a commensal species of fish, is a predominant species in the trout gill and skin microbiome. Since *Flectobacillus* sp. is known to produce high amounts of sphingolipids, it is possible that commensal-derived sphingolipids provide immune benefits to the fish host. Also importantly, we found a very bacterial diverse community present in the skin of trout despite the fact that skin was thought to be mostly sterile (Crouse-Eisnor et al. 1985; Austin 2006). In addition, a rich intraepithelial bacterial community resides in rainbow trout skin. This community accounts for almost 50% of the total bacterial diversity present within the skin. These

new findings expand our knowledge of the mucosal bacterial communities present within rainbow trout, and shed light on potential diversity in other teleost.

Finally, the intraepithelial skin bacterial community was enriched in two bacterial groups, Propionibacterium sp. and Staphylococcus sp., both facultative anaerobes and both known to inhabit the inner layers of human skin and other skin structures. Understanding the relationship rainbow trout have to these commensals can further our knowledge of how human mucosal surfaces interact with these bacteria.

3.2 Final discussion

The topographical map of the mucosal microbiome of rainbow trout we have created scratches the surface of our understanding of bacterial communities within fish, particularly in salmonids. Our findings have important evolutionary and applied implications. By understanding the interactions these bacteria have with the mucosal surfaces in teleost fish, we can better understand the evolutionary process of tolerating beneficial bacteria while fighting pathogens in vertebrate animals. From this study and others, it is clear that vertebrate mucosal surfaces have evolved early during vertebrate evolution, way to specifically permit the colonization of specific commensal bacterial groups at different body sites. In mammals, the current dogma is that commensal bacteria mostly occupy niches outside the host, namely within the mucus layer. However, more and more research indicates that vertebrates' permission may go beyond the mucus layer and that internal body sites within mucosal surfaces are also valuable niches for commensal bacteria. In the case of teleost fish that live in an environment where bacteria

thrive, eliminating most external bacteria in the skin may be necessary. In turn, internal sites within the epidermis appear to be colonized by commensals. The particular benefits that these internal colonizers bring to the fish host will be the subject of future studies.

Secondly, the present study has important implications in the realm of aquaculture. Given our knowledge of the important role commensal bacteria play in competing for niche space (Hibbing et al, 2010) and their ability to secrete antimicrobial peptides (Gallo & Nakatsuji 2011) to inhibit infection by potential pathogens, resistance to disease could be caused by resident microbes. There has been evidence of resistance to potential pathogens within farmed rainbow trout populations (Fevolden et al, 2002; Henryon et al, 2002). While the studies looked at either genetic variation (Henryon et al, 2002) or stress response (Fevolden et al, 2002) as a causative agent for resistance against disease, these resistances could be driven by microbiota present at the mucosal surfaces of rainbow trout. As antibiotic resistance has been found in both pathogenic and environmental bacterial strains present in Danish fish farms (Schmidt et al, 2000), other methods of protecting fish from infections must be researched. The bacterial diversity unraveled by the present study opens up many new avenues for the investigation of commensal-derived products that the fish farming industry can benefit from to increase fish welfare and production.

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