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The functional role of the fish microbiome

A thesis submitted in fulfilment of the requirements

for the degree of Doctor of Philosophy

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Table of Contents

Thesis abstract

Aquaculture is the fastest growing sector of agriculture, currently producing more than half of all seafood. Within Australia, yellowtail kingfish (*Seriola lalandi*) is an emerging fish species farmed in temperate waters. While the production of this species is in constant growth, the development of this industry is not without hurdles. For instance, diseases associated with the mucosal surfaces of the fish (e.g. gut enteritis - an inflammation of the gastrointestinal tract) are a recurrent issue in the production of this species. However, the underlying mechanisms inducing this gut inflammation remain poorly understood. New research has elucidated the importance of microbial communities in mucosal surfaces (microbiota) that may play a key role in this disease. These mucosal surfaces (comprising the gut, skin, gill and olfactory organs) support important functions for the host including digestion and nutrient uptake, osmoregulation and recycling waste products, provide the first line of defence against potential pathogens, and form a barrier – along with the host microbiota. Most fish mucosal diseases are linked to the disruption of these microbial communities, which no longer supports the well-functioning of these mucosal surfaces and therefore influence fish health.

Within this thesis, I synthesise our current understanding of the fish microbiota, in particular in a health and disease context (Chapter I). I also explain how this wealth of information can be of particular value for the aquaculture industry by proposing new prospects to improve the fish resilience to disease. Using the yellowtail kingfish as species model, I explore both changes in the fish microbiota across the gut and skin mucosal surfaces and the evolution of the fish immune response during gut enteritis (Chapter II). By doing so, I also investigate important host-microbiota interactions to further understand the interplay between the fish immune system and its microbiota during disease. Of particular note, I found significant gene expression changes (e.g. upregulation of cytokines related genes) and microbiota perturbations (e.g. loss of diversity) in the skin of fish at the early state of the disease, revealing the sensitivity of this mucosal tissue in response to a gut disease. In Chapter III, I explored the impacts of novel treatment options by modulating the fish microbiota using faecal microbiota transplantation (FMT) in conjunction or in replacement of antibiotic treatment to re-establish a more balanced and healthy fish gut microbiota. This also shed light on the process of microbial repopulation following antibiotic exposure, a feature well under studied though paramount for the successful recovery of the host. In this study, antibiotics

greatly influenced the fish gut microbiota and was marked by a significant decrease in diversity, accompanied by an increase in the relative abundance of an uncultured Mycoplasmataceae sp. in the antibiotic treated fish. The effect of the FMT treatment appeared to vary substantially between individuals, and was associated with stark differences in bacterial diversity, suggesting that modulation of the gut microbiota can only be induced in some individuals and for a short time period. In the final Chapter, I develop a new laboratory protocol using PMA to assess microbial viability in the fish gut microbiota. Such information is particularly relevant when investigating the influence of the microbiota in health and disease to better characterise the activity and likely role of these microbial communities, a feature currently overlooked with the gold standard 16S metabarcoding approach. Using this approach, I found that PMA treatment reduced the microbial diversity and richness from both digesta and mucosal gut samples, as well as induced a loss of important bacterial members considered as beneficial (e.g. lactic acid bacteria).

In essence, my research aimed to explore the involvement of the fish microbiota in the health and fitness of the host and improve our understanding of host-microbiota interactions. Such knowledge would ultimately allow us to better modulate the fish microbiota and develop new treatment options. Overall, my thesis contributes to fish health research by providing context and perspective of the fish microbiota. Even though much more effort is needed, I aimed at producing translational research by demonstrating the importance of such studies for the aquaculture industry to potentially enhance fish resilience to infection/disease and ultimately improve current production systems.

Thesis declaration

I, Thibault Legrand, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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Publications

Journal articles

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Legrand, T.P.R.A., Wynne, J.W., Weyrich, L.S., Oxley, A.P.A (2020) Investigating both mucosal immunity and microbiota in response to gut enteritis in yellowtail kingfish. *Microorganisms* **8**, 1267.
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Legrand, T.P.R.A., Catalano, S.R., Wos-Oxley, M.L., Wynne, J.W., Weyrich, L.S., Oxley, A.P.A (2020) Antibiotic-induced alterations and repopulation dynamics of yellowtail kingfish microbiota. *Animal Microbiome* **2**, 26.
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Legrand, T.P.R.A., Wynne, J.W., Weyrich, L.S., Oxley, A.P.A (2021) Dead or alive: Microbial viability treatment reveals both active and inactive bacterial constituents in the fish gut microbiota. *Journal of Applied Microbiology*.
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"There is no finish line. When you reach one goal, find a new one."

- Chuck Norris

Introduction

Aquaculture: A growing industry

The status of fishery resources is a major concern considering that marine fish stocks are decreasing at an alarming rate. For instance, the fraction of fish stocks that are within biologically sustainable levels has dropped from 90% in 1975 to 66% in 2017 (FAO 2020). This has presented a significant challenge for meeting the increasing demands for sources of protein needed in order to feed the world's rapidly growing population (which reached ~7.6 billion in 2016), and is exemplified by estimates of seafood consumption, which increased from 6.5kg per capita in 1950 to 20.5kg in 2018 (FAO 2020).

In response to this, the farming of aquatic animals has seen a marked increase in growth since 1990 from less than 20 million tonnes to more than 80 million tonnes in 2018 (FAO 2020). In fact, aquaculture is the fastest growing food production sector. Since 2013, more than half of the seafood consumed by humans has come from aquaculture (FAO 2018). This industry was dominated by finfish (54.3 million tonnes) from both inland (47 million tonnes) and marine (7.3 million tonnes) aquaculture, followed by molluscs (17.7 million tonnes) and crustaceans (9.4 million tonnes) (FAO 2020).

In Australia, while there are more than 40 fish species farmed commercially, five account for more than 80% of the total gross value of production (ABARES 2019). The most valuable of these are salmonids (mainly the Atlantic salmon farmed in Tasmania) which have a production value of ~$756 million in 2016-2017 (ABARES 2019). Other large value species include the southern bluefin tuna ($115 million), edible oysters ($112 million), prawns ($86 million) and pearl oysters ($70 million) (ABARES 2019).

Yellowtail kingfish as an emerging high value species

The yellowtail kingfish (*Seriola lalandi*, herein referred as YTK) is an emergent commercial finfish species farmed in Australia. YTK have long bodies that are marked by a blue-green

colour along their back, a white-silver underbelly, and a conspicuous yellow caudal fin (Figure 3). They are powerful swimmers adapted to a pelagic lifestyle that make them a highly prized species for anglers. YTK are found in tropical and temperate waters ranging from Western Australia all the way south and up to Southern Queensland, though some individuals have also been seen in the Northern Territory (Nakada 2008).

Figure 1: Picture of a YTK caught in South Australia

Outside of Australia, YTK are also farmed in New Zealand, Japan, Europe, and North and South America, both in the open in seacages and on land using recirculation water systems. YTK are considered an ideal aquaculture species due to their rapid growth rates and high value. They can reach market size (~4kg) in under 2 years (Kolkovski and Sakakura 2004). In addition, YTK is considered a delicacy in most Asian countries, where it is cooked or eaten raw as sashimi.

While the farming of other *Seriola* species rely on the capture and grow-out of wild-caught juveniles (e.g. Japanese yellowtail, *Seriola quinqueradiata*), the farming of YTK is solely based on hatchery-reared fish. Typically, eggs from domesticated broodstock are collected and incubated in tanks (Figure 4). Once hatched, the larvae are transferred to larval rearing tanks, then into nursery tanks and finally (as juveniles) moved into open seacages for grow-out until they reach market size (~1-4 kg) (Figure 2).

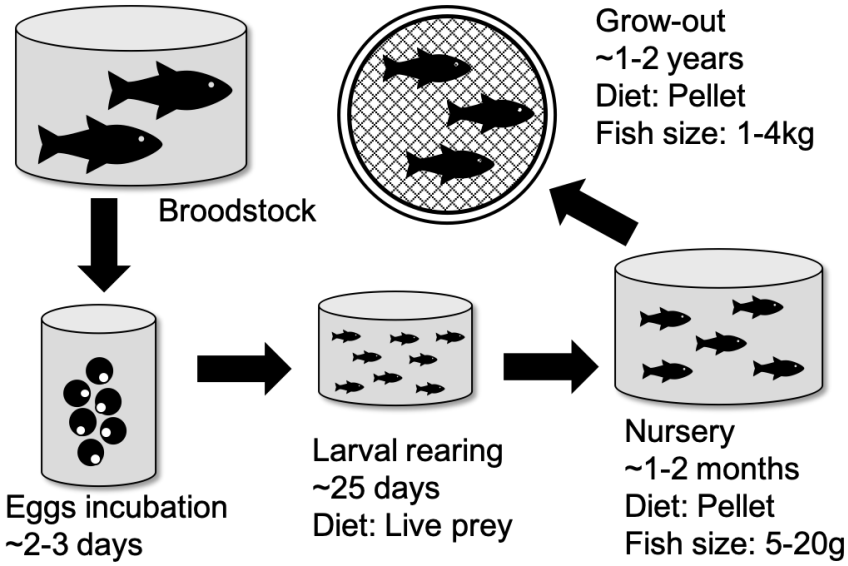

Figure 2: Farming process of YTK

Although the farming of YTK in Australia has seen a marked expansion in recent years, some major drawbacks hinder the further development of the industry. Earlier on, this included the presence of deformities (particularly those of the jaw and skeleton) which became apparent during the larval stage (Cobcroft et al. 2004; Kolkovski and Sakakura 2007). However, recent improvements in larval rearing protocols have significantly reduced the rate of deformities in hatchery-reared fish. In addition to this, YTK can suffer from several diseases of the mucosal tissues associated with the gastrointestinal (GI) tract, skin and gills. For instance, infection with parasitic flatworms (fluke) on the skin (*Benedenia seriolae*) and gill (*Zeuxapta seriolae*) of YTK is a common issue during the grow-out stage. These infections can cause reduced appetite, leading to slower growth and even death due to the loss of osmoregulatory capacity (Sharp et al. 2003; Hutson et al. 2007). Current treatments include the oral administration of praziquantel and bathing in hydrogen peroxide (Mansell et al. 2005; Partridge et al. 2014). In relation to the GI tract, enteritis (also called 'red intestine syndrome' or 'winter gut') is an inflammatory condition which is thought to arise from exposure to sub-optimal water temperatures (usually in winter) and diets comprising plant-based components such as soybean meal, which are sought as a more sustainable feed ingredients (Bansemer et al. 2015). However, to date, there is still no clear understanding of the underlying mechanisms inducing this inflammation. In all cases, the perturbation of these mucosal tissues induced by these conditions often leads to enhanced disease susceptibility and poor growth, resulting in a loss of productivity for the farmers.

The fish mucosal surfaces

The mucosal body surfaces (of the gut, skin, gill and olfactory organs) play important roles in the normal, healthy functioning of fish. Such roles include osmoregulation (within the gut, skin and gills) (Edwards and Marshall 2012), the recycling of waste products (gills) (Evans et al. 2005), and digestion and nutrient uptake (gut) (Grosell et al. 2010). In addition, these surfaces also act as a primary barrier, and are the first line of defense against potential pathogens from the surrounding environment (Salinas 2015). This role is facilitated through the unique physical and chemical properties of the mucosa, as well as the underlying lymphoid tissues (referred to as the mucosa-associated lymphoid tissues or MALT) which are involved in the detection, recognition and defense against pathogen (Salinas 2015). Fish have four MALTs: the gut-associated lymphoid tissues (GALT), the skin-associated lymphoid tissues (SALT), the gill-associated lymphoid tissues (GIALT) and the nasopharynx-associated lymphoid tissues (NALT) (Salinas 2015). They are composed of both innate and adaptive immune cells, whereby T cells are the most abundant (Kelly and Salinas 2017). Furthermore, goblet, club and sacciform cells induce the production of mucus, which is primarily composed of mucins (Reverter et al. 2018). This mucus also contains numerous antimicrobial and immune-related bioactive molecules that limit the growth of pathogens (Reverter et al. 2018). Mucus, alongside the scales and the epithelium, also acts as a physical barrier against the invasion of pathogens (Cabillon and Lazado 2019). Lastly, a complex microbial community (the 'microbiota') colonise this mucus and interact closely with the host, regulating the immune system and directly competing with opportunistic pathogens in order to maintain homeostasis (a balanced microbiota) within these mucosal surfaces (Kelly and Salinas 2017). When these microbial communities are perturbed by disease, infection or environmental changes, a dysbiosis (disturbed or imbalanced microbiota) occurs and increases the host disease susceptibility (Brugman et al. 2018). During recent years, changes in fish microbiota associated with disease and stress were investigated in many studies (Legrand et al. 2020). However, there is still a lack of understanding regarding whether these microbial disturbances are more likely the cause or the result of these conditions and further work is warranted in this regard.

The 'microbiome' concept

Defining the microbiome and its relevance to the host

Despite some contention surrounding the origins of the term 'microbiome' (Prescott 2017), it likely stems from 'microbiota', though refers to the 'entire habitat, including the microorganisms, their genomes, and the surrounding environmental conditions' (Marchesi and Ravel 2015). Insights into the microbiota, the relationships they share with certain hosts, and the processes that drive or govern their dynamics, have thus been an important prelude into our understanding of specific microbiomes. Pioneering studies in humans and animal models have revealed that the host microbiome develops from colonisation of the external body surfaces (including the skin, mouth, nose, digestive system and reproductive tract) by various microbes at birth, leading to the formation of stable core community assemblages (and likely encoded functions) during maturity (Turnbaugh et al. 2007; Huttenhower et al. 2012; Greenhalgh et al. 2016). These assemblages are often highly diverse in nature, comprising various microorganisms including bacteria, archaea, fungi, protozoa and viruses (Gill et al. 2006; Rajilic-Stojanovic et al. 2007; Dave et al. 2012; Hacquard et al. 2015); though bacteria are often the predominant component (Qin et al. 2010). Having forged complex commensal or symbiotic relationships with the host through diverse ecological and evolutionary pressures (Ley et al. 2006), these assemblages are regulated by the host and interact with each other to support the host's healthy development and functioning through diverse contributions to processes ranging from digestion and metabolism to regulation and modulation of the immune and nervous systems (Semova et al. 2012; Hacquard et al. 2015). In this regard, the microbiota is thought to act as an additional body organ (Eckburg et al. 2005; Gill et al. 2006) and has been referred to as the 'second genome' of the host (Grice and Segre 2012).

The significance of the microbiome to the host is evident in its capacity to drive the emergence of specific phenotypes. Seminal work by Turnbaugh et al. (2006) perhaps best exemplifies the importance of such relationships through the experimental transplantation of 'obese' microbiomes into germ-free mice, endowing the recipient animals with the capacity to increase the energy harvested from the diet, leading to the increased accumulation of total body fat and consequently an altered 'obese' phenotype. Though a case for elucidating the specific involvement of the microbiome in the pathophysiology of obesity, other studies have since gone on to establish the relevance of the microbiome in the occurrence of a wide array of other diseases (Young 2017). From these studies, it is evident that compositional imbalances (or

'dysbioses') are primary features, whereby deleterious effects on the host are likely imparted through altered microbial diversity and the concomitant dysregulation of certain functions (Petersen and Round 2014; Valdes et al. 2018). The microbiome, however, does not work alone in the manifestation of such phenotypes, but instead is significantly influenced by host genetics, environmental factors (e.g. diet) and relevant pathogen/s (Hall et al. 2017). In this regard, the microbiome has the capacity to be manipulated, with numerous studies indicating a role for dietary modulation or the utility of probiotics in restoring health and optimal functioning (Valdes et al. 2018).

Strategies for evaluating the microbiome

For a long time, characterisation of the composition and structure of these communities was made using cultured based techniques. Though having informed our current understanding of these systems and being important for e.g. evaluating the physiology or pathogenicity of individual microbes or discovering novel biomolecules (Zengler 2009), these methods are limited in their capacity as <2% of all microorganisms are thought to be readily cultured (Vartoukian et al. 2010). The development of culture-independent molecular technologies, particularly recent advances such as next generation sequencing (NGS), have thus been used over the last decades for this purpose and have led to the proliferation of microbiome studies in various hosts, including fish. In particular, targeted amplicon sequencing procedures (e.g. 16S ribosomal RNA sequencing) have become the gold standard when assessing the structural diversity of the microbiota (Jovel et al. 2016). However, while cost-effective, this technique is usually restricted to the identification of a particular group of microbes (e.g. bacteria or archaea) and does not provide functional information about what each microbe or group of microbes are doing. As a result, metagenomics procedures have also been developed to study both the structural diversity and functional potential of these communities as a whole (Quince et al. 2017). As a further approach, these procedures can be modified to assess the actual functions being expressed by assessing the RNA rather than the DNA through metatranscriptomics (Bashiardes et al. 2016). The type of method implemented by individual studies, of course, being dependent upon the research question being posed (Knight et al. 2018). Due to the tremendous cost associated with such techniques, alternative strategies for assessing the function of the microbiome have been recently developed and include certain

bioinformatics tools (e.g. Tax4Fun, PICRUSt) that can predict functions from 16S amplicon data through comparisons with microbial reference genomes (Ortiz-Estrada et al. 2018).

The fish microbiome

While the concept of microbiome has emerged from human studies, fish microbiome related studies have flourished within the last few years, in particular from 2010 (Figure 3). To date, most of the studies have focused on the microbiome of model species (e.g. zebrafish (*Danio rerio*) and three-spine stickleback (*Gasterosteus culeatus*)) or important aquaculture species (e.g. salmonids and carps) (Lescak and Milligan-Myhre 2017; Legrand et al. 2020; López Nadal et al. 2020). The aims of these studies are often very different depending on the host species, and are often orientated towards answering ecological or medical related questions, typically through evaluation of the associated host-microbe interactions under a given experimental condition/s. For the latter, zebrafish are primarily used due to their body transparency, ease of culture and capacity to be raised and implemented as gnotobiotic (germ-free) model systems (Lescak and Milligan-Myhre 2017). In recent years, however, there has been increasing interest beyond model systems to include various aquaculture species in order to understand how the microbiome may improve productivity by supporting health and nutrition (e.g. through enhanced disease resistance or nutrient absorption) (de Bruijn et al. 2018; Egerton et al. 2018). For a more detailed review of the literature regarding the fish microbiome, please refer to chapter 1.

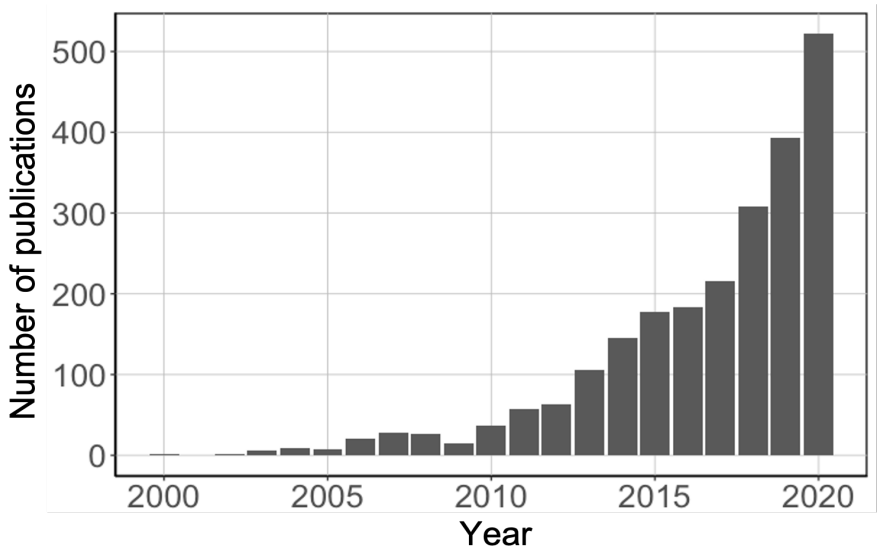

Figure 3: Annual number of publications found in PubMed with the search “fish microbiome” since 2000

Thesis overview

Over the last decade, there has been tremendous interest in the role the microbiome plays in driving the normal, healthy functioning of the vertebrate host. For aquacultured species, such studies are likely to be critical for informing how farms may become more sustainable and productive. The penultimate objective being the capacity to manipulate the microbiome in order to enhance nutrition and improve disease resistance to a range of common conditions (e.g. bacterial, viral or parasitic infections, and gastrointestinal inflammation). However, in order to achieve these far-reaching goals, an improved understanding of the role the microbiome plays in the health and disease of farmed species is required. Collectively, this thesis aims to shed light on the role of the gut and skin mucosal microbiomes in the health and disease of the commercially important species YTK. More specifically, this thesis aims to:

1) Identify if conditions such as gut inflammation perturb the mucosal microbiomes of YTK.
2) Determine bacterial biomarkers of changing health status within the gut and/or skin.
3) Explore host-microbe interactions in a health and disease context.
4) Investigate whether the YTK microbiome could be modulated to re-establish a more balanced microbiome through the use of antibiotic therapies and/or faecal microbiota transplantation (FMT).
5) Assess bacterial viability in the YTK gut microbiome from both digesta and mucosal samples.

The following chapters represent material that has been published or submitted for peer-review, and includes a detailed literature review of the fish microbiome and a series of experiments using a range of current analytical approaches (e.g. 16S rRNA profiling, RNA-seq, shotgun metagenomics) and fish from commercial operations and experimental systems to improve our understanding of the role of the microbiome. While this thesis focuses on YTK, such work has broader relevance to other fish/animal species, making this thesis interesting to a broader audience. New approaches and considerations will be proposed and discussed from the knowledge gained in this thesis.

Chapter 1: A microbial sea of possibilities: current knowledge and prospects for an improved understanding of the fish microbiome

This first chapter reviews the current knowledge of the fish microbiome, in particular in a health and disease context. I review the major microbial constituents found in the fish mucosal microbiomes and the different factors influencing them, notably in farming conditions. I also discuss the limitations of fish microbiome studies to date by exploring a range of different factors influencing the findings and their interpretation. In addition, I review the functional information pertaining to the fish microbiome, as generated from a range of different Next Generation Sequencing (NGS) technologies based on 16S rRNA gene analyses, metagenomics, metatranscriptomics and using germ-free model systems. Furthermore, I discuss the relevance and importance of the regulation of microbial communities by the host (host-microbe interactions) and the competition between the microbiota (microbe-microbe interactions). Finally, I propose different microbiome related management applications for the industry and explain how this wealth of information could be useful for improving productivity in aquaculture systems.

Chapter 2: Investigating mucosal immunity and microbiota in response to gut enteritis in YTK

Conditions such as gut enteritis are a common issue encountered in the farming of several fish species including YTK, and may result in a decrease in productivity due to stock losses. While this condition has been linked with inappropriate diets (typically those enriched with plant-based feed ingredients such as soybean meal) and suboptimal rearing temperatures (< 16ºC for YTK), very little is known regarding the underlying mechanisms inducing this disease. In this chapter, I investigate the gut and skin microbiota responses to the disease by comparing healthy fish and fish displaying signs of early and late stages of gut enteritis. In addition, I constructed RNA-seq libraries from the same samples to explore the host response in these mucosal surfaces with the aim to correlate changes in microbiota structure with changes in host gene expression (particularly those associated with immunity).

Chapter 3: Antibiotic-induced alterations and repopulation dynamics of YTK microbiota

Antibiotic therapy is a common treatment for various microbial infections in the farming of various fish species, including YTK. However, its use is marred by environmental concerns and the development of antimicrobial resistance (as attributed to the acquisition of antibiotic resistance genes or ARGs among pathogens). In addition, most antibiotics have broad-

spectrum activity and are thus a non-targeted approach that depletes bacterial communities as a whole. This can have negative effects for fish health, inducing a perturbation in the microbiome and its associated diversity. Furthermore, the process of microbial repopulation following antibiotic treatment is not well understood, though an important feature for the host recovery following infection. This study aims to improve our understanding of microbial recovery following antibiotic treatment in poor-performing YTK suffering from gastrointestinal disorders. I also investigate the influence of faecal microbiota transplantation (FMT) following the use of antibiotics in order to modulate the fish microbiome and re-establish a more balanced and healthy microbial community within the fish gut.

Chapter 4: Dead or alive: microbial viability treatment reveals both active and inactive bacterial constituents in the fish gut microbiota

Current metagenomics approaches (e.g. 16S rRNA gene and shotgun metagenome sequencing) rely on the sequencing of total DNA samples. As a result, standard approaches explore both the viable and non-viable microbial communities. When investigating the role of the microbiome in health and disease, it appears paramount to delineate the viable microbial cells from the non-viable ones, as only viable cells are likely to interact with the host (as resident rather than transient assemblages) and contribute to key functions for the host. This last chapter explores a novel method that aims to characterise the active microbial components of the YTK gut microbiome in both digesta and faecal samples.

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Article

Investigating Both Mucosal Immunity and Microbiota in Response to Gut Enteritis in Yellowtail Kingfish

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Abstract: The mucosal surfaces of fish play numerous roles including, but not limited to, protection against pathogens, nutrient digestion and absorption, excretion of nitrogenous wastes and osmotic regulation. During infection or disease, these surfaces act as the first line of defense, where the mucosal immune system interacts closely with the associated microbiota to maintain homeostasis. This study evaluated microbial changes across the gut and skin mucosal surfaces in yellowtail kingfish displaying signs of gut inflammation, as well as explored the host gene expression in these tissues in order to improve our understanding of the underlying mechanisms that contribute to the emergence of these conditions. For this, we obtained and analyzed 16S rDNA and transcriptomic (RNA-Seq) sequence data from the gut and skin mucosa of fish exhibiting different health states (i.e., healthy fish and fish at the early and late stages of enteritis). Both the gut and skin microbiota were perturbed by the disease. More specifically, the gastrointestinal microbiota of diseased fish was dominated by an uncultured *Mycoplasmataceae* sp., and fish at the early stage of the disease showed a significant loss of diversity in the skin. Using transcriptomics, we found that only a few genes were significantly differentially expressed in the gut. In contrast, gene expression in the skin differed widely between health states, in particular in the fish at the late stage of the disease. These changes were associated with several metabolic pathways that were differentially expressed and reflected a weakened host. Altogether, this study highlights the sensitivity of the skin mucosal surface in response to gut inflammation.

Keywords: microbiota; immunity; fish; gut; skin; health; mucosa; aquaculture

1. Introduction

The mucosal surfaces of fish, comprising the gut, skin, gills and olfactory organ act as the first lines of defense against pathogens and represent important primary barriers [1,2]. These surfaces are composed of various layers with different physical and chemical properties that protect the host from the environment and potential pathogens. More specifically, the mucosa are coated in a secretion of mucus, mainly composed of mucin, which acts as a physical barrier between the environment and the fish and limits the growth of microbes [3]. This mucus also houses an array of microbes called

microbiota. Recent works on animals including fish have revealed that these microbial communities support important functions including the development and regulation of the immune response, and as such interact closely with the host immune system to fight infections and disease [2,4]. The mucosal surfaces also comprise lymphoid tissues (termed mucosa-associated lymphoid tissues: MALTs) which play an important role in the detection, recognition and defense against potential pathogens [2]. These MALTs contain cells responsible for both the innate and adaptive immune system of fish [5]. Thus, the interactions between these different systems are a fundamental feature in maintaining homeostasis and permeability within the mucosal surfaces.

The relevance of gut health in the farming of finfish has increased within recent years due to the emergence of various gastrointestinal disorders that have hindered the development of the industry [6]. Particular conditions such as enteritis (a gut inflammation) have become especially problematic in the farming of a number of different species such as Atlantic salmon (*Salmo salar*), zebrafish (*Danio rerio*), turbot (*Scophthalmus maximus*), yellowtail kingfish (*Seriola lalandi*), California yellowtail (*Seriola dorsalis*), pearl gentian grouper (*Epinephelus* sp.) and common carp (*Cyprinus carpio* L.) [7–13]. Dietary components including high supplementation of soybean meal in the feed seem to play an important role in the emergence of this disease (or other related inflammatory disorders) in several species [7–10,14,15]. However, it has been reported that this disease can also be induced by various pathogens, either bacterial (e.g., *Aeromonas hydrophila*) or parasitic (e.g., *Enteromyxum leei* and *Pseudocapillaria tomentosa*) [16–18]. Furthermore, studies on zebrafish have shown that the intestinal inflammation is dependent on the microbiota where specific microbiota can predispose an animal to this condition, highlighting the important role of microbial communities in the onset of inflammation [19]. In grass carp (*Ctenopharyngodon idellus*), it was shown that fish with inflammatory intestinal disorders had an altered gut microbiota associated with an increase in diversity in diseased fish [20]. In addition, in yellowtail kingfish suffering from enteritis, alterations in the microbiota of the skin and gills have also been observed, suggesting that broader, body-wide host responses may play a role in the dynamics of these communities [21].

Recent research has greatly enhanced our understanding of host–microbiota interactions in health and disease, in particular in mammalian systems [22]. It is now clear that there is a bidirectional relationship between the microbiota and the host, where the microbiota play an important role in the training and regulation of both the host's innate and adaptive immunity which, in turn regulates and selects for specific bacterial assemblages across the different mucosal tissues [22]. As such, a balanced (homeostatic) state is thought to be required for normal functioning and defense against environmental stress (e.g., diet or antibiotics) [23]. Any dysregulation of this equilibrium is often linked to poorer performance and can ultimately result in disease [22]. However, in fish, host microbiota interactions are poorly understood, and research thus far has been mainly focused on gnotobiotic models such as zebrafish and threespine stickleback (*Gasterosteus aculeatus*) [24].

Studies investigating the fish immune response against diet induced gut inflammation have revealed useful biomarkers at the early stages of the disease including some proinflammatory cytokines and antioxidant enzyme related genes [11,13,25]. However, there is a lack of information regarding the interactions between the host and the microbiota during disease. Considering the importance of host–microbe interactions and the role of the fish microbiome in health and disease [26], further research is required to elucidate the underlying mechanisms leading to the development of the disease. Here, we investigated the influence of gut enteritis on the gut and skin microbiota of yellowtail kingfish and examined the host response using transcriptomics (RNA-Seq) to better understand the effect of a gut disease on the fish mucosal surfaces.

2. Materials and Methods

2.1. Experimental Design and Sample Collection

This study expands on earlier experimental work conducted on yellowtail kingfish that investigated the influence of gut enteritis on the outer surface (skin and gill) microbiota [21]. Specifically, in this study we expand on the earlier bacterial community (16S rDNA) analyses of the outer (skin) surfaces and surrounding environment (seawater) to include a comparison with the microbiota of the hindgut, as well as the assessment of the host response in the skin and hindgut tissues using transcriptomics (RNA-Seq). For this, RNA extracted from samples obtained by Legrand et al. [21] from a total of 36 fish of differing health states was used to generate Next Generation Sequencing (NGS) 16S rDNA amplicon libraries from the hindgut (for comparison with the data generated earlier for the skin (Accession number under the BioProject ID PRJNA396452)), and RNA-Seq libraries from the skin and hindgut. This included samples from 12 healthy fish (referred to herein as the "healthy" group) from a single seacage containing only individuals with no signs of infection, and 24 fish from a nearby seacage (<7 km) exhibiting signs of early and late stages of gut enteritis (herein referred to as the "early" and "late" groups, respectively). All fish were obtained and sampled under the auspices of a commercial aquaculture enterprise according to industry best practice veterinary care, with the health status of each treatment group confirmed by necropsy and histopathological assessment by farm health and veterinary personnel and an external pathology provider. All fish had been fed the same pelleted feed and came from the same hatchery run. For each fish, skin swabs were first collected upon netting by swabbing one side of the fish using FLOQSwabs® (COPAN, Murrieta, CA, USA) and stabilized in tubes comprising RNAlater™ (Ambion, Austin, TX, USA). Fish were then euthanized using a lethal dose of AQUI-S (AQUI-S New Zealand Ltd., Lower Hutt, New Zealand) in seawater, and the gastrointestinal tract was carefully excised with a sterile scalpel. A scraping of the inner mucosal surface of the hindgut region was obtained using a sterile glass microscope slide. The contents of the hindgut were also stabilized in tubes comprising RNAlater™ (Ambion). All RNA samples were treated with the Turbo DNase free™ (Life Technologies, Carlsbad, CA, USA) kit to remove any residual gDNA, and were stored at −80 °C.

2.2. Library Preparation and Sequencing

In order to compare the bacterial (16S rDNA) community composition data obtained earlier from the skin, the same NGS 16S rDNA amplicon library preparation protocol was performed for the hindgut samples as conducted previously [21]. Briefly, purified total RNA extracted from the hindgut samples (as obtained using bead-beating and the RNeasy mini kit (Qiagen, Hilden, Germany) as detailed in Legrand et al. [21]) was converted into cDNA using the Superscript™ III First Strand Synthesis System (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was subsequently concentrated by ethanol precipitation using standard procedures, quantified using the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Indianapolis, IN, USA) and stored at −20 °C. The V1-V2 hypervariable region of the 16S rRNA gene was amplified from the cDNA from all hindgut samples (n = 36 fish) using a multistep approach using universal eubacterial primers 27F and 338R. Following the library preparation, samples were quantified and pooled in equimolar ratios before being sequenced on the MiSeq Illumina platform using 250nt paired-end sequencing chemistry through the Australian Genome Research Facility (AGRF, Melbourne, Australia). Raw demultiplexed sequencing data with sample annotations were deposited in the NCBI SRA data repository under the BioProject ID PRJNA637190.

For assessing host gene expression, transcriptomic (RNA-Seq) libraries were prepared from purified RNA extracts from a total of 9 skin and 9 hindgut samples (n = 3 per treatment group per sample type) following initial quality assessment using the LabChip System (Caliper Life Sciences, Inc., Hopkinton, MA, USA). Libraries were generated using the ScriptSeq™ Complete Gold Kit (Epidemiology) (Illumina, San Diego, CA, USA), which included the initial depletion of the rRNA using

the Ribo-Zero™ Gold rRNA Removal Kit (Epidemiology) (Epicentre, Madison, WI, USA). For each library, a minimum of 100 ng of rRNA depleted RNA was used in each reaction according to the manufacturer's instructions, and the libraries were purified using the MinElute™ PCR Purification Kit (Qiagen). Potential contaminating primer dimers were removed by Exonuclease I treatment (Illumina) and further size selection of fragments (~200–600 bp) using the SPRIselect Reagent Kit (Beckman Coulter, Brea, CA, USA). Fragments were then assessed for quantity and quality using the Quant-iT™ picogreen™ dsDNA Assay Kit (Invitrogen) and the LabChip System (Caliper Life Sciences, Inc.). Libraries were pooled in equimolar ratios, and 6 samples were multiplexed per lane and sequenced on the Illumina HiSeq4000 platform (Illumina) using 150nt paired-end sequencing chemistry through the Murdoch Children's Research Institute (MCRI)—Translational Genomics Unit (Melbourne, Australia). Raw demultiplexed sequencing data with sample annotations were deposited in the NCBI SRA data repository under the BioProject ID PRJNA639544.

2.3. *Bioinformatics and Statistical Analysis*

The raw 16S rDNA sequence reads obtained from 33 hindgut samples (3 samples failed in library preparation which were fish #114, #128 and #129) were paired using PEAR (v. 0.9.5), and the primer regions were removed [27]. These trimmed sequence reads were subsequently merged with the 36 skin and 2 seawater sample fastq files obtained in the earlier study of Legrand et al. [21] (NCBI SRA accession numbers under the project PRJNA396452) and were processed and analyzed together using the QIIME2 (v. 2019.1) pipeline [28]. Demultiplexed paired-end sequence reads were truncated to a length of 320 bp, quality filtered and denoised into amplicon sequence variants (ASVs) using the DADA2 plugin [29]. A total of 3,183,303 demultiplexed paired-end sequence reads were assigned to 9155 ASVs features from a total of 71 samples. The number of reads ranged from 16,255 to 135,287 with a median of 40,799 per sample. Following the denoising and removal of reads associated with chloroplast, mitochondria and eukaryotes (after assigning taxonomy), a total of 3,116,835 reads were obtained for downstream analysis. Each sample was rarefied to a depth of 16,255 reads, resulting in a total of 7863 ASVs in the dataset. Alpha rarefaction showed sufficient coverage of the samples (Figure S1). Taxonomy was assigned to the ASVs using the q2-feature-classifier against the Silva 132 99% OTUs (Operational Taxonomic Units) reference sequences resource [27]. Alpha-diversity metrics (Shannon's diversity, Pielou's evenness and Chao1 richness), beta diversity metrics (Bray–Curtis), and Principle Coordinate Analysis (PCoA) using both the weighted and unweighted Unifrac distance matrix were estimated using q2-diversity. Statistical differences for alpha diversity were assessed using the Kruskall–Wallis test with Benjamini-Hochberg correction for pairwise comparison. Statistical differences for the ASV dataset as a whole were identified using PERMANOVA. QIIME artifacts were imported into R using the package Qiime2R, and plots were made using Phyloseq and ggplot2 [30]. Statistical differences for each ASV (differential abundance) were assessed using Deseq2 [29], as suggested recently for the analysis of microbiome data with a small number of replicates per treatment (<20) [31,32].

Sequencing of the transcriptomic libraries from the 9 hindgut and 9 skin samples yielded ~1064 million reads, with an average of 59 ± 17 million reads per sample (Table S1). Reads were quality filtered to remove low quality reads and Illumina adapters using Trimmomatic (v0.38) with the parameters ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10 LEADING:10 TRAILING:10 SLIDINGWINDOW:4:20 MINLEN:40 [33]. Then, rRNA sequence reads were removed from the dataset using SortmeRNA (v. 2.1) by using the default settings, which included interrogation against the SILVA rRNA database [34]. A total of 476 million cleaned paired-end reads (average of 26 ± 13 million reads per sample) were subsequently obtained and, in the absence of an annotated reference genome for *S. lalandi*, were mapped to the genome from the related species *Seriola dumerili* (accession number GCA_002260705.1 in ensembl.org) using STAR (v. 2.5.3a) [35]. Reads were aligned back to the genome and counted with Subread (v. 1.6.2) using the function featureCounts [36]. Approximately 80 ± 2% of the reads was able to be mapped to the *S. dumerili* genome (a similar mapping rate around ~80% was found using the *S. lalandi* reference genome). The resultant count data were used to initially

identify biological outliers via ordination of the relative abundances of the transcript sequence reads using Deseq2 (wherein one hindgut sample from the early group was removed from the downstream analysis (Figure S2)), and Deseq2 was used to calculate differential gene expression [29]. Genes were identified as significantly differentially expressed when p-adj < 0.05 and log2 fold-change> or <1.5. Differential pathway analysis was performed using Voronto [37] and the R package clusterProfiler [38] using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database [39].

3. Results

3.1. Analysis of the Gut and Skin Microbiota

To explore whether the impact of gut enteritis on bacterial community dynamics is similarly reflected across the mucosal surfaces of yellowtail kingfish, hindgut samples from fish belonging to three different health states (n = 12 fish per health state) were compared with data obtained earlier from the skin of the same fish and environmental (seawater) samples [21].

The overall gut microbiota was significantly different from the skin samples based on the Bray–Curtis similarity matrix (Pseudo-F = 38.80, p = 0.002). In addition, the gut microbiota had a significantly lower Shannon diversity than the skin (p < 0.001) and significantly lower ASV richness (p < 0.001) with 40 ± 20 ASVs when skin samples had an average of 545 ± 296 ASVs per sample.

3.1.1. Influence of Gut Enteritis on the Global Gastrointestinal and Skin Mucosal Microbiota

We investigated the effect of gut enteritis on the gastrointestinal and skin mucosal bacterial communities. We found that the health status had a significant influence on the global gut (Figure 1a; Pseudo-F = 4.43, p = 0.003) and skin (Figure 1b; Pseudo-F = 4.55, p = 0.003) bacterial communities based on the weighted Unifrac distance matrix. More specifically, the gut microbiota of healthy fish were significantly different when compared to fish at the early (Pseudo-F = 7.05, p = 0.045) and late stage of the disease (Pseudo-F = 4.99, p = 0.045). However, the gut microbiota of the fish at the early and late stage of the disease were not significantly different (Pseudo-F = 0.44, p = 0.615). In contrast, no global bacterial communities differences were found when using the unweighted Unifrac distance (Figure S3, Pseudo-F = 1.15, p = 0.268). On the other hand, the skin microbiota were significantly different between all health states using both weighted Unifrac (p < 0.05 for all pairwise comparisons) and unweighted Unifrac distances (Figure S4, p < 0.001 for all pairwise comparisons).

The alpha-diversity (Shannon index) in the gut microbiota of the fish at the late stage of the disease was lower than both healthy and early fish, though not significantly different (Figure 1c; p = 0.123 and p = 0.291, respectively). Similarly, the microbial community evenness (Pielou's index) was lower in the fish at the late stage of the disease but was not significant, while the richness (Chao1 index) was fairly consistent across all three health status (Figures S5 and S6). Unlike the gut, fish at the early stage of the disease exhibited a significant loss of Shannon's diversity in the skin compared to both healthy and fish at the late stage of the disease (Figure 1d; p = 0.007 and p = 0.020, respectively). This was associated with a significant loss of both evenness and richness in the fish at the early stage of the disease when compared to both healthy and early stages of the disease (Figures S7 and S8).

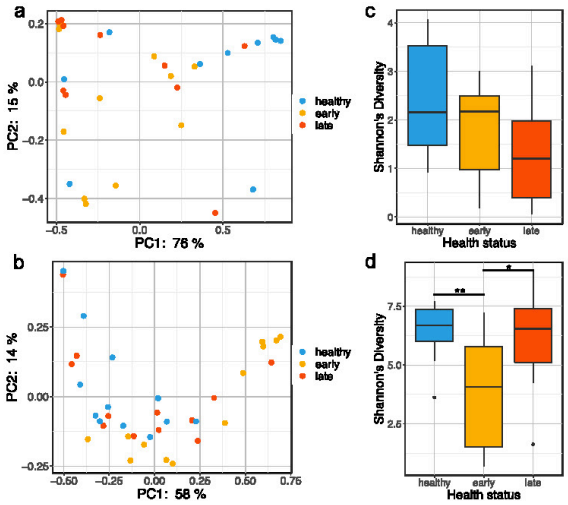

Figure 1. Global bacterial community changes associated with gut enteritis. PCoA plot based on the weighted Unifrac distance matrix showing clustering of gut (**a**) and skin (**b**) microbiota samples by health status (e.g., healthy, early stage of enteritis and late stage of enteritis); boxplot representing the Shannon's diversity of the gut (**c**) and skin (**d**) microbiota for the different health status. Statistical differences were assessed using a Kruskall–Wallis test, with the levels of statistical significance between groups denoted by asterisks, with alpha set at 0.05.

3.1.2. Taxonomic Composition and Potential Biomarkers of Gut Enteritis in the Gut and Skin Microbiota

The gut microbiota was dominated by a few bacterial members, including *Mycoplasmataceae*, Aliivibrio, Photobacterium and Brevinema (Figure 2a). The most dominant ASV was associated with an uncultured *Mycoplasmataceae* sp. and represented 54 ± 34% of the total relative abundance in the gut samples. This ASV was significantly less prevalent in healthy fish than both fish at the early and late stage of the disease (Figure 2b; $p = 0.021$ and $p = 0.013$, respectively, using the Kruskall–Wallis test). At the genus level, the other most dominant members were Photobacterium (13% of the total relative abundance), Aliivibrio (11%), Brevinema (10%) and Vibrio (9%). In contrast, the skin microbiota was more diverse and dominated by other bacterial lineages in both health states. At the order level, the skin microbiota was dominated by Flavobacteriales members, representing 46% of the total relative abundance (Figure 2c). Other important members were related to Alteromonodales (10%), *Rhodobacterales* (9%), Oceanospirillales (5%) and Synechococcales (3%).

To further characterize the change of gut and skin microbial communities associated with the disease, we performed some differential abundance analyses to identify potential biomarkers within these mucosal tissues. In the gut, we found 12 ASVs that were significantly differentially abundant between healthy fish and fish at the early stage of the disease (Table S2). All of them were less abundant in the fish at the early stage, with four associated with Aliivibrio and four associated with Photobacterium. Within the fish at the late stage of the disease, we found 11 ASVs that were differentially abundant when compared with healthy fish (all less abundant in diseased fish) (Table S3). Among these, we found the same ASVs related to Aliivibrio and Photobacterium, indicating that these ASVs are less abundant in diseased fish, regardless of the stage of the disease. Finally, only one ASV was significantly different between fish at the early and late stage of the disease, which was Gammaproteobacteria (Table S4).

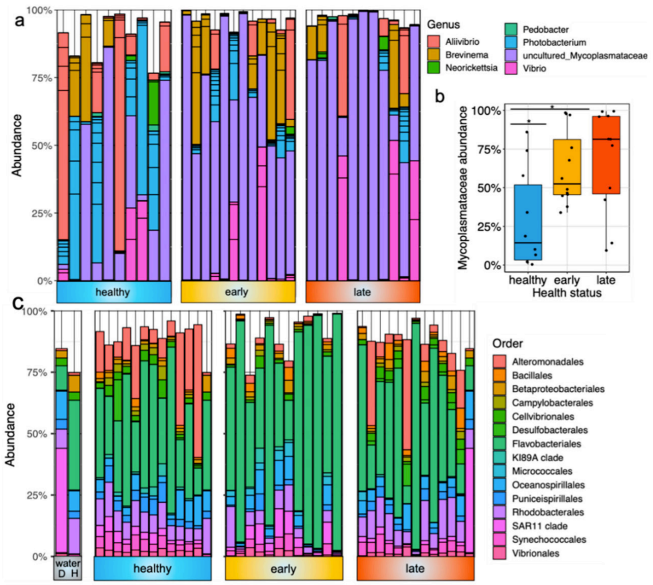

Figure 2. Taxonomic composition of the fish gut and skin microbiota with different health states: (**a**) barplot representing the relative abundance of the top 30 most abundant ASVs in the gut microbiota of fish exhibiting different health states (e.g., healthy, early stage of enteritis and late stage of enteritis); (**b**) boxplot representing the relative abundance of an uncultured *Mycoplasmataceae* sp. in the gut microbiota of fish exhibiting different health states; statistical differences were assessed using Wilcoxon test, with the levels of statistical significance between groups denoted by asterisks, with alpha set at 0.05; (**c**) barplot representing the relative abundance of the top 15 most abundant order in the skin microbiota of fish exhibiting different health status as well as seawater bacterial communities (D = diseased cage and H = healthy cage).

In stark contrast to the gut, we found more differentially abundant ASVs associated with disease within the skin. Interestingly, while most of the differentially abundant ASVs in the gut were found in the two diseased states (both early and late), those found in the skin were mainly in the early condition only. More specifically, 195 and 263 ASVs were found differentially abundant between fish at the early stage of the disease and healthy and late condition, respectively (Tables S5 and S6). However, despite being housed in two different cages with relatively distinct water bacterial communities (Figure 2c), we only found 18 differentially abundant ASVs between healthy fish and fish at the late stage of the disease (Table S7). At the genus level, Alteromonas, Pseudoalteromonas, Glaciecola, Halomonas, Marinobacter, Cobetia, Idiomarina, Arcobacter, SAR 92 clade, Synechococcus CC9902 and Litoricola were all found to be significantly depleted in the fish at the early stage of the disease when compared to healthy fish or fish at the late stage of the disease.

3.2. Analysis of the Transcriptomics Data

In order to evaluate the mucosal immunity across both the gut and skin mucosal surfaces of yellowtail kingfish in response to gut enteritis, we performed some differential gene expressions on selected samples representing the three different conditions (i.e., healthy, early and late stage of enteritis; $n = 3$ per condition). We found <100 significantly differentially expressed genes (DEGs) in the gut when comparing all treatment groups (Table 1). On the other hand, more DEGs were found in the skin, in particular in the group at the late stage of the disease (1467 DEGs when compared to healthy and 2068 DEGs when compared to early).

Table 1. Table representing the number of differentially expressed genes (DEGs) in the gut and skin of fish exhibiting different health states (H = healthy, E = early stage of enteritis and L = late stage of enteritis).

Sample Type	Gene Expression	HvsE	HvsL	EvsL
gut	upregulated	15	31	18
	downregulated	52	57	1
	total	67	88	19
skin	upregulated	54	481	552
	downregulated	130	986	1516
	total	184	1467	2068

3.2.1. Differential Expression in the Gut of Fish Exhibiting Different Health States

Of particular interest, we found a number of genes associated with the intestinal immune network for immunoglobulin production (H-2 class II histocompatibility antigen and HLA class II histocompatibility antigen associated genes) and the Toll-like receptor signaling pathway (TIR domain containing adaptor protein and Toll-like receptor 2 type-2) downregulated in the fish at the late stage of the disease (Table S7). In addition, we found a neutrophil related gene (*ncf4*) downregulated in these late fish (log2fold = −1.58); this gene group plays a role in the phagosome pathway and a procathepsin H-like gene involved in the lysosome and apoptosis pathway. In contrast, some genes related to glycerolipid metabolism (patatin-like phospholipase) and glycine metabolism (glycine dehydrogenase and glycine decarboxylase) were found upregulated in the fish at the late stage of the disease (Table S8).

In the fish at the early stage of the disease, we found an upregulation of the histone H2A-like gene involved in the necroptosis pathway (log2fold = 6.15, Table S9). On the other hand, two genes involved in the apoptosis pathway (procathepsin H-like and inositol 1,4,5-trisphosphate receptor type 1-like) were downregulated in these early group fish (Table S8). Overall, due to the low number of DEGs found in the gut, no pathways were significantly differently expressed between groups.

3.2.2. Differential Gene Expression and Associated Pathways in the Skin of Fish at the Late Stage of the Disease

Due to the high number of DEGs found in the skin, we used Voronoi tessellation diagrams (using Voronto) to represent the expression of the different pathways between the different health states. In the fish at the late stage of the disease, the immune system pathway was downregulated compared to healthy fish (Figure 3). In fact, all six pathways at level 3 of the KEGG database (including but not limited to the Toll-like receptor signaling pathway, NOD-like receptor signaling pathway and intestinal immune network for immunoglobulin production pathway) were downregulated. In addition, we found a downregulation of the cytokine–cytokine receptor interaction pathway including the downregulation of cytokines (such as *il1*, *il8*, *il12*, *il17* and *il23*) and chemokines (Figure 3, Table S10). In contrast, the ECM (extracellular matrix)–receptor interaction and focal adhesion pathways were upregulated in these fish at the late stage of the disease (Figure 3).

We then investigated the significantly differentially expressed pathways in these fish at the late stage of the disease. Using clusterProfiler, we identified six significantly downregulated and four significantly upregulated pathways (Figure 4). The most significant pathways were ECM–receptor interaction and focal adhesion (both upregulated) and the cytokine–cytokine receptor interaction pathway (downregulated). The upregulation of the ECM-receptor interaction was characterized by an upregulation of collagen, laminin, reelin, thrombospondin, fibronectin and tenascin (Figure S9).

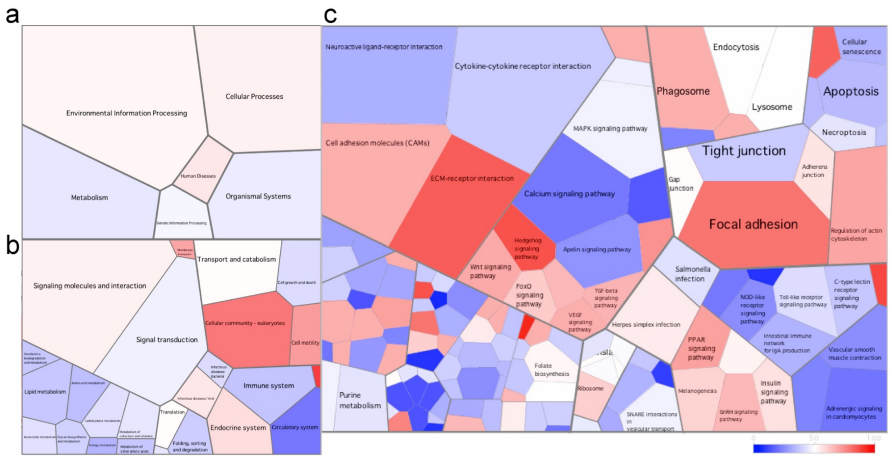

Figure 3. Voronoi tessellation diagrams representing differentially expressed pathways at (**a**) level 1, (**b**) level 2 and (**c**) level 3 of the KEGG database in the skin of the fish at the late stage of the disease. Each polygon represents an ontology term, with their size corresponding to the numbers of genes involved in the associated pathway. To further explore the pathways differentially expressed between health states, we imported all DEGs (and associated logs values) to generate the Voronoi tessellations. Pathways colored in red are upregulated, and those in blue are downregulated in the fish at the late stage of the disease when compared to healthy fish. A quantile color scale was used to show differential expression.

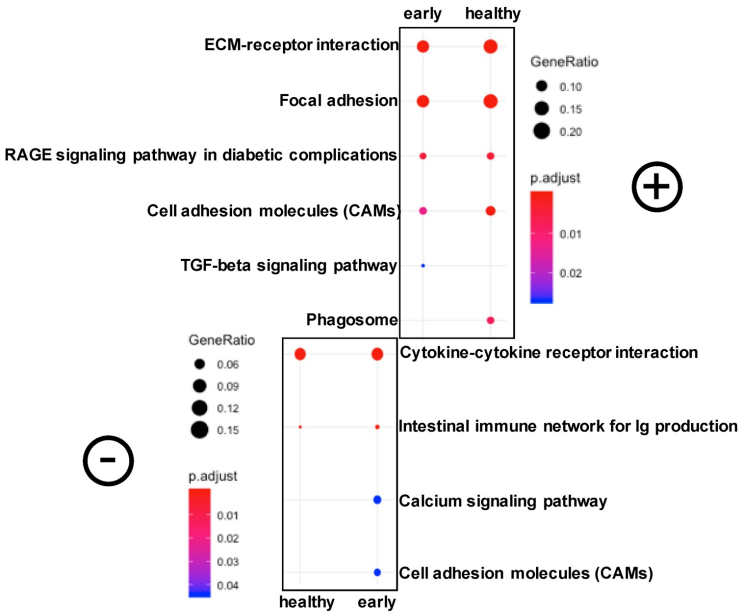

Figure 4. A representation of significantly differentially expressed pathways in the skin of fish at the late stage of the disease compared to both healthy and early conditions using clusterProfiler. Symbols were used to designate upregulated (+) and downregulated (–) pathways.

3.2.3. Differential Gene Expression and Associated Pathways in the Skin of Fish at the early Stage of the Disease

Although no significant differentially expressed pathways were identified, numerous genes were differentially expressed in the fish at the early stage of the disease compared to healthy fish (Table S11). In contrast to the fish at the late stage of the disease, we found that the cytokine–cytokine receptor interaction pathway was upregulated in the early stage fish compared to healthy fish (Figure 5). The immune system (including four out of five pathways at level 3) was also upregulated in the fish at the early stage of the disease. Furthermore, we found a notable upregulation of the foxO signaling pathway due to the strong upregulation of the recombination-activating gene (*rag1*, log2fold = 6.74). The cellular community pathways were downregulated (including all four associated pathways at level 3 comprising focal adhesion, tight junction, gap junction and adherens junction) in these early fish.

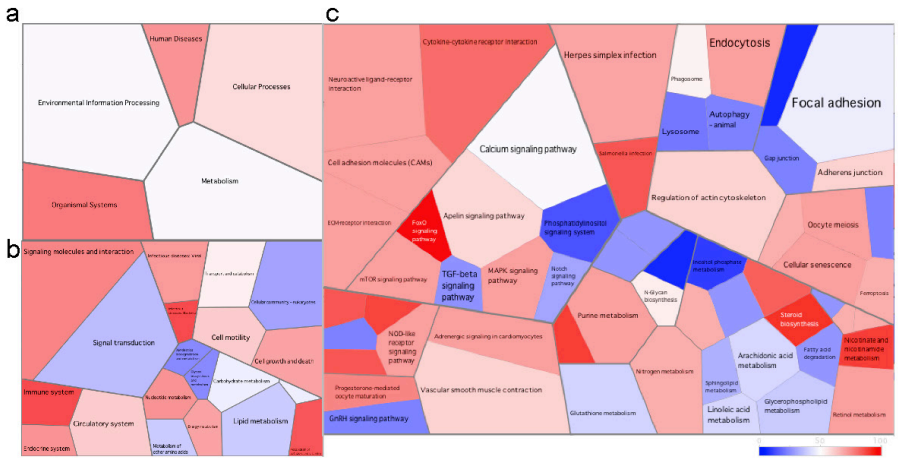

Figure 5. Voronoi tessellation diagrams representing differentially expressed pathways at (**a**) level 1, (**b**) level 2 and (**c**) level 3 of the KEGG database in the skin of the fish at the early stage of the disease. Each polygon represents an ontology term, with their size corresponding to the numbers of genes involved in the associated pathway. To further explore the pathways differentially expressed between health states, we imported all DEGs (and associated logs values) to generate the Voronoi tessellations. Pathways colored in red are upregulated, and those in blue are downregulated in the fish at the early stage of the disease when compared to the healthy fish. A quantile color scale was used to show differential expression.

4. Discussion

To date, enteritis remains a major issue in the farming of numerous carnivorous species fed a diet partly constituted of soybean meal. Numerous strategies have been deployed to mitigate soybean-induced inflammation in fish. These include the supplementation of glutamine, arginine, resveratrol, microalgae, bacteria grown on natural gas and lactoferrin in the feed [40–44]. Fermentation of soybean meal prior to feeding has also been tested in turbot with encouraging results, suppressing the intestinal inflammation and enhancing the intestinal integrity [45]. Furthermore, efforts in selecting resistant fish with increased tolerance to plant diets have been made in some species such as rainbow trout (*Oncorhynchus mykiss*) [46]. In yellowtail kingfish, efforts in finding alternatives to soybean meal showed that poultry byproduct, faba bean and lupin kernel meals represent good protein sources compared to corn gluten and blood meals [47]. Here, we investigate both the microbiota and gene expression across the gut and skin mucosal surfaces of healthy fish and fish at different

stages of enteritis, identifying important microbial changes and differentially expressed host genes and associated pathways that reflect the health status of the fish.

The gastrointestinal microbiota of diseased farmed yellowtail kingfish was dominated by an uncultured *Mycoplasmataceae* sp. Members of this family are typically found in the gut of farmed fish including Atlantic salmon, rainbow trout and common carp [48–50]. In our study, we found that this member was prevalent in diseased fish (both at the early and late stage of gut enteritis) when compared to healthy fish, indicating a potential harmful effect of these bacteria. In zebrafish, Mycoplasma was prevalent in fish exposed to a parasite (*Pseudocapillaria tomentosa*) and positively correlated with hyperplesia [18]. It was hypothesized that this member was responsible for the lesions in the fish, confirming a potential harmful role of certain *Mycoplasma* sp. In fact, this genus already includes known fish pathogens, such as *Mycoplasma mobile*, a bacterium colonizing the gills of freshwater fish [51]. In contrast, recent genome reconstruction analysis revealed a mutualistic lifestyle of new *Mycoplasma* species isolated from Atlantic salmon and hadal snailfish (*Pseudoliparis swirei*) [50,52]. In our study, it is unclear whether this bacterium played a role in the disease, or was the result of the poor overall health of the fish. Thus, functional analyses (e.g., shotgun metagenomics or metatranscriptomics) may be further required to elucidate the role of this bacterium in yellowtail kingfish health.

The overall gut bacterial community was significantly different between healthy and diseased fish, and was associated with a loss of diversity in the fish at the late stage of the disease. This is supported by the literature, where numerous investigations reported a loss of microbial diversity in fish exposed to stress or disease [26]. In addition, the overall skin microbiota was significantly different between all three health states, indicating that this gut disease not only influences the gut microbiota but also the outer surface microbiota, as previously shown by our group [21]. We found the largest number of differentially abundant ASVs between fish at the early stage of the disease and fish at the late stage of the disease, even though these fish were housed in the same cage. In contrast, very few differentially abundant ASVs were detected between healthy and fish at the late stage of the disease despite them being housed in two different cages separated by almost 7 km. While the fish skin microbiota has been shown to be a lot more sensitive to the environment than the gut [53], our results shows that health status can also play a major role in shaping the fish skin microbiota regardless of the surrounding environment. Interestingly and in contrast to the gut microbiota, we found a drastic loss of skin microbial diversity and evenness in the fish at the early stage of the disease when compared to healthy fish and fish at the late stage of the disease. Loss of diversity and evenness are characterized by a reduced resilience and functional capacity of the microbial communities, indicating that the gut and skin microbiota of diseased fish may have lost important functions including resistance to opportunistic pathogens [54].

To better understand this loss of bacterial diversity in the fish skin at the early stage of the disease, we investigated the immune response through transcriptomic (RNA-Seq) analysis. Firstly, we found that the immune system pathway was upregulated in the fish at the early stage. More specifically, the cytokine–cytokine receptor interaction pathway was upregulated in this condition compared to healthy fish, and significantly upregulated when compared to fish at the late stage of the disease. In this pathway, interleukin 8-like, regakine 1-like and CXC (Cystine-X-Cystine, where X is any amino acid) chemokine receptor type2-like genes were significantly upregulated in the fish at the early stage of the disease compared to the other two health states. These genes could potentially be used as biomarkers for the early detection of gut enteritis in yellowtail kingfish, although this requires further validation. Interleukin 8 was found to modulate the early cytokine immune response in rainbow trout, and its upregulation at the early stage of the disease indicates a proinflammatory response in the fish skin [55]. This same gene was found to be upregulated in the skin of rainbow trout after Ich infection, suggesting that its expression could be a potential useful biomarker for the detection of a proinflammatory response in fish [56]. CXC motif chemokine receptor 2 (*cxcr2*) upregulation has been associated with numerous inflammations and is known to induce the recruitment of neutrophils, supporting the idea of an immune response at this stage of the disease [57]. In addition, we found

a strong upregulation of the foxO signaling pathway in the early stage fish, driven by a significant upregulation of recombination activating gene 1 (*rag1*). The protein encoded by this gene is involved in antibody and T-cell receptors and as such, plays an important role in the recognition of pathogen and immunoregulation. In fact, *rag1* is a known gene marker for the early development of the fish immune system, and therefore, the upregulation of this gene at the earliest stage of the disease confirmed an immune response in the skin mucosal surface [58]. In support of this, a recent study showed that *rag1*-deficient zebrafish did not develop intestinal inflammation when fed an inflammatory diet constituted of soybean, in contrast to normal fish [11]. This highlights the role of adaptive immunity in the response against enteritis, a feature confirmed in yellowtail kingfish. Altogether, these changes in gene expression suggest a strong immune response located in the fish skin. Some changes also highlight compromised functions within this mucosal surface. For instance, we observed a downregulation of the adherens junction, gap junction and tight junction—three pathways extremely important for barrier function [59]. Considering the vital role of the skin as a physical barrier to prevent the intrusion of potential pathogens, these changes could lead to the dysfunction of this organ, resulting in an increased disease susceptibility [60].

In the skin of fish at the late stage of the disease, we observed a significant upregulation of the ECM–receptor interaction and focal adhesion pathways. The ECM–receptor interaction pathway plays important functions in the host immune system including cell proliferation, differentiation and survival, intercellular communication and the regulation of leukocytes into inflamed tissues [61]. The upregulation of several proteins involved in this pathway likely reflect the advanced stage of the disease in which the host is developing a strong response to repair tissues. The upregulation of the focal adhesion pathway is not surprising considering its close interaction with the ECM–receptor interaction pathway. Indeed, focal adhesion also plays a role in the regulation of cell cycle progression and its dysregulation has been shown in numerous human diseases such as cancer and Alzheimer's disease [62,63]. Within this pathway, we found an upregulation of integrin alpha subunit (ITGA) associated genes. These integrins are also involved in the ECM pathway and can be characterized as signaling molecules controlling cell differentiation, growth and survival [64]. In contrast to the fish at the early stage of the disease, fish at the late stage showed a downregulation of numerous cytokines (e.g., chemokines and interleukins) in the skin. This would suggest that the fish have passed the acute immune response phase and is reflective of a very weakened host. The downregulation of the immune system across the six KEGG pathways at level 3 confirms this hypothesis.

Although we investigated the influence of a gut disease, few genes were differentially regulated during disease in the gastrointestinal tract. Recently, it was shown that grass carp can exert different responses along the intestinal tract to induce inflammation when fed soybean ß-conglycinin [65]. More specifically, while the midgut and hindgut showed signs of inflammation, no changes were found in the foregut. Similarly, it was found that rainbow trout exhibits different microbial and immune responses across different regions of the digestive tract (e.g., mouth, pharynx, stomach, foregut, midgut and hindgut) following viral infection [66]. In this study, we only investigated the hindgut gene expression and microbiota, and therefore, other parts of the intestinal system could have had differential gene expression and/or microbial changes that went undetected.

Recently, the concept of the hologenome has gained popularity. This concept argues that the genome of the host and its associated microbial communities (microbiome) are in constant interaction, and as such, cannot be viewed independently [67]. It is well known that fish regulate their microbiota across all mucosal surfaces through mucus production, immune related cells and antimicrobial peptides [2]. Here, we speculate that the loss of microbial diversity observed in the skin of fish at the early stage of the disease could be linked with the upregulation of the immune system. More specifically, the upregulation of *rag1*, *il8* and *cxcr2* may be in part responsible for the observed changes in the skin microbiota. Moreover, *cxcr2* (also called *il8rb*) encodes a protein which is a receptor for *il8*. As such, *il8* and *cxcr2* interact closely and the upregulation of them both is not surprising. Since *il8* is associated with antimicrobial responses [66,68–70], its upregulation in the skin of fish at the early

stage of the disease may have played a role in this observed loss of microbial diversity. Furthermore, the microbiota is known to modulate the host immune response [71]. More specifically, the production of several inflammatory cytokines can be driven by specific microbiota, highlighting the importance of host–microbiota interactions [72]. In this study, the number of replicates was insufficient in order to correlate the host gene expression with microbial diversity and further work should be performed in order to better understand host–microbe interactions, in particular in a health and disease context.

5. Conclusions

In this study, we found that gut enteritis perturbed the yellowtail kingfish gut microbiota, with an enrichment of an uncultured *Mycoplasmataceae* sp. in diseased fish. We observed profound changes within the skin microbiota, highlighting the sensitivity of this mucosal surface in relation to the host health. More specifically, fish at the early stage of the disease had a significant loss of microbial skin diversity when compared to both healthy fish and fish at the early stage of the disease. Surprisingly, gene expression within the gut did not widely differ between health conditions. In contrast, numerous differentially expressed pathways and genes were found in the skin, particularly in the fish at the late stage of the disease where several metabolic pathways were differentially expressed.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/9/1267/s1. Figure S1: Rarefaction plot of all samples used in this study showing sufficient sequencing depth at 16,255 reads/sample. Figure S2: PCoA plot representing the gene expression of both gut and skin samples of all fish used in this study. Figure S3: Boxplot representing the Pielou's evenness of the gut microbiota for the different health status. Statistical differences were assessed using a Kruskall–Wallis test. Figure S4: Boxplot representing the Pielou's evenness of the skin microbiota for the different health status. Statistical differences were assessed using a Kruskall–Wallis test. Figure S5: Representation of genes involved in the ECM–receptor interaction pathway in the fish at the late stage of the disease. Genes in red were upregulated, in blue were downregulated and in green were not statistically differentially expressed. Figure S6: Boxplot representing the Chao1 richness in the gut microbiota for the different health status. Statistical differences were assessed using a Kruskall-Wallis test. Figure S7: Boxplot representing the Pielou's evenness in the skin microbiota for the different health status. Statistical differences were assessed using a Kruskall-Wallis test. Figure S8: Boxplot representing the Chao1 richness in the skin microbiota for the different health status. Statistical differences were assessed using a Kruskall-Wallis test. Figure S9: Representation of genes involved in the ECM-receptor interaction pathway in the fish at the late stage of the disease. Genes in red were upregulated, in blue were downregulated and in green were not statistically differentially expressed. Table S1: Number of RNA-seq reads retained for analysis following each step of the bioinformatic pipeline. Table S2: Differential abundant ASVs found in the gut microbiota of fish at the early stage of the disease when compared to healthy fish. Table S3: Differential abundant ASVs found in the gut microbiota of fish at the late stage of the disease when compared to healthy fish. Table S4: Differential abundant ASVs found in the gut microbiota of fish at the late stage of the disease when compared to fish at the early stage of the disease. Table S5: Differential abundant ASVs found in the skin microbiota of fish at the early stage of the disease when compared to healthy fish. Table S6: Differential abundant ASVs found in the skin microbiota of fish at the late stage of the disease when compared to fish at the early stage of the disease. Table S7: Differential abundant ASVs found in the skin microbiota of fish at the late stage of the disease when compared to healthy fish. Table S8: Differentially expressed genes (DEGs) found in the gut of fish at the late stage of the disease when compared to healthy fish. Table S9: Differentially expressed genes (DEGs) found in the gut of fish at the early stage of the disease when compared to healthy fish. Table S10: Differentially expressed genes (DEGs) found in the skin of fish at the late stage of the disease when compared to healthy fish. Table S11: Differentially expressed genes (DEGs) found in the skin of fish at the early stage of the disease when compared to healthy fish.

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Chapter 3: Antibiotic-induced alterations and repopulation dynamics of yellowtail kingfish microbiota

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Contribution to the Paper	Designed and performed the experiment, performed data processing, analysed and interpreted the data, wrote the manuscript.		
Overall percentage (%)	65		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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RESEARCH ARTICLE Open Access

Antibiotic-induced alterations and repopulation dynamics of yellowtail kingfish microbiota

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Abstract

Background: The use of antibiotics in aquaculture is a common infection treatment and is increasing in some sectors and jurisdictions. While antibiotic treatment can negatively shift gut bacterial communities, recovery and examination of these communities in fish of commercial importance is not well documented. Examining the impacts of antibiotics on farmed fish microbiota is fundamental for improving our understanding and management of healthy farmed fish. This work assessed yellowtail kingfish (*Seriola lalandi*) skin and gut bacterial communities after an oral antibiotic combination therapy in poor performing fish that displayed signs of enteritis over an 18-day period. In an attempt to promote improved bacterial re-establishment after antibiotic treatment, faecal microbiota transplantation (FMT) was also administered via gavage or in the surrounding seawater, and its affect was evaluated over 15 days post-delivery.

Results: Antibiotic treatment greatly perturbed the global gut bacterial communities of poor-performing fish – an effect that lasted for up to 18 days post treatment. This perturbation was marked by a significant decrease in species diversity and evenness, as well as a concomitant increase in particular taxa like an uncultured *Mycoplasmataceae* sp., which persisted and dominated antibiotic-treated fish for the entire 18-day period. The skin-associated bacterial communities were also perturbed by the antibiotic treatment, notably within the first 3 days; however, this was unlike the gut, as skin microbiota appeared to shift towards a more 'normal' (though disparate) state after 5 days post antibiotic treatment. FMT was only able to modulate the impacts of antibiotics in some individuals for a short time period, as the magnitude of change varied substantially between individuals. Some fish maintained certain transplanted gut taxa (i.e. present in the FMT inoculum; namely various *Aliivibrio* related ASVs) at Day 2 post FMT, although these were lost by Day 8 post FMT.

Conclusion: As we observed notable, prolonged perturbations induced by antibiotics on the gut bacterial assemblages, further work is required to better understand the processes/dynamics of their re-establishment following antibiotic exposure. In this regard, procedures like FMT represent a novel approach for promoting improved microbial recovery, although their efficacy and the factors that support their success requires further investigation.

Keywords: 16SrRNA, Microbiome, FMT, Antibiotics, Fish

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Introduction

Aquaculture is the fastest growing sector in the food animal industry [1]. However, its development is not without challenges. Due to the intensive methods used in production, diseases are common and often require the use of therapeutics. While there are a range of alternative treatment options available (e.g. probiotics, prebiotics, synbiotics, postbiotics, phytobiotics, phage therapy, or quorum sensing interference), antibiotics remains the most common therapy used in some aquaculture sectors to treat microbial infections [2, 3]. Worldwide, 67 antibiotic compounds were reported to be used in 11 of the top 15 highest producing countries, with oxytetracycline, sulphadiazine, and florfenicol the most commonly used in the industry [4]. In some countries, usage has increased substantially in recent years. For instance, antibiotic use doubled over a three-year period (from 143 to 382 tons between 2013 and 2016) in the Chilean salmon industry, which was largely attributed to the increased use of florfenicol and oxytetracycline for combatting *Piscirickettsia salmonis* infection [5]. While antibiotic use varies significantly between countries due to different laws and regulations [4, 6], there is widespread concern regarding the development of antimicrobial resistance in the global aquaculture industry and their broader impacts on the environment [5, 7–10].

The risks posed by the use of antimicrobials also include changes in an animal's microbiota. Many of the antibiotics used in the aquaculture industry are considered to be broad-spectrum, and may indiscriminately act on both the pathogenic and commensal constituents [11]. Perturbation of the gut microbiota following antibiotic exposure has been reported in various fish species [8, 12, 13] and may be associated with changes in microbial enzymatic activity, gene expression, and protein and metabolite synthesis [14]. In humans and other animals, antibiotic use may have prolonged effects on the gut bacterial composition, leading to widespread perturbations and the extinction of some species [15]. Considering the importance of the microbiota in nutrient metabolism, digestion, and disease resistance [16], antibiotic-induced changes may be functionally detrimental, impacting the health and fitness of the animal. Knowledge of the specific impacts caused by select antibiotics as well as strategies that seek to minimise their effects on the fishes' microbiota are thus likely to be a critical feature for supporting optimal performance and productivity of the system.

Prospects for overcoming or improving the inherent effects that antibiotics impose on the microbiota, or for optimising the overall health and fitness of fish in a production context, are increasing in demand and have been extensively studied within the last decade. This includes common strategies that aim to modulate the fish microbiota through the diet in order to improve disease resistance, nutrient digestibility, tolerance to stress, and reproduction [17]. More recently, however, procedures such as faecal microbiota transplantation (FMT) have be touted as a prospective, more holistic approach that has the capacity to improve outcomes by modulating the entire microbial community and facilitating the re-establishment of defunct species [18, 19]. First developed in 1958 to cure pseudomembranous enterocolitis in humans [20], FMT has since been used to successfully treat a range of other conditions including, among others, *Clostridioides difficile* infection, inflammatory bowel disease (IBD), and obesity [21]. Its role in mitigating the effects of antibiotics has also been recently demonstrated in humans and mice and has been shown to be more effective than treatment with probiotics, which instead resulted in a delayed or incomplete reconstitution of the microbiota [22]. In animal production systems, similar findings have also been reported for chickens, alongside improvements in nutritional capacity [23]. To the best of our knowledge, FMT has thus far not been investigated in fish in response to antibiotic-induced microbiota alterations, although experiments in African turquoise killifish (*Nothobranchius furzeri*) have demonstrated the power of the approach, revealing its capacity to restore bacterial diversity in old fish and influence longevity [24].

In Australia, the commercial production of valuable species, such as yellowtail kingfish (*Seriola lalandi*), is impeded by a variety of diseases including fluke infestation and gut enteritis [25, 26]. The latter is known to occur when fish are farmed at suboptimal temperatures and fed with a high proportion of soybean meal, although the mechanisms underlying this disease remain poorly understood and have limited treatment options available beyond antibiotics [26]. Such conditions have also been reported to be accompanied by changes in the bacterial diversity of the outer mucosa (skin and gills), suggesting a body-wide response [27]. An improved understanding of the effects of treatments, as well as new strategies that ameliorate treatment effects on the microbiota of fish suffering from gut disease, are warranted. Here, we investigated the influence of a novel antibiotic combination-therapy formulated for broad spectrum activity against a range of microorganisms (comprising commonly used oxytetracycline, as well as erythromycin and metronidazole) on the gut and skin mucosal microbiota of poor performing yellowtail kingfish (i.e. those suffering from enteritis); and the prospective role of FMT in gut microbiota repopulation.

Methods

Study design and experimental set-up

To assess the impacts of antibiotics and FMT on the gut and skin microbiota of yellowtail kingfish exhibiting

symptomatic features of gut disease (as characterised by low body condition and weight loss), a total of 217 fish with a mean weight of ~ 1.6 kg were obtained from a single seacage (comprising fish of the same cohort, though of mixed genetics) under the auspices of a commercial aquaculture enterprise from temperate waters of southern Australia according to industry best practice veterinary care. Of these, 10 fish were randomly sampled to provide baseline bacterial community composition data. Fish were transported in a water tanker (with oxygen supplementation) to a research facility and housed in 5000 L tanks. Tanks were supplied with seawater at ambient temperature (12.7–14.0 °C; see Additional file 1: Table S1) from a flow-through system with mechanical filtration (drum filter). Additional water parameters were also recorded during the length of the experiment such as dissolved oxygen (94–115% saturation; see Additional file 1: Table S2), pH (7.59–7.73; see Additional file 1: Table S3), salinity (36–37‰; see Additional file 1: Table S4), ammonia concentration (< 0.25 ppm; see Additional file 1: Table S5), and CO_2 concentration (always below detection level). Fish were fed to satiation once a day with the same proprietary feed formulation used in the commercial operation and were allowed to acclimatise for 3 weeks prior to the investigation. Tanks were flushed once a day to eliminate faeces at the bottom of the tanks. During acclimation, 15 fish were randomly sampled for histopathological examination to confirm their condition, revealing mild enteritis (as conducted by an external fish pathologist). After acclimation (~3 weeks), a total of 144 fish were distributed among 12 tanks (n = 12 fish/tank). The lengths and weights of all fish were recorded following brief sedation in 14 mg/L AQUI-S (AQUI-S New Zealand Ltd.) solution in surrounding seawater as described previously [26]. The total fish weight for each tank was recorded with an attempt to make these as even as possible (see Additional file 1: Table S6).

The experimental design comprised 6 treatment groups: (1) no antibiotic treatment with no FMT (A^-/FMT^-); (2) antibiotic treatment with no FMT (A^+/FMT^-); (3) no antibiotic treatment with FMT via oral gavage (A^-/FMT^G); (4) antibiotic treatment with FMT via oral gavage (A^+/FMT^G); (5) no antibiotic treatment with FMT via water (A^-/FMT^W); and (6) antibiotic treatment with FMT via water (A^+/FMT^W). Each treatment was replicated across two tanks of 12 fish (Fig. 1). Antibiotic treatment was administered by oral gavage 3 days prior to FMT treatment and consisted of a combination therapy comprising oxytetracycline (200 mg/kg), erythromycin (50 mg/kg) and metronidazole (50 mg/kg) (Sigma-Aldrich), which was prepared the morning of administration in polypropylene glycol (Sigma-Aldrich). Dose was determined in consultation with veterinary staff based on existing knowledge from Seriola or other species and was formulated to maximise the depletion of various types of gram positive and negative bacteria.

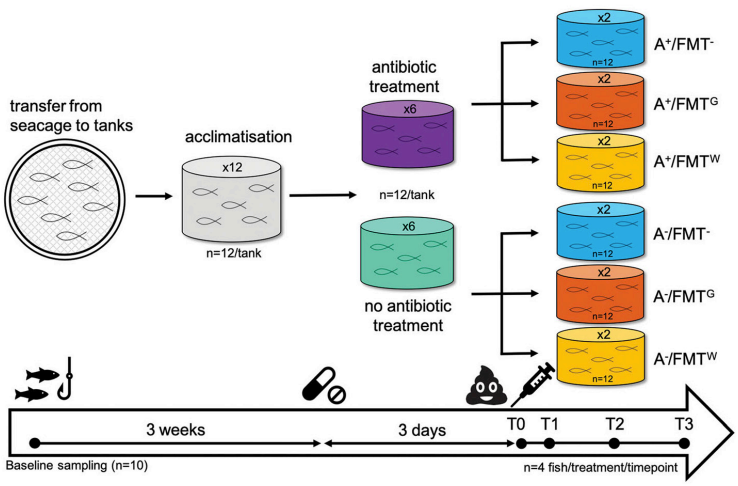

Fig. 1 Overview of the experimental design of the study. A total of 6 treatment groups were compared and included no antibiotic treatment with no faecal microbiota transplantation (A^-/FMT^-); antibiotic treatment with no FMT (A^+/FMT^-); no antibiotic treatment with FMT via gavage (A^-/FMT^G); antibiotic treatment with FMT via gavage (A^+/FMT^G); no antibiotic treatment with FMT via water (A^-/FMT^W); and antibiotic treatment with FMT via water (A^+/FMT^W). Each treatment was replicated across 2 tanks. Antibiotic treatment was administered by oral gavage 3 days prior to FMT, with sampling conducted at: T0 = immediately prior to FMT; T1 = 2 days post FMT; T2 = 8 days post FMT; and T3 = 15 days post FMT

The FMT inoculum comprised the gut contents from 102 x ~ 3.5–4.5 kg healthy fish from a "healthy" seacage, where fish showed no signs of disease. The gut contents were obtained by the manual stripping of fish on-site at the commercial operation, which was immediately transported back to the research facility on ice for use in the trial on the same day. A total of 110 mL of feacal material was obtained and was made up to 400 mL in filter-sterilised seawater (as prepared using a 0.22 μm Nalgene™ Rapid-Flow™ filter unit, Thermo Fisher Scientific), and mixed thoroughly by vortexing. The inoculum was subsequently split into two parts, where one part (200 mL) was inoculated with one capsule (containing ~ 10 billion cells, $\sim 5 \times 10^7$ cells/mL) of the commercial probiotic *Lactobacillus rhamnosus* GG (LGG®) (Inner Health), a strain previously reported to have protective effects against pathogen infection [28], while the other part was left untreated; these are herein referred to as 'spiked' and 'unspiked' inoculum respectively. FMT was administered by oral gavage or in the surrounding seawater of the tanks 3 days post-antibiotic treatment, as described below.

For the treatment groups that received antibiotics and/or FMT via gavage, fish were first sedated in seawater comprising 14 mg/L AQUI-S (as described above) and then administered the treatment via a sterile 5 mL syringe fitted with a soft silicone tube [ø 5 mm] (Gecko Optical Scientific Equipment, Australia) that was just long enough to enter the stomach (~ 17 cm), as guided through a larger [ø 10 mm] flexible PVC tube. Care was taken to minimise stress by placing the fish on a cushioned surface, covering with a seawater saturated cloth, and gently restraining the fish during the brief procedure. A total of 1.5 mL of antibiotics and/or 3 mL of FMT inoculum was administered to each fish during the respective treatments.

For the treatment groups that received the FMT inoculum within the surrounding seawater, the water level of the tanks was dropped to ~ 1500 L. The tanks were cleaned to remove any accumulated faecal material and then 60 mL of the spiked or unspiked inoculum sample was added to a 5 liter bucket of seawater which was then added to the respective treatment tanks. Fish were then allowed to bath in the FMT inoculum treated water with no exchange (though with oxygen supplementation) for 3 hours before being refilled to full capacity.

Sampling of fish

Alongside the 10 fish collected for baseline analyses, four fish per treatment/time point (n = 2 fish/replicate tank) were sampled over an 18 day period (i.e. at T0 [3 days post antibiotic treatment]; T1 [5 days post antibiotic treatment and 2 days after FMT]; T2 [11 days post antibiotic treatment and 8 days after FMT]; and T3 [18 days post antibiotic treatment and 15 days after FMT]) (Fig. 1). From each fish, a swab of the skin and a scraping of the hindgut was collected. Swabs of the skin were conducted as detailed earlier using sterile FLOQSwabs® (COPAN) [27]. For the hindgut, the gastrointestinal tract was first removed, separated from the fore and midgut, an incision made along its length with a sterile scalpel blade to expose the inner mucosal surface, and a scraping of the entire region obtained using a sterile glass microscope slide (with care taken to avoid excessive faecal material). Samples were stabilised immediately in RNAlater™ (Ambion) and stored at − 20 °C until downstream RNA extraction. In addition, 500 μL aliquots of the spiked and unspiked inoculum sample were placed in 15 mL tubes with 1.5 mL of RNAlater™ and stored at − 20 °C until downstream nucleic acid extraction. Finally, 1 liter of seawater was also taken from the seacage at the time of the initial fish collection, and 1 liter from a tank at the start and end of the experiment (i.e. at T0 and T3).

Nucleic acid extraction, library preparation and Illumina sequencing

To investigate the active and thus likely resident bacterial community constituents, RNA was extracted from hindgut scrapings, skin swabs and spiked and unspiked FMT inoculum as described previously [27]. In brief, samples were placed into lysing matrix B tubes (MP Biomedicals) containing 1 mL of ice-cold RLT buffer supplemented with 1% β-mercaptoethanol v/v (Sigma-Aldrich). Bead-beating was performed to disrupt the samples using the FastPrep-24™ 5G instrument (MP Biomedicals) at an intensity setting of 5.5 for 45 s. Samples were then placed on ice, disrupted a second time using the same settings, and centrifuged at 14,000×g for 10 min at 4 °C. RNA was then extracted from the supernatant using the RNeasy mini kit (Qiagen) following the manufacturer's instructions. The Turbo DNA-free™ kit (Life Technologies) was used to remove any contaminating gDNA. RNA extracts were then converted to cDNA using the Superscript™ III First Strand Synthesis System (Life Technologies) according to the manufacturer's instructions.

To evaluate the contribution of the surrounding environmental bacterial consortia on the fish microbiota, DNA was extracted from the seawater samples following filtration onto 0.22 μM Nalgene™ Rapid-Flow™ filters (Thermo Fisher Scientific) using the FastDNA™ Spin Kit for Soil (MP Biomedicals) according to the manufacturer's instructions. In addition, DNA was also extracted from the spiked and unspiked FMT inoculum samples using the same kit to evaluate the contribution of any taxa not represented in the RNA extracts. All samples were subsequently concentrated by ethanol precipitation

using standard procedures, quantified using the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific) and stored at − 20 °C prior to downstream library preparation.

The V1-V2 region of the 16S rRNA gene was amplified from the extracted cDNA and DNA extracts using universal eubacterial primers 27F and 338R, as described previously [27], and in conjunction with positive and negative (no template) PCR reagent controls. Briefly, two μL of cDNA and five μL of each sample were first subjected to 20 cycles of PCR, whereby one μL of this mixture from the first round was used as template in a further 15 cycles of PCR for incorporating individual barcodes and Illumina specific adaptors. Finally, one μL of the resultant mixture was used as a template in a final 10-cycle PCR for incorporating the Illumina multiplexing sequencing and index primers. Libraries were then purified using Agencourt AMPure XP beads (Beckman Coulter) and quantified using the Quant-iT™ Picogreen® dsDNA kit (Life Technologies) before being pooled in equimolar ratios and sequenced on the MiSeq platform (Illumina, San Diego, CA, United States) using 250 nt paired-end sequencing chemistry through the Australian Genome Research Facility (AGRF, North Melbourne, VIC, Australia).

Bioinformatics and statistical analysis

Sequence reads were paired using PEAR (v0.9.5) where adapter sequences were also removed [29]. The merged fastq files were then processed and analysed using the QIIME2 (v2019.1) pipeline [30]. Demultiplexed paired-end sequence reads were truncated to a length of 300 bp, quality filtered and denoised into amplicon sequence variants (ASVs) using the DADA2 plugin [31]. Sequencing resulted in a total of 15,187,504 demultiplexed paired-end reads from 214 samples (average of 65,463 reads/sample, range 7254 – 159,600). Subsequent denoising, removal of reads associated with mitochondria, filtering of Eukaryote and unclassified Kingdom (after assigning taxonomy) sequences, and removal of samples with low coverage (< 9848 reads), resulted in 12,116,464 reads across 211 samples for downstream analysis. Each sample was rarefied to a depth of 9848 reads resulting in a total of 8255 ASVs in the dataset. Alpha rarefaction revealed sufficient sequencing coverage of the remaining samples (Additional file 2: Figure S1). Taxonomy was assigned to each ASV using the q2-feature-classifier against the Silva 132 99% OTUs reference sequences resource [32]. Alpha-diversity metrics (Shannon's diversity, Pielou's evenness, Faith's phylogenetic diversity and total observed ASVs as a measure of richness) were estimated using q2-diversity. QIIME artefacts were imported into R using the package Qiime2R and plots were made using Phyloseq and ggplot2 [33]. Beta diversity metrics (Bray-Curtis dissimilarity matrix) and Principle Coordinate Analysis (PCoA) using the Bray-Curtis matrix were performed with Phyloseq. To investigate the influence of antibiotic and FMT treatment on the bacterial assemblages, read abundances for each ASV in the feature table were square-root transformed to down-weight the impact of a few extremely dominant ASVs. Statistical differences for the univariate measures, such as, alpha diversity were performed using 2-way ANOVA, accounting for both the treatment (i.e. antibiotic or FMT treatment) and time. For multivariate measures, significant differences between a priori predefined groups of samples were evaluated using both two-way and one-way permutational multivariate analysis of variance (PERMANOVA), allowing for type III (partial) sums of squares, fixed effects of sum to 0 for mixed terms, and exact *p*-values generated using unrestricted permutation of raw data [34], using the Adonis function in R. The function *pairwise.adonis* with Bonferroni correction was used to investigate the significance between timepoints when time was a significant factor from the PERMANOVA analysis. Differential abundance was assessed using Deseq2 with p-value corrected using the default Benjamini-Hochberg false discovery rate method, as suggested recently for the analysis of microbiome data with a small number of replicates per treatment (< 20) [35, 36]. In some cases (e.g. for some ASVs), the data distribution was assumed to not follow a normal distribution, so the non-parametric version of 2-sample or *k*-sample tests were performed (e.g. the Mann-Whitney U test or Kruskal-Wallis H test). In order to quantify the change in magnitude in the bacterial communities after antibiotic and FMT treatment, pair-wise comparisons between each pair of samples were made using the Bray-Curtis similarity algorithm, where a higher value indicates that samples share more ASVs of a similar abundance.

Results

Impact of acclimatisation on the gut and skin microbiota

In order to evaluate the effects of antibiotics and FMT on the gut and skin bacterial communities of yellowtail kingfish with poor gut health, fish suffering from a putative enteritis were first translocated from an offshore seacage to a series of onshore treatment tanks where they were allowed to acclimatise for 3 weeks prior to commencing the trial. The impact of this change was initially assessed by comparing a subset of fish sampled at the time of collection from the seacage ($n = 10$) to a group of fish sampled from the tanks 3 weeks post acclimatisation ($n = 12$). Ordination of the samples based on Bray-Curtis dissimilarity matrix revealed independent clustering of the skin and gut samples (Fig. 2a) (one-way PERMANOVA: Pseudo-F = 38.3, $p < 0.01$), as expected.

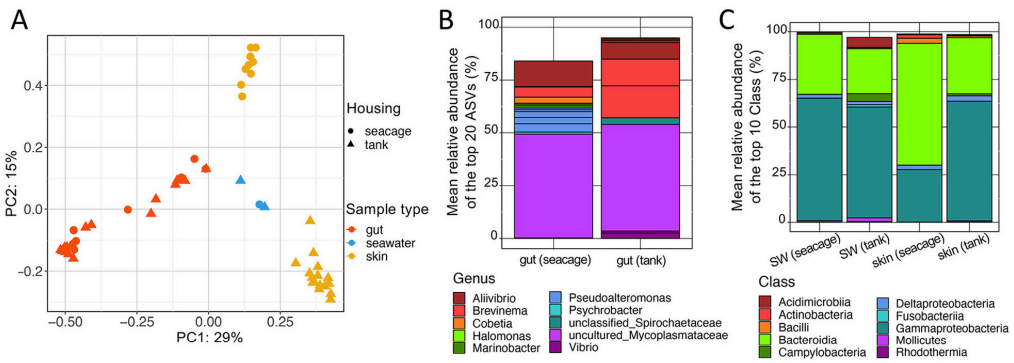

Fig. 2 Impact of acclimatisation on the gut and skin microbiota of yellowtail kingfish. **a** PCoA plot representing Bray-Curtis similarities comparing global bacterial assemblages between sample types (gut, skin and seawater) from fish housed in seacages and those relocated and acclimatised in tanks. **b-c** Stacked barplots presenting the mean relative abundance (%) of the top 20 most abundant bacterial ASVs found in the gut of fish and the top 10 bacterial Classes found in the seawater (SW) and on the skin of fish, respectively, comparing fish housed in seacages and those relocated and acclimatised in tanks

However, the acclimatisation process had no effect on the global gut bacterial communities (one-way PERM ANOVA: Pseudo-F = 2.5, p = 0.063) (Fig. 2a), with both the seacage and tank fish comprising similar mean relative abundances of the most dominant ASVs (namely uncultured *Mycoplasmataceae* and *Allivibrio*) (Fig. 2b). However, for these and many of the other ASVs detected in this study, species level assignment could not be determined. The gut bacterial assemblages of tank fish comprised a number of additional notable ASVs, representing various genera particularly *Brevinema*, *Vibrio*, and an unclassified *Spirochaetaceae*, while seacage fish also comprised *Pseudoalteromonas*, *Cobetia* and *Halomonas* (Fig. 2b). Differential abundance analysis identified a total of 17 ASVs that were differentially abundant, whereby two were enriched in tank fish (namely *Brevinema* and *Allivibrio*) vs. 15 in seacage fish (which included eight that were associated with *Pseudoalteromonas*) (Fig. 2b, Additional file 1: Table S7).

In evaluating the effect of acclimatisation on the skin bacterial communities, a significant difference was linked to acclimatisation (one-way PERM ANOVA: Pseudo-F = 14.8, p < 0.001, Fig. 2a), as the fish acclimatised in tanks showed a shift in the ratio of *Proteobacteria:Bacteroidetes* (P:B ratio) with the *Gammaproteobacteria* becoming the more dominant Class (Fig. 2c). Specifically, the mean P:B ratio changed from < 0.5 to > 2 after fish were translocated from seacages and acclimatised in tanks for 3 weeks (Additional file 2: Figure S2). The seawater samples taken from the seacage and the tanks comprised similar bacterial assemblages and clustered independently to those taken from the skin and gut (Fig. 2a).

Effect of antibiotics on the gut and skin microbiota

To explore the impact of the antibiotic combination therapy on the gut and skin associated bacterial communities, fish from two tanks treated with antibiotics were compared with fish from two untreated tanks over a period of 18 days (i.e. at days 3, 5, 11 and 18 days post antibiotic treatment). Antibiotic treatment had a significant effect on the global gut bacterial communities (two-way PERMANOVA: Pseudo-F = 4.4, p < 0.001), with no significant difference over time (Pseudo-F = 1.39, p = 0.085) and no significant interaction effect between antibiotic treatment and time (Pseudo-F = 0.86, p = 0.669) (Fig. 3a), indicating that the antibiotic effect lasted for up to 18 days. This corresponded with a loss of ASV diversity and evenness within the gut bacterial assemblages of fish treated with antibiotics (Fig. 3b and Additional file 2: Figure S3a), as based on measures of Shannon's diversity (two-way ANOVA: F = 5.36, p = 0.029) and Pielou's evenness (F = 10.98, p = 0.003) respectively. No significant differences were observed for these diversity metrics over time, indicating that the community did not recover over the 18-day period (p > 0.05). However, this did not correspond to a loss in ASV richness or phylogenetic diversity (two-way ANOVA: p > 0.05; Fig. 3c and Additional file 2: Figure S3b), indicating that ASVs were diminished but not completely eliminated following antibiotic treatment. To further explore which microbes were susceptible to the antibiotics, differential abundance analysis of ASVs was performed. Three ASVs were significantly reduced in the gut of antibiotic treated fish: two associated with *Brevinema* (Fig. 3d) and one associated with *Aliivibrio* (Additional file 1: Table S8). Moreover, an unclassified *Mycoplasmataceae* related ASV became substantially more dominant in fish

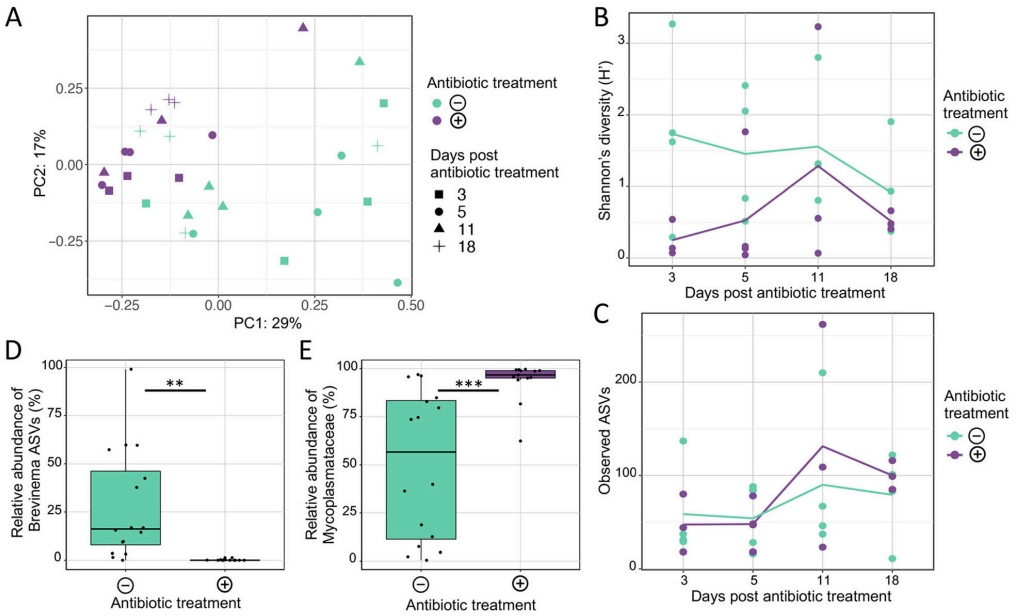

Fig. 3 Effect of antibiotics on the gut microbiota of yellowtail kingfish. **a** PCoA plot representing Bray-Curtis similarities comparing the change in global gut bacterial assemblages after treatment with antibiotics (+) over 18-days, with those fish that did not receive treatment (–). **b-c** Means plots showing the change in mean value of Shannon's index of diversity and total observed ASVs (as a measure of richness), respectively, over the 18-day treatment period (from $n = 4$ fish). **d-e** Boxplots presenting the median and IQR of the relative abundances of the summed *Brevinema*-associated ASVs and a single ASV associated with *Mycoplasmataceae* (ASV1452), respectively, comparing fish treated and not treated with antibiotics. The level of significant difference is denoted by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ or $^{***}p \leq 0.001$, following the Mann-Whitney U test

exposed to antibiotic treatment (Fig. 3e). While its abundance in control fish varied considerably, this ASV was always dominant in fish administered antibiotics.

Antibiotic treatment also had a significant impact on the global skin bacterial communities (two-way PERMANOVA: Pseudo-F = 2.74, $p < 0.01$), with the most notable differences occurring up to 3 days post-treatment (Additional file 1: Table S9; Additional file 2: Figure S4). Changes in the global bacterial assemblages were also observed over time, irrespective of antibiotic treatment (two-way PERMANOVA: Pseudo-F = 8.41, $p < 0.001$). Despite some disparity in the clustering of samples from antibiotic treated and control fish, there was a lack of notable difference in the diversity, evenness, richness or phylogenetic diversity, which is likely due to variation observed among fish within some treatment groups (Additional file 2: Figure S5a-S5d). Eight of the total 2672 skin ASVs were significantly more abundant in antibiotic treated fish compared to the control. These included *Tenacibaculum*, *Oleiphilus*, *Glaciecola*, *Paraglaciecola* and an uncultured *Saccharospirillaceae* (Additional file 1: Table S10).

Impact of FMT on the gut microbiota

To measure the effect of FMT on antibiotic perturbed and unperturbed gut associated bacterial communities of yellowtail kingfish, fish from 4 tanks administered FMT via gavage and from 4 tanks administered FMT via water bathing were compared with and without antibiotic pre-treatment (2 tanks per treatment group). In addition, these treatment groups were also compared to fish from untreated tanks and tanks that only received the antibiotic treatment (serving as controls). Fish were end-point sampled at 0, 2, 8 and 15-days post FMT treatment. In addition, half of FMT-treated tanks were spiked with a specific-*Lactobacillus* strain as an internal control (i.e. *L. rhamnosus* GG or LGG®). However, this organism was not established or detected in fish receiving this treatment despite its predominance in the spiked inoculum (Fig. 4a). The unspiked FMT inoculum was also sampled to discern the global catalogue and active bacterial constituents by sampling the DNA and RNA respectively. A total of 95 ASVs were detected from DNA, 41 from RNA, and 27 that were detected in both (Additional file 2: Figure S6). To exclude the influence of the environment (seawater) on FMT treatment, ASVs

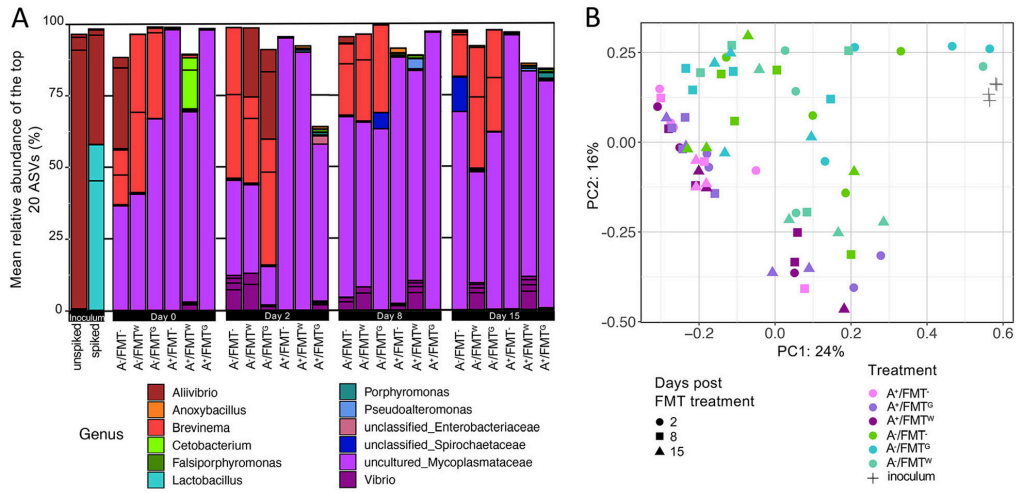

Fig. 4 Impact of FMT on antibiotic perturbed and unperturbed gut microbiota. **a** Stacked barplot presenting the mean relative abundance (%) of the top 20 bacterial ASVs found in the healthy donor inoculum compared to the fish from the different treatment groups; A^-/FMT^-, A^+/FMT^-, A^-/FMT^G, A^+/FMT^G, A^-/FMT^W and A^+/FMT^W. Mean from $n = 4$ fish. **b** PCoA plot representing Bray-Curtis similarities, comparing global gut bacterial communities between the treated (antibiotic and/or FMT) and untreated fish to the donor inoculum, comparing treatment effects for 15 days post FMT

from the unspiked inoculum were also compared with those from the seawater. Of the 562 ASVs detected in seawater, only 13 occurred in DNA, one in RNA and two occurred in both the DNA and RNA inoculum samples (Additional file 2: Figure S6). These two shared ASVs belonged to an unclassified *Lactobacillus* sp. and *Allivibrio* and were predominant in the unspiked inoculum, with the seawater samples only comprising minute counts, indicating that they likely represent host rather than environmental associated ASVs. The low numbers of host-associated (gut-derived inoculum) ASVs in seawater highlights the independent nature of the sampled niches, and is reflected in the independent clustering of the samples as detailed above and depicted in Fig. 2a.

Fish that received FMT following antibiotic treatment clustered independently away from the donor inoculum as well as to those fish that received FMT without antibiotic treatment, indicating that the antibiotics had a prolonged effect on the global gut bacterial assemblages and FMT establishment (Fig. 4b). This is evident by a clear separation of the A^+/FMT^G and A^+/FMT^W samples (purple shaded symbols) from the A^-/FMT^G and A^-/FMT^W samples (green shaded symbols). There were no significant differences between the global bacterial communities of fish who received FMT via water or gavage to fish who did not receive FMT, in either the antibiotic treated cohort (two-way PERMANOVA: Pseudo-F = 1.23, p-value = 0.183) or the non-antibiotic treated cohort (two-way PERMANOVA: Pseudo-F = 0.97, p-value > 0.486). However, there was a significant difference in respect to time post FMT in the non-antibiotic treated cohort (two-way PERMANOVA: Pseudo-F = 2.26, p-value < 0.01), indicating that any slight modifications to the global bacterial communities in this treatment group were not static. Indeed, the fish gut microbiota was significantly different in T1 compared to both T2 ($p = 0.018$) and T3 ($p = 0.018$) although it did not change from T2 to T3 ($p = 0.176$). In contrast, time had no influence on the bacterial communities on the antibiotic-treated cohort (two-way PERMANOVA: Pseudo-F = 1.34, p-value = 0.132).

There was notably high variation between treated fish within the same treatment group/tank. For example, of the four fish sampled from each treatment at Day 2, two fish from the A^-/FMT^G and one fish from the A^-/FMT^W had global gut bacterial assemblages better resembling the donor inoculum as indicated by the three symbols clustering closely to the grey crosses on Fig. 4b. This suggests that FMT treatment via gavage and water did have some impact on some individual fish (despite the PERMANOVA results on the global bacterial communities above indicating that the effect of FMT was minor). To best quantify the effect of FMT on establishment within treated fish, the change in resemblance to the healthy donor inoculum was calculated using the Bray-Curtis (BC) similarity algorithm. This gives a

percent similarity between pairs of samples. First, the mean similarity between the 16 control fish that received no antibiotic or FMT treatment to the unspiked inoculum was 10.8% (median of 6.5%). This indicates that the healthy donor fish and the poor performing fish do not share the same ASVs or that the relative ASV abundance of shared ASVs differ vastly between these fish cohorts. At the RNA level, the inoculum was dominated by an ASV associated with *Aliivibrio* (see Fig. 4a), while the remaining ASVs belonged to uncultured *Mycoplasmataceae*, *Brevinema*, *Aliivibrio*, *Vibrio*, and *Lactobacillus*.

By comparing the global gut bacterial communities of both treated and untreated fish to the healthy donor inoculum, there was a clear shift in both BC% value and diversity in some treated groups at Day 2 post FMT (Fig. 5a-b). For example, there was an increase in diversity in some fish from the FMT via gavage treatment group, especially in fish treated with antibiotics (A^+/FMT^G)(Fig. 5b). This increased diversity was also associated with an increased richness in some individuals (Additional file 2: Figure S7). The two fish with high diversity and richness were sampled from different tanks, excluding a tank effect during the experimental period. As noted above, the fish with greatest similarity to the donor inoculum belonged to the A^-/FMT^G and A^-/FMT^W groups, with similarities to the inoculum of 44–64% (Fig. 5a), a marked difference from the median value of ~ 6% for control fish. However, the other fish within these treatment groups did not have such high similarities to the inoculum (6–8%), suggesting that FMT only works on some animals (Fig. 5a). Furthermore, the lower BC% at later timepoints (8 and 15 days post FMT) indicates that FMT only induced short-lived changes in the bacterial communities.

Of the 79 healthy-donor (RNA/DNA) inoculum ASVs that were not detected in any control fish (A^-/FMT^-) or seawater, 17 were detected at least once in the FMT^+ treatment groups (11 ASVs in the A^-/FMT^+ and 6 ASVs in the A^+/FMT^+). Sixty-two ASVs were only detected in the inoculum and were not found in any fish from any treatment group or from seawater. A deeper look into those ASVs that were able to be transplanted or

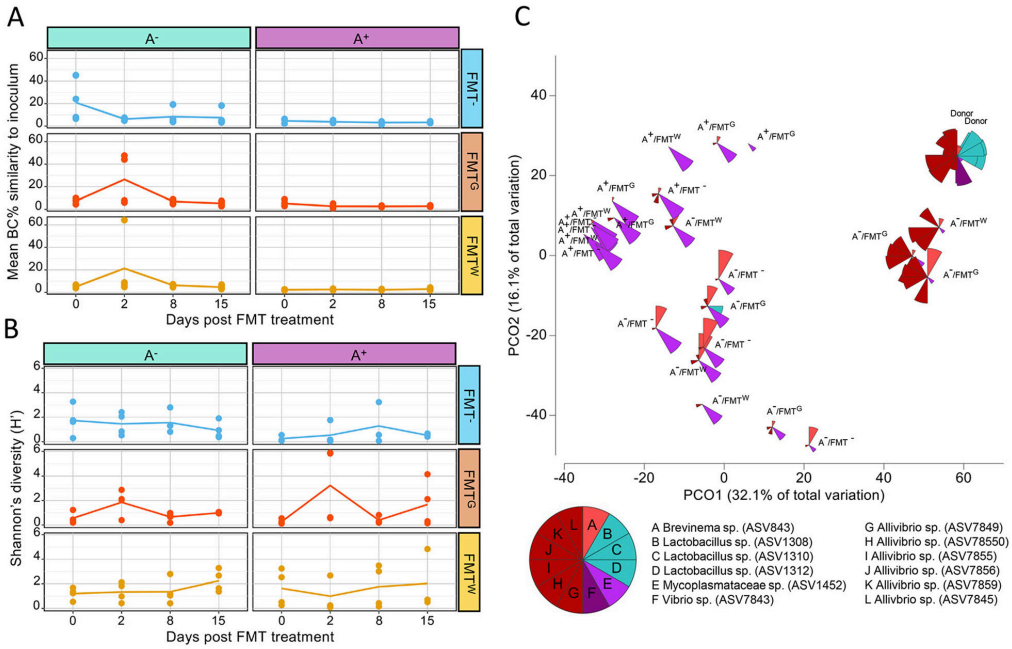

Fig. 5 Impact of FMT on antibiotic perturbed and unperturbed gut microbiota. **a-b** Line plot presenting the mean Bray-Curtis similarity to the donor inoculum and mean Shannon's index of diversity, respectively, for the different treatment groups; A^-/FMT^-, A^+/FMT^-, A^-/FMT^G, A^+/FMT^G, A^-/FMT^W and A^+/FMT^W over 15 days post FMT (mean from $n = 4$ fish). **c** PCoA plot representing Bray-Curtis similarities, comparing global gut bacterial communities between the treated (antibiotic and/or FMT) and untreated fish to the donor inoculum at Day 2 post FMT, with superimposed wedges indicating the relative abundance of the 12 most abundant ASVs in the RNA component of the donor inoculum. Wedge size is proportional to its rank order across all samples, whereby large wedges indicate those ASVs that had the greatest ranked abundance between samples, and the smallest wedges having the lowest ranked abundance between samples

enriched at Day 2 post FMT in these fish that resembled the inoculum included several *Aliivibrio* ASVs such as ASV7859, ASV7856, ASV7855, ASV7850 and ASV7849, and a *Lactobacillus* ASV (ASV1312) (Fig. 5c).

Discussion

In commercial aquaculture operations, marine fish species are raised either in open water seacages or on land in tanks. Inherent variations exist between these systems and may have an impact on the fishes' associated microbiota. For example, the gut bacterial assemblages of Atlantic salmon (*Salmo salar*) raised in a recirculation system compared to those from open commercial cages, have been shown to vary and are associated with the occurrence of unique species in each system [37]. In this study, however, no significant differences were observed in the global gut bacterial communities between seacage fish and those that were translocated from seacages and allowed to acclimate in tanks for 3 weeks. Given that both groups of fish originated from the same seacage and were maintained on the same pelleted diet, this is not surprising and suggests that any potential stresses imposed on the translocated fish (e.g. transport and variations in water quality) did not impact their gut communities prior to the commencement of the trial. In contrast, the global skin bacterial communities differed between seacage fish and translocated seacage fish acclimated in tanks, where a shift in the ratio of *Proteobacteria:Bacteroidetes* (P:B ratio) was apparent due to the increased abundance of *Gammaproteobacteria* in fish acclimated in tanks. Given the occurrence of similar types of environmental (seawater) bacterial assemblages in both systems, it is likely then that other factors may have contributed to the selection of particular taxa in this instance. This may include factors that contribute to the physiological stress of the animal (e.g. stocking density, current, swimming and oxygen availability), which in turn may impact the way in which they respond and regulate their microbiota [15]. This was recently demonstrated in work reporting on the skin P:B ratio as a biomarker for performance in yellowtail kingfish, where in comparing wild to farmed healthy and diseased seacage fish, low P:B ratios of < 2 were associated with fish with early stages of disease (compared to > 10 in wild) [26]. In this work, the mean P:B ratio changed from < 0.5 to > 2 after fish were translocated from seacages and acclimatised in tanks for 3 weeks and may suggest a positive change in the balance of the bacterial communities. However, further work would be required to elucidate whether this corresponds to improvements in the health status of these fish.

In poor performing yellowtail kingfish in this trial, the gut bacterial assemblages were dominated by a number of ASVs relating to uncultured *Mycoplasmataceae* and *Allivibrio*. Some additional ASVs were more abundant in either tank or seacage fish and included, among others, *Brevinema* in tank fish, and *Pseudoalteromonas* in the seacage fish. As the dominant constituent here in poor performing fish, the occurrence of a single ASV related to an uncultured *Mycoplasmataceae* raises questions around its association with disease. As a member of this bacterial family, *Mycoplasma* have been previously identified in the gut of other fish such as Atlantic salmon, Chinook salmon (*Oncorhynchus tshawytscha*), zebrafish (*Danio rerio*), common carp (*Cyprinus carpio*), largemouth bronze gudgeon (*Coreius guichenoti*) and rainbow trout [38–44]. While recent genome reconstruction indicates a mutualistic lifestyle of this organism in the intestine of certain species like Atlantic salmon and hadal snailfish (*Pseudoliparis swirei*) [45, 46], for others it has been associated with disease [42]. Members of the genus *Allivibrio* largely form part of the natural gut microbiota of various fish (e.g. cods, Atlantic salmon) [47, 48], though certain species have also been found to be pathogenic (e.g. *A. wodanis*, *A. salmonicida*, *A. fischeri*) [49–51]. *Brevinema* has been found in the gut of Atlantic salmon and Atlantic cod (*Gadus morhua*) [52, 53] as well as in rainbow trout (*Oncorhynchus mykiss*) with genetic susceptibility to particular pathogens (e.g. *Flavobacterium psychrophylum*) [54]. Some members of the genus *Brevinema* found in Atlantic salmon have, however, been reported to produce butyrate [53], which may support intestinal barrier function and mucosal immunity [55, 56]. With other dominant constituents found here (e.g. *Pseudoalteromonas*) also reported to comprise both pathogenic [57] and beneficial (probiotic-like) species [58–61] questions thus remain around their role and changes in abundance between cultivation systems and requires further elucidation, particularly given the inability to resolve many of the ASVs to a species level in this study.

Antibiotic treatment (consisting of a combination therapy comprising oxytetracycline, erythromycin and metronidazole) had a notable impact on these taxa (e.g. uncultured *Mycoplasmataceae* and *Brevinema*), and the bacterial communities more broadly. Specifically, in the gut, a shift in the global bacterial assemblages was evident immediately in response to the treatment and was marked by a loss of species (ASV) diversity and evenness, which did not recover over the 18 day period. Despite this, the species (ASV) richness did not change substantially over this period, indicating that while antibiotic treatment had a significant effect on these assemblages, many of the species were likely diminished but not completely eliminated. This raises questions around whether these populations have the capacity to return to their 'original' composition over a prolonged period (in this case beyond 18 days), or whether they are likely to

remain in (and continue to evolve from) an altered state after antibiotic treatment. Studies from other fish species have also demonstrated the notable effects that antibiotics may have on the gut microbiota. For example, a loss of gut diversity was observed in Atlantic salmon following oxytetracycline treatment [62], in channel catfish (*Ictalurus punctatus*) following florfenicol treatment [63], in fathead minnow (*Pimephales promelas*) after triclosan use [64] and in zebrafish following olaquindox treatment [65]. In contrast, in some cases, it has been reported that antibiotic treatment may even increase or cause a shift in species diversity, as shown for Atlantic salmon and zebrafish [12, 13], and pacu (*Piaractus mesopotamicus*) [8] respectively. This is pertinent given that changes in diversity and evenness within the gut has been suggested to influence functional capacity and disease resilience [66]. While contentious, the consequences of this may be significant, particularly where the communities fail to recover over an extended period, as observed here. Attempts to investigate the functional changes in these communities in response to antibiotic treatment (e.g. using metagenomics or metatranscriptomics) is thus warranted.

Antibiotic treatment also had a significant impact on the global skin bacterial communities, with the most notable differences occurring up to 3 days post-treatment. Unlike the gut though, global changes were also observed to occur with time (irrespective of antibiotic treatment), indicating that while antibiotic treatment may have immediate, broader effects outside of the gut, the skin communities are also inherently more dynamic. This was further exemplified by the notable variation observed between individuals, which obscured any apparent differences in the diversity, evenness, richness or phylogenetic diversity. Instead, several ASVs were found to be significantly enriched in the skin of antibiotic treated fish, particularly *Tenacibaculum*. This is a concern, as this genus encompasses numerous pathogenic species which have the capacity to cause serious ulcerative disease (tenacibaculosis) in a wide range of marine fish species [67–69]. In other fish, treatment with antibiotics has also been shown to have negative effects that extend across the mucosal surfaces. For example, rifampicin exposure (via bathing rather than oral administration) led to the reduction of both the skin and gut associated microbial diversity in western mosquitofish (*Gambusia affinis*) [70] and led to an increase in the susceptibility to certain opportunistic pathogens and stressors, and a failure to thrive over a prolonged period. Furthermore, it was shown that while these communities stabilise during recovery, they do not appear to return to their original state in the short-term (~ 1 week). In some fish such as sea bass (*Dicentrarchus labrax*), it has also been shown that recovery over the longer term (~ 3 weeks following oral administration of oxytetracycline) may vary between the different mucosal surfaces, with the communities associated with the skin reported to be more resilient to those of the gills [71]. In support of this, we also observed here a greater disparity in the effects of antibiotic treatment on the gut rather than the skin bacterial communities. While we cannot exclude the possibility that variations in dosing may have contributed to this finding (e.g. from partial or complete regurgitation of the administered antibiotics), it is likely that this was due to the mode in which the antibiotics were delivered, whereby in this case initial exposure and uptake occurred first in the gut followed by its subsequent dissemination through the body and into the outer mucosal surfaces. Further variations in the specific pharmacokinetics of the antibiotics, however, may also be a contributing feature, particularly given the low level of absorption (< 3% of the administrated dose) reported for antibiotics like oxytetracycline in other fish species [72]. The approach to antibiotic administration and treatment should thus be extended to include varied and alternative dosing regimens.

To further assess for which taxa were affected by the antibiotics, differential abundance analysis was performed, revealing three ASVs that were significantly reduced in the gut of antibiotic treated fish (two associated with *Brevinema* and one associated with *Aliivibrio*) and one that became substantially more dominant (namely an unclassified *Mycoplasmataceae* sp.). As stated above, the antibiotic treatment administered in this study comprised a combination of agents (namely oxytetracycline, erythromycin and metronidazole), which together have the capacity to target a wide range of both gram-positive and gram-negative organisms. Given that both *Brevinema* and *Allivibrio* are gram-negative (microaerophilic or facultative anaerobic) bacteria [73, 74], their depletion following treatment was not surprising. What was unexpected, however, was the increase in dominance of an ASV representing an unclassified *Mycoplasmataceae* species. As a member of this bacterial family, *Mycoplasma* are characterised by a lack of cell wall around their membrane which makes them resistant to antibiotics targeting cell wall synthesis such as beta-lactams, glycopeptides and fosfomycin [75]. However, oxytetracycline (a tetracycline) is known to be an effective treatment for *Mycoplasma* infections as it targets protein rather than cell wall synthesis [76]. In addition, erythromycin (a macrolide) and metronidazole (a nitroimidazole) are also both inhibiters of protein synthesis [77, 78], thus their mode of action should presumably have contributed to the depletion (rather than the increase in abundance) of *Mycoplasma*. Despite this, it has been found that this genus can quickly develop resistance to both macrolides and tetracyclines [79]. While it

is tempting to postulate then that such mechanisms may have led to its increase in abundance here, it is important to note that it could have equally been depleted following antibiotics but remained at a high relative abundance because of the depletion of other taxa. Nevertheless, given the inherent parasitic nature of the *Mycoplasma* [80], it would be prudent to further investigate changes in their actual abundance in response to antibiotic treatment (e.g. using qPCR) in a farm setting more broadly, as well as the likely resistance mechanisms encoded within its genome, and would support an improved understanding of its role in yellowtail kingfish health.

Since antibiotic exposure can perturb the microbiota and may have possible consequences for the health of the animal, attempts have been made in helping the microbiota recover to re-establish homeostasis. Traditionally, this has included, among others, the use of various probiotic microorgansims. For instance, within black molly (*Poecilia sphenops*) the administration of native probiotics (namely *Phaeobacter inhibens* and *Bacillus pumilus*) following antibiotic exposure led to improved disease resistance to pathogenic *Vibrio* species [81]. In this study, we attempted to introduce a purported probiotic *Lactobacillus* species (*L. rhamnosus* GG or LGG®, of human origin) in conjunction with FMT, which was previously used to improve disease resistance and the immune response in other fish species [82]. However, this organism was not detected in any of the samples here. While this suggests that this strain may thus not be able to colonise the mucosal surfaces of yellowtail kingfish, further validation using more sensitive approaches like qPCR would be required. Despite this, other differentiable *Lactobacillus* related ASVs were detected in the gut samples following FMT treatment, indicating that these organisms may naturally occur in yellowtail kingfish as part of a broader group of other lactic acid bacteria (LAB) reported in finfish [83]. In this regard, the use of autochthonous probiotics for this species would be more appropriate and would likely improve the prospect of successful establishment within the gastrointestinal tract. Recently, a total of 11 isolates (including members of *Shewanella*, *Psychrobacter*, *Acinetobacter*) from yellowtail kingfish was discovered but further work is required to evaluate their potential benefits in the farming of this species [84].

As an alternative, more holistic biological approach for modulating the gut microbiota, FMT was also investigated in this study and was administered to a total of 96 poor-performing yellowtail kingfish. Alongside groups of fish that solely received or were administered FMT following antibiotic pre-treatment, two approaches to FMT were evaluated. As strategies used previously for fish [24, 85, 86], this included the direct delivery of a single FMT inoculum via oral gavage, and the indirect delivery of this inoculum via bathing in a reduced volume of seawater for a prolonged period (~3 hours). To elucidate the effects of FMT and the approach to its administration, end-point samples of both the gut and skin were evaluated over a 15-day period (i.e. at 0, 2, 8 and 15-days post FMT administration). Although no significant differences in the global bacterial communities were observed between the FMT treated and control groups of fish (regardless of the route of administration), any broad effect of FMT was masked by the notable variation apparent between individuals. Despite this, samples from several of the oral-gavage and seawater bathed FMT-treated fish appeared to cluster more closely to the FMT donor inoculum samples, indicating some level of impact. Indeed, for some fish, a similarity with the donor inoculum of up to 64% was observed (compared to only ~ 6% for the control fish) and was most notable at Day 2 post FMT treatment. Much lower similarities were observed, however, at the later time points (i.e. at 8 and 15 days post FMT delivery) and for many of the other fish, thus indicating that FMT may only induce short-lived changes in certain individuals. While it is unclear why this was observed, likely explanations may include variations in the "colonisation resistance" of the respective gut communities to the introduction of exogenous microorganisms; a feature that has been suggested for humans and rodents and purported to be exacerbated by treatment with antibiotics [87, 88]. Further investigations into the mechanisms that contribute to resilience would thus be pertinent for improving the efficacy of FMT.

While it is not possible to completely exclude variations in the initial composition of the microbiota between fish in the individual treatment groups (due to end-point sampling), the gut bacterial assemblages of the poor-performing control fish at the beginning of the trial were markedly different to those from the healthy donor inoculum (as derived from 102 healthy seacage fish), and suggests that other factors may have contributed to the higher similarities observed for these select individuals. As postulated in studies using FMT to modulate the gut microbiota of killifish [24], such findings may also include variations in the fish's immune response and its capacity to influence establishment. Furthermore, genetic diversity is also known to shape the selection of the host microbiota [89–91], potentially resulting in the varied responses to FMT among the same population of fish. Considering that these fish came from a cohort comprising mixed genetics, this may in part also explain why only a small proportion of the total ASVs detected in the inoculum (i.e. 17/79) were observed in the FMT treated fish and may reflect a limitation of the current approach. Nevertheless, given that certain dominant constituents prone to the antibiotic treatment were able to be transferred to select individuals (e.g. *Allivibrio* spp.), this suggests that FMT has some

capacity to influence the microbiota (irrespective of whether it is delivered directly or indirectly) and warrants further investigation. This should include strategies used elsewhere to improve and prolong its effects in other animals, e.g. through the administration of multiple consecutive doses of the FMT inoculum and by pre-treating the inoculum to support the survival of potentially fastidious constituents [92] or by using material derived from wild (rather than healthy farmed) individuals for restoring potentially 'extinct' autochthonous taxa [93]. In addition, given the profound impacts FMT mediated gut microbiota alterations can have on the animal's health (as recently demonstrated in Pacific white shrimp, *Litopenaeus vannamei* [94]), further work is also required to elucidate the role of FMT in modulating the health outcomes in yellowtail kingfish.

Conclusion

In this study, the impacts of antibiotics on the gut and skin microbiota of a commercially important farmed finfish species *Seriola lalandi* (yellowtail kingfish) was investigated. The oral administration of a broad-spectrum antibiotic combination therapy in poor-performing fish significantly perturbed the global gut and skin bacterial assemblages and led to the loss of key constituents and the concomitant enrichment of potentially opportunistic species. Unlike the gut where a prolonged effect was observed, the bacterial assemblages of the skin appeared to be dynamic and inherently more resilient to the treatment (likely due to the varied pharmokinetics of the compounds), and shifted towards a more 'normal' (though disparate) state over the recovery period. Given the increasing awareness of the role the microbiota plays in supporting host health, attempts to restore changes exerted by the antibiotic treatment on these bacterial communities was undertaken using faecal microbiota transplantation (FMT). As derived from a population of healthy farmed yellowtail kingfish, FMT was delivered both directly (via oral gavage) and indirectly (through the surrounding seawater). Despite the lack of notable global changes in the gut bacterial communities in FMT treated fish, for some individuals the effect was profound (regardless of the mode of delivery) and was associated with a change in the bacterial composition that more closely resembled that of the donor inoculum. Though short-lived, this suggests the potential of FMT for modulating these communities. Further work is required, however, to improve the approach (e.g. using more varied, pre-screened inoculums) and to evaluate its capacity to influence health in yellowtail kingfish. To this end, metagenomic and/or metatranscriptomic methods would represent useful tools for supporting an improved understanding of the global functional changes in these communities in response to these treatment regimens.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s42523-020-00046-4.

Additional file 1: Table S1. Water temperature (°C) recorded during the experiment. **Table S2:** Oxygen concentration (%) recorded during the experiment. **Table S3:** pH recorded during the experiment. **Table S4:** Salinity (‰) recorded during the experiment. **Table S5:** Ammonia concentration (ppm) recorded during the experiment. **Table S6:** Characteristics (weight and length) of the fish stocked in the 12 experimental tanks prior the start of the experiment. **Table S7:** Differential abundant ASVs found in the gut microbiota of fish housed in seacage and tanks. **Table S8:** Differential abundant ASVs found in the gut microbiota of fish treated with antibiotic and non-treated with antibiotic. **Table S9:** Pair-wise PERMANOVA results investigating the influence of time on the skin microbial communities. **Table S10:** Differential abundant ASVs found in the skin microbiota of fish treated with antibiotic and non-treated with antibiotic.

Additional file 2: Figure S1. Rarefaction plot of all samples analysed in this study. **Figure S2:** Stacked barplots presenting the mean relative abundance (%) of the top 10 bacterial Phylum found in the seawater (SW) and on the skin of fish, comparing fish housed in seacages and those relocated and acclimatised in tanks. **Figure S3:** Means plots showing the change in mean value of Pielou's evenness (a) and Faith's phylogenetic diversity (b) in the gut bacterial communities, over the 18-day treatment period (from $n = 4$ fish). **Figure S4:** PCoA plot representing Bray-Curtis similarities comparing the change in global skin bacterial assemblages after treatment with antibiotics (+) over 18-days, with those fish that did not receive treatment (–). **Figure S5:** Means plots showing the change in mean value of Shannon's index of diversity (a), Pielou's evenness (b), total observed ASVs (as a measure of richness) (c) and Faith's phylogenetic diversity (d) in the skin bacterial communities, over the 18-day treatment period (from $n = 4$ fish). **Figure S6:** Venn diagram showing the distribution of unique and shared ASVs in the seawater, and the DNA and RNA inoculum samples. The total number of ASVs within each group are denoted in parentheses. **Figure S7:** Mean plot presenting the mean number of observed ASVs for the different treatment groups; A^{-}/FMT^{-}, A^{+}/FMT^{-}, A^{-}/FMT^{G}, A^{+}/FMT^{G}, A^{-}/FMT^{W} and A^{+}/FMT^{W} over 15 days post FMT (mean from $n = 4$ fish).

Abbreviations

16S rRNA: 16 Svedberg ribosomal ribonucleic acid; ARG: Antimicrobial resistant gene; ASV: Amplicon sequence variant; cDNA: Complementary DNA; FMT: Faecal microbiota transplantation; NCBI: National Centre for Biotechnology Information; PCoA: Principle Coordinate Analysis; PCR: Polymerase Chain Reaction; PERMANOVA: Permutational Multivariate Analysis Of Variance

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Authors' contributions

APA conceived the study; APA, SRC and TPRAL designed the experiment; APA, SRC and TPRAL performed the experiment; SRC performed the laboratory work; TPRAL analysed the data; TPRA, MLWO and APA wrote the manuscript; LSW and JWW provided overall guidance. All authors read and approved the final manuscript.

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Availability of data and materials
Sequencing reads from the demultiplexed samples analysed in this study have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession PRJNA602789. Scripts used to analyse the 16S rRNA gene sequence data using phyloseq have been deposited in GitHub at https://github.com/aoxley1975/Kingfish

Ethics approval and consent to participate
This study was exempt from ethics approval as the fish were privately owned and were sampled under the auspices of a commercial aquaculture enterprise according to industry best practice veterinary care.

Consent for publication
Not applicable.

Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 4: Dead or alive: microbial viability treatment reveals both active and inactive bacterial constituents in the fish gut microbiota

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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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ORIGINAL ARTICLE

Dead or alive: microbial viability treatment reveals both active and inactive bacterial constituents in the fish gut microbiota

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Abstract

Aims: This study evaluated the microbial viability of fish gut microbiota in both digesta (faecal) and mucosal samples using a modified propidium monoazide (PMA) protocol, followed by 16S ribosomal RNA (rRNA) gene sequencing.

Methods and results: Digesta and gut mucosal samples from farmed yellowtail kingfish (*Seriola lalandi*) were collected and a modified PMA treatment was applied prior to DNA extraction to differentiate both active and nonviable microbial cells in the samples. All samples were then sequenced using a standard 16S rRNA approach. The digesta and mucosal samples contained significantly different bacterial communities, with a higher diversity observed in digesta samples. In addition, PMA treatment significantly reduced the microbial diversity and richness of digesta and mucosal samples and depleted bacterial constituents typically considered to be important within fish, such as Lactobacillales and Clostridales taxa.

Conclusions: These findings suggest that important bacterial members may not be active in the fish gut microbiota. In particular, several beneficial lactic acid bacteria (LAB) were identified as nonviable bacterial cells, potentially influencing the functional potential of the fish microbiota.

Significance and impacts of the study: Standardizing the methods for characterizing the fish microbiota are paramount in order to compare studies. In this study, we showed that both sample type and PMA treatment influence the bacterial communities found in the fish gut microbiota. Our findings also suggest that several microbes previously described in the fish gut may not be active constituents. As a result, these factors should be considered in future studies to better evaluate the active bacterial communities associated with the host.

Introduction

Nucleic acid sequence-based techniques have greatly improved our understanding of microbial communities living in and around animals. More specifically, these approaches have elucidated the involvement of the microbiome in the health and disease of numerous hosts, including fish (Chow *et al.* 2010; Kostic *et al.* 2013; de Bruijn *et al.* 2018; Legrand *et al.* 2020b; Wynne *et al.* 2020). In addition to supporting the host immune system functions and combatting pathogens, the fish microbiota has been shown to be involved in numerous other

functions, such as nutrient metabolism, digestion, reproduction and the recycling of waste products (van Kessel *et al.* 2016; Banerjee and Ray 2017; Butt and Volkoff 2019). This wealth of information is of particular value for the fish farming industry, as such knowledge can be applied to improve fish health and performance (Legrand *et al.* 2020b).

Next generation sequencing (NGS) techniques are not without caveats, and despite efforts in developing standardized approaches (Vatsos 2017; Poussin *et al.* 2018), care needs to be taken when drawing conclusions from fish microbiome related studies. For instance, sample collection, laboratory procedures, sequencing and data analysis can widely differ between studies, limiting reproducibility and resulting in differing conclusions (Pollock *et al.* 2018; Poussin *et al.* 2018; Legrand *et al.* 2020b). Different sample types (e.g. mucosa and digesta) also produce distinct microbiota; different samples from Atlantic salmon (*Salmo salar*) (Gajardo *et al.* 2016) and rainbow trout (*Oncorhynchus mykiss*) (Lyons *et al.* 2017) have been shown to exhibit different bacterial communities. Furthermore, metagenomics techniques are often performed using genomic DNA (gDNA) from the collected samples, limiting the ability to differentiate viable and nonviable microbial cells.

Several techniques have been developed to differentiate between viable and nonviable cells when using NGS. For instance, RNA instead of gDNA can be used to generate libraries and thus better characterize the active constituents of the host gut microbiota (De Vrieze *et al.* 2018; Legrand *et al.* 2020a; Legrand *et al.* 2020c). Alternative methods that assess microbial viability of gDNA in samples can also be utilized, such as molecular viability testing (MVT) and viability PCR (vPCR) (Cangelosi and Meschke 2014). The latter is by far the most studied method, which assesses viability based on cell envelope impermeability where samples are pretreated with a membrane-impermeative reagent such as propidium monoazide (PMA) (Nocker and Camper 2009; Cangelosi and Meschke 2014). During treatment, this reagent covalently binds to free DNA and nucleic acids in cells that do not have intact cell membranes which, following photoactivation, enables them to be separated from viable cells with intact membranes prior to DNA extraction and PCR amplification, and thus interfering with downstream sequencing (Cangelosi and Meschke 2014). Due to this advantageous property, PMA has thus been used on a wide range of samples to discriminate between viable and nonviable bacterial cells and/or spores in microbiome-related studies, including human saliva, human sputum, human stool, rex rabbits gut contents and water (Rawsthorne *et al.* 2009; Rogers *et al.* 2013; Li *et al.* 2017; Young *et al.* 2017; Fu *et al.* 2018; Marotz *et al.* 2018; Papanicolas *et al.* 2019). Recently, a study investigating the gut microbiota of Atlantic salmon (*Salmo salar*) using PMA showed that up to 9·1% of the sequencing reads came from nonviable bacterial cells (Dvergedal *et al.* 2020). However, this study only investigated the influence of PMA treatment on digesta samples, thus the application of PMA treatment on other fish tissues, such as mucosal samples with high levels of resident microbes, remains unknown.

Here, we characterized the intestinal bacterial communities in both gut contents and mucosal samples from farmed yellowtail kingfish (*Seriola lalandi*) using next generation 16S rRNA gene sequencing. We also assessed the microbial viability of yellowtail kingfish gut microbiota in both sample types using a modified PMA treatment protocol. The gastrointestinal tract is a physically perturbed and acidic environment, with food constantly moving through, epithelial cells being shed and microbial cells competing with each other for space and nutrients. As such, we hypothesized that numerous microbial cells (e.g. originating from the environment or food) become nonviable when exposed to this dynamic environment. Therefore, the intestinal content and gut mucosa would harbour complex bacterial communities comprising both viable (and likely active) and nonviable (not being able to survive in the gut environment) microbial cells.

Materials and methods

Sample collection

Five fish with a mean weight of 1·3 ± 0·1 kg and a mean length of 47 ± 1 cm were collected on the 18th of November 2019 from a single offshore seacage under the auspices of a commercial aquaculture enterprise of southern Australia according to industry best practice veterinary care. Fish originated from the same cohort and were fed the same proprietary diet prior to sampling. Fish were immediately transported on ice to a laboratory for dissection. Within 4 h post-mortem, the body cavity of the fish was opened, and the digestive tract was extracted. The digesta was then collected by squeezing the gastrointestinal tract into a sterile 15 ml falcon tube and immediately placed on ice. Then, an incision was made along the length of the hindgut and midgut sections using a sterile scalpel blade to expose the inner mucosal surface. The exposed mucosa was then collected using a sterile glass microscope slide as described previously (Legrand *et al.* 2020c) and placed in a 50-ml falcon tube. Gloves were used and changed between the collection of each sample type to avoid contamination.

For all digesta samples, 5 ml of digesta was placed into 15 ml falcon tubes containing 10 ml of phosphate

buffered saline (Invitrogen™ PBS, ThermoFisher Scientific, San Jose, CA, USA), and all samples were homogenized by vortexing. Samples were then centrifuged at 500 ***g*** for 3 min to remove debris using the Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany). The supernatant was collected using a sterile pipette for each sample and transferred into a new 15 ml falcon tube. The clean samples were then centrifuged at 12 000 ***g*** for 8 min. The supernatant was subsequently collected and discarded. Finally, the cell pellet was resuspended in 2 ml of PBS, vortexed and split in two 1 ml aliquots in 1·5 ml centrifuge tubes. The samples were then placed back on ice until PMA treatment. For all mucosal samples, 3 ml of PBS was added to each 50 ml falcon tube while removing the glass microscope slide from the tube. Each tube was then vortexed vigorously and hand shaken until all the mucosa was well mixed with the PBS. Next, 1 ml of this solution was transferred into two 1·5 ml tubes (1 ml per tube) and placed on ice until PMA treatment.

PMA treatment

Prior to PMA treatment, all samples were left at room temperature for 5 min. Then, half of the digesta and mucosal samples were placed back on ice and were used as nontreated (control) samples to investigate the influence of PMA on the resultant bacterial community composition. For the other half of the samples, 50 μl of a solution containing 0·2 mmol l^{-1} of PMA (PMAxx™, Biotium Inc, Hayward, CA, USA) was added in order to obtain a final concentration of 10 μmol l^{-1} of PMA, as described previously (Marotz *et al.* 2018). Samples were then gently vortexed and incubated in the dark at room temperature for 5 min. Next, samples were laid horizontally on ice at <30 cm from a light source comprising two 500W halogen globes (Philips Plusline S 500W R7s) for a period of 25 min, with brief mixing of the samples every 5 min. Samples were then placed back on ice with the control samples. All samples were subsequently stored at −20°C until DNA extraction.

DNA extraction, library preparation and sequencing

Prior to DNA extraction, all 20 samples (10 PMA-treated and 10 controls from 5 digesta and 5 mucosal samples) were thawed at air temperature and mixed with light vortex. Then, 1 ml of each sample was used as input for DNA extraction using the MP Bio Fast DNA Spin Kit for faeces (MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions. All DNA extracts were subsequently purified by ethanol precipitation using standard procedures and quantified using the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific) and stored at −20°C.

Sample DNA extracts were sent to the Australian Genome Research Facility (AGRF, Melbourne, Australia) for Illumina NGS library preparation. The V3–V4 hypervariable region of the 16S rRNA gene was amplified from the purified DNA samples using the universal eubacterial primers 341F/806R (Takahashi *et al.* 2014). PCR products were then indexed using Nextera XT indexes, and libraries were normalized and pooled in equimolar concentrations for sequencing on the Miseq platform (Illumina, San Diego, CA, USA) using 300 bp paired-end sequencing chemistry. Raw demultiplexed sequencing data with sample annotation were deposited in the NCBI SRA data repository under the BioProject ID PRJNA681418.

Bioinformatics and statistical analysis

Sequencing of the 20 samples resulted in a total of 2 602 389 paired end reads (130 119 ± 34 150 per sample). Demultiplexed sequences were processed using QIIME2 (v.2019.10) (Bolyen *et al.* 2019). First of all, paired-end reads were imported using the Casava 1·8 format. Then, forward reads were truncated to 297 bp and reverse reads to 223 bp to remove low-quality sequences and denoised into amplicon sequence variants (ASVs) using the DADA2 plugin (Callahan *et al.* 2016). This resulted in a total of 2 134 733 merged reads retained for downstream analysis. Taxonomy was assigned to each ASV using the q2-feature-classifier against the Silva 132 99% OTUs (Operational Taxonomic Units) reference sequences resource (Quast *et al.* 2013). ASVs with <10 reads, as well as those which were unassigned or which represented mitochondria, chloroplast, eukaryote sequences, were removed from the dataset. Samples were rarefied to an even depth of 40 000 reads, resulting in a total of 1709 ASVs. Alpha rarefaction revealed sufficient sequencing coverage (Fig. S1). The plugin q2-diversity was used to measure both alpha diversity metrics (Simpson diversity and observed ASVs) and beta diversity metrics (e.g. weighted and unweighted UniFrac). Functional profiles of the microbiome were predicted with Tax4Fun2 based on the KEGG database (Wemheuer *et al.* 2020). For univariate measures, statistical differences were assessed using the Wilcoxon rank-sum test. For multivariate measures, the function permdisp was first used to check the assumption of homogeneous dispersion between groups. When the assumption was met, a PERMANOVA was conducted using the function Adonis (allowing for type III (partial) sums of squares, fixed effects of sum to 0 for mixed terms, and exact *P*-values generated using unrestricted permutation of raw data) to measure for

statistical differences between groups, accounting for both the treatment group (e.g. sample type or PMA treatment) and fish id (Anderson 2001). Due to the relatively low number of replicates between groups, differential abundance for each ASV was assessed using Deseq2 with Benjamini-Hochberg false discovery rate method applied to correct the *P*-values (Love *et al.* 2014; Weiss *et al.* 2017).

Results

Sample type influences the fish gut microbiota

We first investigated the influence of sample type on the fish gut bacterial communities using the 10 control samples (five digesta and five mucosa samples). Using the unweighted Unifrac matrix, we found that the bacterial community composition was significantly different according to sample type (F.model = 2·26, $P = 0{\cdot}008$) (Fig. 1a). This indicates that different microbial communities populate the mucosa of fish, compared to the digesta.

To further characterize this change in bacterial communities, we compared the alpha diversity in the two sample types. Simpson's diversity was higher in digesta than mucosal samples though not significant ($P = 0{\cdot}056$, Fig. S2a). This was supported by significantly higher richness (observed Amplicon Sequence Variants ASVs, $P = 0{\cdot}008$; Fig. 1b) and evenness (Pielou's evenness, $P = 0{\cdot}016$; Fig. S2b) in the digesta samples compared to mucosal samples. This suggests that the fish mucosal samples contain fewer microbial species than digesta samples.

We also explored taxonomic differences between the two sample types (Fig. 2a). While *Ralstonia* was the most prevalent genus in both sample types, both sample types contained distinct bacterial taxa (Fig. 2a). Specifically, 23 differentially abundant ASVs were identified between these sample types (Table S1). *Brevinema*, *Aliivibrio* and *Vibrio* ASVs were significantly more prevalent in mucosa samples (Fig. 2b, Table S1). Clostridiales and Lactobacillales ASVs were more abundant in digesta samples (Fig. 2b, Table S1). Overall, these findings indicated that the type of biological sample examined can influence the microbiota signatures in the yellowtail kingfish gut microbiota.

The prediction of the functional profiles of all samples was generated using an average of 73 ± 22% of all sequences. However, in some samples, predictions were made using as low as 20% of the sequences (as observed in a mucosal sample, Table S2). In total, Tax4Fun2 was able to generate predictions for 356 KEGG pathways (Table S3). However, no significant differences in the functional profiles were found between digesta and mucosal samples.

Sample treatment with PMA has an impact on the resulting microbiota in both digesta and mucosal samples

PMA treatment of digesta samples

Since both digesta and mucosal samples comprised distinct bacterial communities, we evaluated the influence of PMA treatment on these two sample types separately. Using the unweighted Unifrac distance matrix, PMA treatment had a significant effect on the global bacterial communities of digesta samples (F.model = 2·98, $P = 0{\cdot}013$) (Fig. 3a).

PMA treatment also had a slight, yet nonsignificant, impact on the bacterial alpha diversity of digesta samples, as marked by a lower Simpson diversity in the PMA-

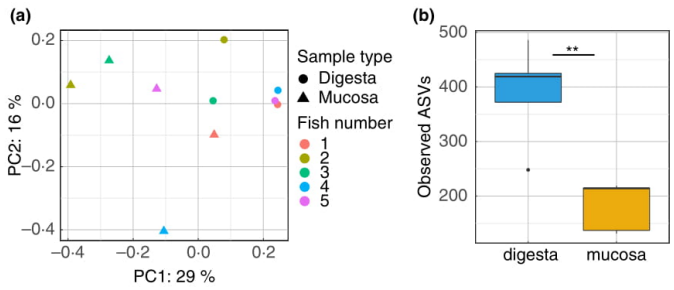

Figure 1 Impact of sample type on the global bacterial communities associated with the fish gut. (a) PCoA plot representing unweighted Unifrac distances comparing the change in bacterial communities found in digesta and mucosal samples for all five fish replicates used in this study. (b) Boxplot presenting the median and IQR of the number of observed amplicon sequence variants (ASVs) identified in digesta and mucosal samples. The levels of significant difference are denoted by $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$ and $***P \leq 0{\cdot}001$, following the Wilcoxon rank-sum test.

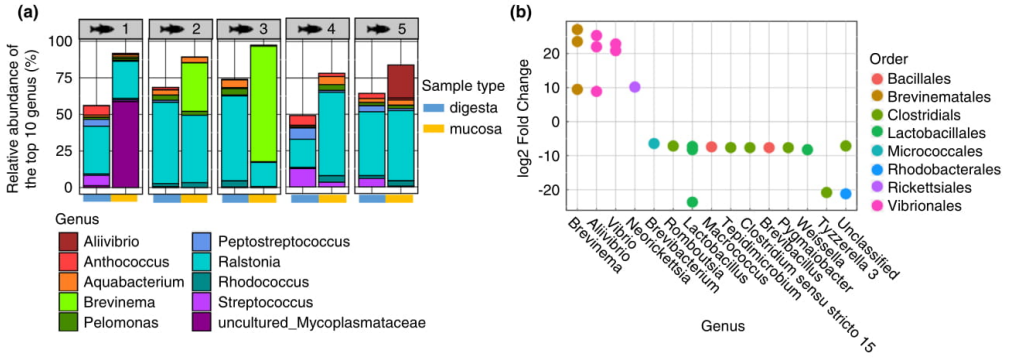

Figure 2 Impact of sample type on the taxonomical composition of the fish gut microbiota. (a) Stacked barplot presenting the relative abundance (%) of the top 10 most abundant bacterial genus found in the gut microbiota of all five replicates, comparing digesta and mucosal samples. (b) Dotplot showing significantly differentially abundant amplicon sequence variants (ASVs) between digesta and mucosal samples, as identified using Deseq2.

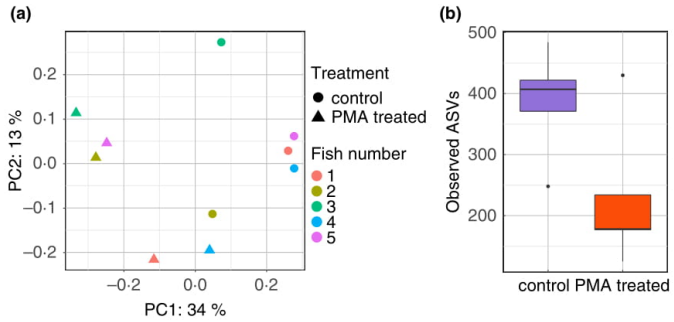

Figure 3 Impact of propidium monoazide (PMA) treatment on the global bacterial communities associated with the fish digesta. (a) PCoA plot representing unweighted Unifrac distances comparing the change in bacterial communities found in PMA-treated and control samples for all five digesta replicates used in this study. (b) Boxplot presenting the median and IQR of the number of observed amplicon sequence variants (ASVs) identified in PMA-treated and control digesta samples. The levels of significant difference are denoted by $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$ and $***P \leq 0{\cdot}001$, following the Wilcoxon rank-sum test.

treated samples ($P = 0{\cdot}056$, Fig. S3a). Untreated samples exhibited higher Pielou's evenness ($P = 0{\cdot}056$, Fig. S3b) and higher numbers of observed ASVs ($P = 0{\cdot}095$, Fig. 3b) although not significant. This indicates that several ASVs were detected from nonviable cells in digesta samples.

The taxonomic composition of the samples post PMA treatment was changed (Fig. 4a). Typically, *Ralstonia* was more abundant in PMA-treated digesta samples (Fig. 4a). Some taxa were not observed in PMA samples such as *Anthococcus*, *Streptococcus*, *Peptostreptococcus* and *Vagococcus* (Fig. 4a). In total, 153 AVS were found to be significantly differentially abundant between PMA-treated and control digesta samples (Table S4). Among those, only two taxa were more prevalent in PMA-treated samples and were *Brevibacillus* and *Staphylococcus* ASVs from the Bacillales order (Fig. 4b). Most of the ASVs found to be more abundant in control samples were associated with Bacillales, Clostridiales and Lactobacillales (Fig. 4b). Of particular note, 12 ASVs associated with *Lactobacillus*, 12 with *Enterococcus*, 5 with *Lactococcus*, 16 with *Streptococcus*, 8 with *Vagococcus* and 3 *Methanobrevibacter* were significantly less abundant in PMA-treated samples. While the total relative abundance of Lactobacillales-related ASVs in control samples was about 22%, the relative abundance of the same ASVs decreased to 0% in PMA-treated samples (Fig. S4). No significant differences were observed in the functional profiles between PMA-

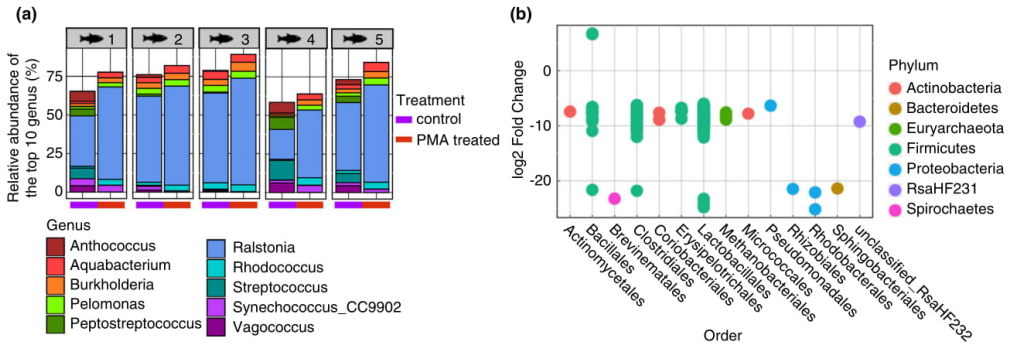

Figure 4 Impact of propidium monoazide (PMA) treatment on the taxonomical composition of the digesta-associated microbiota. (a) Stacked barplot presenting the relative abundance (%) of the top 10 most abundant bacterial genus found in the digesta of all five fish replicates, comparing PMA-treated and control samples. (b) Dotplot showing significantly differentially abundant amplicon sequence variants (ASVs) between PMA-treated and control digesta samples, as identified using Deseq2.

treated and nontreated digesta samples. Overall, this suggests that PMA treatment impacts composition, but not diversity, of gut digesta samples.

PMA treatment of mucosal samples

Next, we explored the impact of PMA treatment on the mucosal samples. Similar to the digesta samples, the bacterial community composition of mucosal samples were significantly different after PMA treatment when using the unweighted Unifrac distance matrix (F.model = 2·19, $P = 0{\cdot}002$) (Fig. 5a). This indicates that PMA treatment significantly impacted the bacterial composition of gut mucosal samples.

PMA treatment was found to significantly decrease the number of ASVs identified in mucosal samples (observed ASVs, $P = 0{\cdot}008$) (Fig. 5b). However, PMA treatment did not affect the Simpson's diversity (Simpson diversity, $P = 1$; Fig. S5a) or evenness (Pielou's evenness, $P = 1$; Fig. S5b) of the mucosal samples. This suggests that similarly to digesta samples, a number of ASVs detected in mucosal samples originated from nonviable cells.

PMA treatment impacted the downstream taxonomical composition of the mucosal samples. Although the relative abundance of the most dominant taxa (e.g. *Ralstonia*, *Brevinema* and uncultured Mycoplasmataceae) remained similar between PMA-treated and control samples, some ASVs were not detected in PMA-treated samples (Fig. 6a). More specifically, 21 ASVs were significantly reduced in PMA-treated samples (Fig. 6b, Table S5). Most of these ASVs were associated with Clostridiales, Lactobacillales and Vibrionales (Fig. 6b). Similarly to digesta samples, ASVs associated with *Enterococcus*,

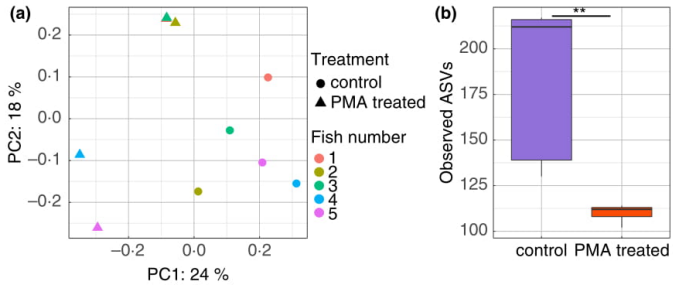

Figure 5 Impact of propidium monoazide (PMA) treatment on the global bacterial communities associated with the fish gut mucosa. (a) PCoA plot representing unweighted Unifrac distances comparing the change in bacterial communities found in PMA-treated and control samples for all five mucosal replicates used in this study. (b) Boxplot presenting the median and IQR of the number of observed amplicon sequence variants (ASVs) identified in PMA-treated and control mucosal samples. The levels of significant difference are denoted by $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$ and $***P \leq 0{\cdot}001$, following the Wilcoxon rank-sum test.

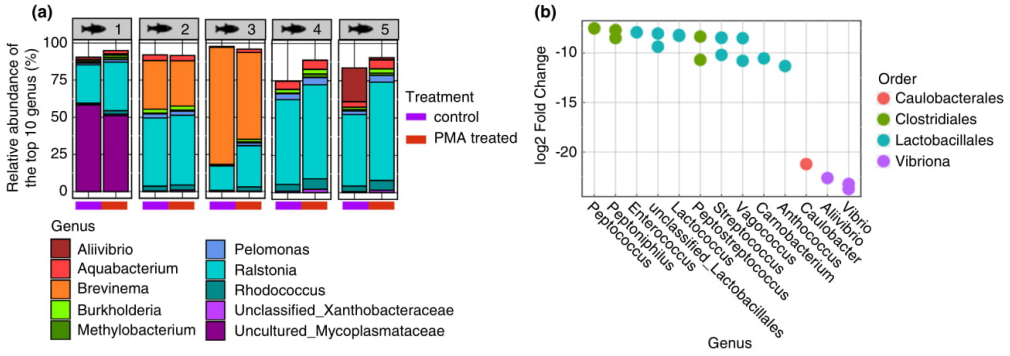

Figure 6 Impact of propidium monoazide (PMA) treatment on the taxonomical composition of the gut mucosa associated microbiota. (a) Stacked barplot presenting the relative abundance (%) of the top 10 most abundant bacterial genus found in the mucosa of all five fish replicates, comparing PMA-treated and control samples. (b) Dotplot showing significantly differentially abundant amplicon sequence variants (ASVs) between PMA-treated and control mucosal samples, as identified using Deseq2.

Lactococcus, *Streptococcus* and *Vagococcus* were lost after PMA treatment. Furthermore, the total relative abundance of Lactobacillales related ASVs decreased from 4% in control samples to 0% in PMA-treated samples (Fig. S6), although these species were not as abundant as in digesta samples. No significant differences were observed in the functional profiles between PMA-treated and nontreated mucosal samples. Overall, this suggests that PMA treatment significantly influenced the downstream microbial composition and diversity of gut mucosal samples.

Discussion

Faecal (digesta) material is often used as a proxy in animal gut microbiome investigations due to it being a noninvasive method in contrast to collecting the gut mucosa (Tang *et al.* 2020). However, there are major drawbacks when using this sample type such as incomplete separation between faecal bacteria and mucosal microbiota, homogenization of the sample and the effect of storage method (Tang *et al.* 2020). For instance, studies have shown that the mucus layer and intestinal lumen host distinct intestinal microbial niches with different biological roles in humans and other animals like dairy cattle and mice (Li *et al.* 2015; Mao *et al.* 2015; Ringel *et al.* 2015). In yellowtail kingfish, studies investigating the gut microbiota have used digesta, as well as a wide range of other sample types, including whole larvae, whole intestinal tract and intestinal mucosa (Wilkes Walburn *et al.* 2018; Horlick *et al.* 2020; Legrand *et al.* 2020c). In this study, we first investigated whether the microbial communities of digesta samples were different to those associated with mucosal samples. Based on both alpha and beta diversity indices, the two sample types exhibited distinct bacterial communities. We found a higher diversity and richness in digesta samples when compared to mucosa, a feature also identified in Atlantic salmon (Gajardo *et al.* 2016). However, this contrasts with previous results found in yellowtail kingfish, where a higher microbial diversity and richness were found in mucosal samples when compared to digesta samples (Horlick *et al.* 2020). Such variation within the same fish species can be explained with differences in environmental conditions (e.g. surrounding water, temperature), diet and number of replicates (Legrand *et al.* 2020b; Panteli *et al.* 2020). Taken together, this highlights the need to select the right sample type in relation to the research question when investigating fish gut microbiome. Typically, digesta is often collected when exploring transient (allochthonous) microbes that are influenced by environmental factors (e.g. diet or surrounding water) (Legrand *et al.* 2020b). In contrast, the mucosa contains more resident (autochthonous) microbes that are more influenced by the host and therefore more closely interact with the host mucosal surfaces (Ringo *et al.* 2016).

Another major limit of current fish microbiome studies is the inability to differentiate viable and nonviable microbial cells. There are several methods available to explore the bacterial viability in gut samples (e.g. plate counts, fluorescence approaches, staining of dead/viable cells), but these techniques are rarely used in current studies because of the expense of sequencing approaches (Hammes *et al.* 2010). While sequencing techniques focusing on RNA, proteins and metabolites (e.g. metatranscriptomics, metaproteomics and metabolomics)

provide information on active microbial communities, they are not commonly used in studies exploring the fish microbiota due to their high cost and limited bacterial cells in specific sample type (e.g. mucosa) (Ghanbari *et al.* 2015; Legrand *et al.* 2020b). Instead, DNA-based techniques (e.g. 16S rRNA gene and shotgun sequencing) are often used, but these methods cannot determine the viability and thus likely activity of microbial communities. This is of particular interest for studies that aim to assess the potential functional roles of the fish microbiome using predictive tools, such as PICRUSt or Tax4Fun, as sequencing data from total DNA will result in predicting the role of both active and non-active microbial communities (Langille *et al.* 2013; Asshauer *et al.* 2015). As such, we investigated the use of PMA treatment in order to estimate the viability of the microbial communities found in digesta and mucosal samples.

In this study, we used a modified PMA treatment protocol and found that there were significant changes in bacterial communities between PMA-treated and untreated digesta and mucosal samples. In both sample types, the composition of bacterial communities was significantly different after PMA treatment, as shown with the unweighted Unifrac matrix. In addition, we found a lower microbial richness and evenness in PMA-treated samples, indicating that some microbes were not detected in PMA-treated samples. More specifically, we found some bacterial lineages that were significantly reduced in PMA-treated samples, which would imply that these microbes are not viable (and thus not active) in the fish gut microbiota. For instance, several ASVs associated with Bacillales, Clostridiales and Lactobacillales were significantly less abundant in digesta samples following PMA treatment. This trend was also observed in mucosal samples, where several Clostridiales and Lactobacillales were significantly less abundant in PMA-treated samples. This is of particular interest to therapeutic treatments in aquaculture, as these lactic acid bacteria (LAB) are often considered as favourable micro-organisms due to their beneficial roles in enhancing immune responses, disease resistance, digestive functions and mucosal tolerance (Ringo *et al.* 2018). Interestingly, several LAB-associated genera that were depleted after PMA treatment (such as *Carnobacterium*, *Streptococcus*, *Enterococcus* and *Lactococcus*) are also known to contain potential pathogens (Ringo *et al.* 2018). As 16S rRNA gene sequencing is limited in its taxonomic resolution, further work should be implemented to better characterize the role of these important microbes found in the yellowtail kingfish gut microbiota.

While some bacterial lineages seem to be associated with nonviable cells, this study characterized the viability of the fish gut microbiota at only one point in time. Recently, it was revealed that time following feeding is an important driver of fish microbiota structure and functionality, as shown in clownfish (*Premnas biaculeatus*) and coral trout (*Plectropomus leopardus*) (Mekuchi *et al.* 2018; Parris *et al.* 2019). In this experiment, fish were collected in the morning, and their gastrointestinal tract contained leftover food from the previous day. As a result, bacterial viability could have been different if sampling occurred at a different time following feeding. Thus, the influence of feeding retention on bacterial viability requires further elucidation. In addition, it is unknown whether the nonviable bacteria detected in this study came from the environment/food or were already established in the fish gastrointestinal tract. In Atlantic salmon, it was demonstrated that diet and seawater derived bacteria were found in the fish hindgut (Zarkasi *et al.* 2016). It is therefore possible that the nonviable bacteria found in the yellowtail kingfish gut microbiota originate from feed or the surrounding environment. Despite this, LAB are typically occurring in the fish gut microbiota, as observed in numerous fish species (Ringo *et al.* 2018; Wang *et al.* 2018). In this study, LAB was more abundant in digesta samples than mucosal samples. This suggests that LAB are prevalent in the fish allochtonous bacterial communities and therefore not in close interactions with the host, in contrast to the autochtonous micro-organisms. Considering that the gastrointestinal tract is a complex environment where microbes are constantly under pressure (e.g. host-microbe and microbe–microbe interactions) (Perez *et al.* 2010; Legrand *et al.* 2020b), it remains unclear if these non-viable cells were previously viable before sampling. In addition, no differences in the predicted functional profiles were found between sample type and PMA treatment. This result could be explained by the low number of replicates used in this study (5 per treatment group) and poor level of prediction in some samples, as no reference genome for some of the bacteria found in the gut microbiota of yellowtail kingfish are available in the database used to generate the predictions. Therefore, further studies including the collection of samples at different time points, as well as exploring the gene expression and metabolite profile of these communities, are required to better elucidate the role of these micro-organisms within the fish gastrointestinal tract.

Overall, this study highlights important caveats found in fish microbiota related studies. Here, we showed that the digesta and gut mucosa contain distinct bacterial communities. As such, care should be taken when selecting sample type to investigate the fish gut microbiota. While collecting digesta has the advantage of being a non-invasive method, collecting the gut mucosa seems more appropriate if the overall aim of the study was to explore the micro-organisms that are in closer interaction

with the host. In addition, the microbial activity of the fish gut microbiota is likely to have an influence on the resultant role of these communities in disease resistance and nutrient digestibility, and ultimately fish health and performance. As a result, characterizing the active microbial communities found in the fish microbiota is paramount. In this regard, PMA treatment can be a very useful, cost effective tool. This simple, rapid and cost-effective method can easily be applied to better characterize and understand the contribution of dominant fish gut microbiota constituents.

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Authors' contributions

T.P.R.A.L. conceptualized the study, collected samples, performed laboratory work, analysed the data and wrote the manuscript. A.P.A.O. wrote the manuscript and supervised the study. M.L.W. contributed to data interpretation and edited the manuscript. J.W.W. provided reagents, edited the manuscript and supervised the study. L.S.W. edited the manuscript and supervised the study.

Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Rarefaction plot of all samples analysed in this study.

Figure S2 Boxplot presenting the median and IQR of (a) Simpson's diversity and (b) Pielou's evenness in digesta and mucosal samples. The levels of significant difference are denoted by $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$ and $***P \leq 0{\cdot}001$, following the Wilcoxon rank-sum test.

Figure S3 Boxplot presenting the median and IQR of (a) Simpson's diversity and (b) Pielou's evenness in PMA-treated and control digesta samples. The levels of significant difference are denoted by $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$ and $***P \leq 0{\cdot}001$, following the Wilcoxon rank-sum test.

Figure S4 Boxplot presenting the median and IQR of the relative abundances of the summed Lactobacillales associated ASVs found in PMA-treated and control digesta samples. The levels of significant difference are denoted by $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$ and $***P \leq 0{\cdot}001$, following the Wilcoxon rank-sum test.

Figure S5 Boxplot presenting the median and IQR of (a) Simpson's diversity and (b) Pielou's evenness in PMA-treated and control mucosal samples. The levels of significant difference are denoted by $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$ and $***P \leq 0{\cdot}001$, following the Wilcoxon rank-sum test.

Figure S6 Boxplot presenting the median and IQR of the relative abundances of the summed Lactobacillales associated ASVs found in PMA-treated and control mucosal samples. The levels of significant difference are denoted by $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$ and $***P \leq 0{\cdot}001$, following the Wilcoxon rank-sum test.

Table S1 Differentially abundant ASVs found in digesta and mucosal samples.

Table S2 Amount of sequences used to generate the prediction of microbial functions using Tax4Fun2.

Table S3 Prediction of the functional profiles of all samples used in this study.

Table S4 Differentially abundant ASVs found in PMA-treated and control digesta samples.

Table S5 Differentially abundant ASVs found in PMA-treated and control mucosal samples.

Discussion and conclusion

Preamble

The study of the fish microbiome provides a new lens through which to understand and improve the overall health and performance of the host. This is of particular interest for the fish farming industry, where this wealth of information can be used to enhance the animal's ability to digest and absorb nutrients, resist pathogens, reproduce, or even recycle waste products (Banerjee and Ray 2017b; Butt and Volkoff 2019; Legrand et al. 2020b). The contribution of the microbiome to immune function is of particular interest, and is thought to play a primary role in the fish's response to infection or disease (Gomez and Balcazar 2008; Perez et al. 2010). In addition, the microbiome can also directly compete against exogenous microorganisms and pathobiont invasion through the "colonisation resistance" effect, where commensal microbes can resist colonisation of pathogens by competing for space and nutrients (Perez et al. 2010). Specific microbes can also produce antimicrobial compounds that limit the propagation of select microbial constituents (Austin 2006; Balcazar et al. 2006). As a result, elucidating the structure of the fish microbiome and improving our understanding of their role/s, and how they interact with the host is key to developing new management strategies and treatment options for the industry in order to improve farm productivity and sustainability.

In this work, the role of the microbiome in the health and disease of YTK (as a model farmed species) from commercial operations and experimental systems was investigated using Next Generation Sequencing (NGS)-based 'omics' approaches. Specifically, the dynamics of the microbiome and its contribution to the immune response in YTK, as well as the application of conventional and novel treatment options were explored to gauge the effects and recovery of the microbiome in the host health and disease. In addition, a new analytical approach was also evaluated in order to better characterise the active constituents of the fish microbiome, and thus those likely to be involved in the overall health of the fish. Altogether, this thesis sought to gain insight into four keys areas as follows:

1. To provide context and perspective to microbiome science from the field of aquaculture to improve production systems.
2. To investigate the interactions between the fish and its microbiome during health and disease.

3. To explore the utility of novel treatment options (as an alternative to conventional therapies) in common gut related fish diseases.
4. To develop and evaluate a new protocol to investigate the active microbial constituents of the fish microbiome.

In this section, I begin with a chapter-by-chapter synthesis of the main findings arising from this thesis, and demonstrate their contributions and significance to the field/s of research. This is followed by a discussion of the broader contributions of the results, as well as the current limitations of the work and potential future directions.

Overview and main findings from Chapter 1

This chapter provides a descriptive review of the current knowledge of the fish microbiome. In recent years, a considerable amount of research has been dedicated to the study of the fish microbiome, in particular using NGS-based 'omics' technologies. Here, I summarise the microbial constituents associated with the mucosal surfaces of fish (i.e. from the gut, skin and gill), the different factors that likely influence the structure of the fish microbiome (particularly within a farm setting), and our current understanding of the functional involvement of the microbiome. I also discuss the interplay between the host and the microbiome, how this knowledge can be applied to the fish farming industry, identify current gaps in knowledge, and propose future directions in order to improve our understanding of the fish microbiome.

Overview and main findings from Chapter 2

Although the interactions between the fish microbiome and immune system during disease is central to understanding its etiology and in order to develop new management strategies and/or treatment options, this area of research remains poorly understood. In this chapter, I investigate changes in both the fish microbiota and immune system across the gut and skin mucosal surfaces in response to gut enteritis, a common disease in the farming of YTK. This work demonstrated that despite the disease being located in the fish gastrointestinal tract, the biggest changes (in terms of both microbial composition and host-gene expression) were found within the skin mucosa, highlighting the sensitivity of this organ in regard to changes in host health.

Overview and main findings from Chapter 3

The most typical, conventional treatment approach to combat disease in aquaculture is the administration of antimicrobials such as antibiotics. However, these compounds are non-targeted and may cause the depletion of both pathogens and commensals (leading to an altered or 'dysbiotic' microbiome state). In order to determine the broader impacts of these conventional therapies, it is thus fundamental to determine what microbes are depleted and whether they are able to recolonise and repopulate within the fish microbiota following treatment. In addition, with growing concern over the use of antibiotics on the environment and human health, alternative treatment options are eagerly sought, and extend to include approaches that seek to modulate the microbiome. Beyond probiotics, this includes approaches such as faecal microbiota transplantation (FMT), which have shown promise in the treatment of gut disease in humans and mammal model systems. This chapter represents the first study to investigate the influence antibiotics on the gut and skin microbiota of YTK, and explores the efficacy of FMT in the re-establishment of the microbiota following antibiotic exposure.

Overview and main findings from Chapter 4

It is well known that many biases can be introduced in metagenomic studies, potentially leading to inaccurate results and the misinterpretation of findings. When investigating the microbiome in health and disease, it appears paramount to differentiate both viable and non-viable microbial cells to better understand the influence of the microbiome on the health of the host. In this study, a novel approach was used to investigate the viable constituents of the fish microbiome from gut mucosa and digesta samples. Significant differences between the two approaches (with or without viable cell differentiation) were observed, indicating that non-viable microbial cells are naturally occurring in the fish gut microbiota. Such results are extremely valuable when investigating the functional role of the microbiome, as only viable microbial cells will likely contribute to pertinent host functions such as digestion, nutrient metabolism or colonisation resistance.

In summary, this thesis highlights the broad interdisciplinary nature of microbiome studies and their importance for the aquaculture industry. In demonstrating the interactions between the fish immune system and the fish microbiome, as well as exploring new treatment options for diseased fish, this thesis has been able to contribute valuable peer-reviewed information pertaining to the microbiome of fish and which has broader implications for

extending knowledge within the fields of host microbial ecology, fish immunology, veterinary medicine and aquaculture.

Discussion

Fish microbiome perturbations and fish immune response: who is behind the wheel?

Within the last few years, there has been considerable research effort in evaluating changes in the microbiome of various fish species in relation to stress and diseases (de Bruijn et al. 2018; Legrand et al. 2020b). However, it remains difficult to disentangle the effect that disease may have on the microbiome with those that may arise from the host and its immune response. For instance, in the case of a change in the fish microbiome towards a more "pathogenic" state and a concurrent activation of immune pathways, it is often unknown which came first. In Chapter 2, it was observed that the skin microbiota of fish suffering from gut enteritis was significantly different to healthy individuals, particularly at the early stages of disease where the community diversity was substantially lower (Legrand et al. 2020a). In addition, key genes related to the host immune response (notably several cytokines and related genes) were significantly differentially expressed in fish at the early stages of disease. This indicates that both microbial composition and host gene expression of the skin are perturbed in response to an underlying gut disease, and highlights the prospect of skin as a useful (non-invasive) sample type for exploring changes in both microbiota composition and gene expression in relation to the overall health of the animal. From this, two scenarios may be proposed: 1) changes in the expression of immune related genes drive the microbial perturbations; or 2) the alteration of the fish microbiota (as marked by a decrease in diversity and an increase in the abundance of opportunistic pathogens) lead to the increased gene expression of key immune related genes. In order to resolve these scenarios, and in order to develop new therapeutic strategies, further work is required to better understand the underlying mechanisms contributing to the emergence and progression of the disease itself. To do this, temporal studies involving experimentally infected fish could be used to track alterations in the microbiota and host gene expression across different stages of the disease (i.e. from its onset to establishment to the animals deterioration). Techniques that complement or enhance the metatranscriptomic approach used here (e.g. dual RNA-seq, metaproteomics or metabolomics), would be useful in this regard to better

disentangle the complex interactions between the pathogen, host and the microbiota (Le Luyer at. 2020).

The need to better understand the complex interplay that likely exists between the host and its microbiota and how such interactions drive disease, is particularly important for farmed systems where the microbiota of both healthy and diseased fish may also comprise opportunistic pathogens, as was recently shown in Atlantic salmon (*Salmo salar*) (Wynne et al. 2020). These organisms may occur year round in the surrounding water, regardless of the system (Rudi et al. 2017) and may become virulent under specific conditions, e.g. when fish are under stress induced by environmental changes or farming practices (Conte 2004; Mateus et al. 2017). Consequently, there is a need for microbiome related studies to go beyond characterising changes in the composition of the microbiota to include strategies that seek to identify the factors that contribute to or trigger the infection process, thereby improving the prospect of developing new intervention or targeted therapeutic strategies (e.g. vaccines).

Modulation of the fish microbiome

In recent years, growing awareness of the microbiome's role in promoting host health and nutrition (as highlighted by it being termed an additional 'organ' system in humans and other animals (Eckburg et al. 2005; Gill et al. 2006)) has led to a renewed interest in better understanding how current treatments may affect its structure and function, and whether alternative approaches may exist to support its optimisation. This is of particular importance given the overwhelming dependence on antibiotic use in some countries, despite the development of antimicrobial resistance and environmental related threats these therapies pose alongside the broader non-targeted effects they have on the commensal constituents of the microbiota (Perez-Sanchez et al. 2018; Lulijwa et al. 2020; Vincent et al. 2019; Schar et al. 2020). This was exemplified in Chapter 3, where an antibiotic combination therapy was administered to poor performing YTK and resulted in a loss of bacterial diversity in the gut for up to 18 days following treatment (Legrand et al. 2020c). This is a major limitation for the use of antibiotics, as the depletion of beneficial microbes may increase disease susceptibility as shown for Atlantic salmon, western mosquitofish (*Gambusia affinis*) and zebrafish (*Danio rerio*) (Navarrete et al. 2008; Gaulke et al. 2016; Carlson et al. 2017). Interestingly though, the influence of antibiotics on the fish gut microbiota can differ, as recently shown in European

seabass (*Dicentrarchus labrax*) where the gut microbiota persisted following treatment, indicating its stability to the use of particular antibiotics (Kokou et al. 2020). While these results could be explained by fish species differences, prior exposure to antibiotic can also play a role in the resistance to antibiotic. Indeed, fish microbiota that had been exposed to antibiotic in the past may harbour already resistant microbes to specific antibiotic, potentially resulting in the absence of observed changes in the fish microbiota following antibiotic exposure. Nevertheless, the resilience of these specific microbes under different types of antibiotics still remains to be explored. Antibiotic treatment has also been associated with profound changes in the overall bacterial communities of other mucosal tissues including the skin and gills (Rosado et al. 2019; Legrand et al. 2020c). This highlights the need to further explore the influence of antibiotic on the integrity and resilience of the outer mucosal surfaces, which act as important primary barriers for the host (Kelly and Salinas 2017; Cabillon and Lazado 2019). Furthermore, there is a lack of information regarding the recovery of the microbiota following antibiotic treatment. In Chapter 3, FMT was explored as a strategy for the repopulation of the fish gut microbiota following antibiotic treatment. However, while several bacteria from the inoculum were successfully transferred to select individuals, the delivery and efficacy of FMT on repopulating the fish gut microbiota remain elusive and this requires further investigation.

Other strategies can be used to modulate the fish microbiota and include changes in diet, probiotics, prebiotics, synbiotics, postbiotics, phytobiotics, quorum sensing inhibitors, phage therapy and biofilters to favour slow-growing specialists (K-strategists) over fast-growing specialists (r-strategists) (Defoirdt et al. 2011; Perez-Sanchez et al. 2018; Derome and Filteau 2020). To date, modification of the dietary components is the most studied method to modulate the fish microbiome with the aim to improve nutrient absorption and/or increase disease resistance (Ringo et al. 2016). This includes changes in the protein/lipid/carbohydrate source and the addition of vitamins, essential amino acids, bioactive compounds (e.g. sodium butyrate), dietary acidifiers and metals in the diet (Ringo et al. 2016; Piazzon et al. 2017). Tremendous research efforts have been dedicated to the use of various probiotics (notably lactic acid bacteria - LAB) in cultivated fish species with the aim to improve nutrient absorption and/or increase disease resistance (Merrifield and Ringo 2014; Banerjee and Ray 2017a; Hoseinifar et al. 2018). However, despite their use as prospective beneficial microbes, many of the current probiotics have been shown to have limited or species-specific effects. To improve the utility of these strategies, independent research focusing on individual fish species is required to identify prospective probiotics and their acceptance (colonisation) within the host

(Ringo et al. 2018; Wanka et al. 2018). To this end, current knowledge from microbiome (metagenomic) studies could be used to support culture-based investigations targeting putative probiotics through the identification of traits that may better support their isolation. In a study of YTK, several strains of *Shewanella*, *Psychrobacter* and *Acinetobacter* were isolated from wild individuals as prospective autochthonous probiotics, though further work is warranted to confirm their beneficial role to the host (Ramírez et al. 2019).

Investigating the functional role of the fish microbiome

Studies of the fish microbiome have mainly focused on structure (microbial diversity) rather than on function. However, in order to elucidate whether the microbiome may be modulated to improve outcomes that may be of benefit for the farming industry (e.g. improved disease resistance or growth), it is fundamental to also understand the functional involvement of these microbial communities. In Chapters 2, 3 and 4, it was shown that the gastrointestinal microbiota of YTK is frequently dominated by only a few bacterial species. For instance, an uncultured Mycoplasmataceae species has been found to be a primary constituent in the gut mucosal surface, in particular in the diseased or weakened host (Legrand et al. 2020a; Legrand et al. 2020c). Interestingly a *Mycoplasma* sp. was found to be associated with diseased zebrafish but in Atlantic salmon and hadal snailfish (*Pseudoliparis swirei*), genome reconstruction revealed a potential symbiotic relationship between *Mycoplasma* sp. and the host (Gaulke et al. 2019; Jin et al. 2019; Lian et al. 2020). Genome sequencing is a useful tool to investigate the role of particular microbes, revealing pathogen-gene clusters and ultimately helping understand their strategies to invade and develop within the host (Sudheesh et al. 2012). As such, to elucidate the potential involvement of the uncultured Mycoplasmataceae species in YTK, and in the absence of suitable reference sequences, further work should be implemented to generate the genome of this specific microbe. During my candidature, attempts were made in performing metagenomic assembled genomes (MAGs) for a number of key species from DNA extracts from gut mucus and faeces. However, not enough microbial DNA was recovered in order to assemble the genomes of these bacteria. As a result, this work was not included in this thesis.

Microbial functionality can also be investigated using other omics-based approaches including metatranscriptomics, metaproteomics and metabolomics (Ghanbari et al. 2015). In

Chapter 2, a metatranscriptomic approach was used to sequence the RNA from gut and skin samples (following the depletion of rRNA) to investigate the gene expression of both the host and microbial constituents. However, despite a high sequencing depth (~60 million sequence reads per sample), insufficient numbers of microbial sequences (< 1% of the total reads) were generated to facilitate the reliable analysis of the gene expression from these constituents. Instead, a high proportion of host RNA (~79% of the total reads) was sequenced, allowing some unique insights into host gene expression. Despite this shortcoming, metatranscriptomics has been successfully used elsewhere to investigate the role of the gut microbiome in other species (Wu et al. 2015; Ngugi et al. 2017; Parris et al. 2019). In these studies, intestinal contents (rather than the mucosa) were sampled, suggesting that a higher microbial cell content is more likely in these samples compared to those from the mucosa (as used here). While such results are useful, given that intestinal contents comprise a high proportion of transient (rather than resident) organisms which are less likely to have a close association with the host (Ringo et al. 2016), caution should be taken in their interpretation. Nonetheless, metatranscriptomics remains a powerful tool for exploring the role of the host microbiome in health and disease, and modified approaches should be investigated in the future to recover more microbial RNA from mucosal samples.

Limitations of fish microbiome research and future directions

NGS technologies have revolutionised the way in which we are able to study the ecology of microorganisms in comparison to traditional culture-dependent methods. Due to the constant decrease in sequencing cost and emergence of new bioinformatics tools, NGS is nowadays the most commonly used approach in microbiome investigations of fish as well as other host and environmental systems. However, despite encouraging standardised approaches, many biases can be introduced in the generation and analysis of sequencing data, ultimately limiting reproducibility and comparison between studies (Vatsos 2017; Poussin et al. 2018). For instance, the experimental design, choice of sample type, nucleic acid extraction method, library preparation and bioinformatics pipeline can all influence the results and interpretation of the data. In Chapter 4, the two most commonly used sample types in fish microbiome studies were evaluated (i.e. intestinal contents and mucosa), highlighting the differences that exist in their associated microbial communities (Legrand et al. 2021). As a result, comparisons between studies using different sample types cannot be performed. Since most fish microbiome related

studies seek to identify those constituents that are more likely to interact with the host, it is recommended that studies use mucosal samples instead of intestinal contents. In addition, the mucosal microbial communities are less likely to be influenced by environmental factors (e.g. surrounding environment) and diet, thus rendering comparison between studies more suitable. In Chapter 4, the influence of bacterial viability was also investigated using a combination of PMA treatment and 16S rRNA sequencing approaches (Legrand et al. 2021). Here, it was found that several bacterial lineages were identified from non-viable cells. Indeed, by sequencing all DNA from the samples, both viable and non-viable microbial cells are included in the dataset and thus do not reflect the activity of the microbial constituents. As such, if the aim of the study is to characterise the viable (and thus likely active) microbial constituents, methods such as PMA treatment or the generation of libraries from RNA (rather than DNA) are required and may support more reliable findings (Legrand et al. 2018; Legrand et al. 2021; Dvergedal et al. 2020).

Recent bioinformatics tools can generate functional predictions from phylogenetic inference of 16S rRNA sequencing data such as PICRUSt and Tax4Fun (Langille et al. 2013; Asshauer et al. 2015). Numerous fish microbiome related studies have used these tools to predict the involvement and likely role of the fish microbiome (Ortiz-Estrada et al. 2018; Legrand et al. 2020b). However, these tools rely on already established microbial genome databases. In fish, especially in species not well studied, the vast majority of microbes detected using 16S rRNA sequencing are poorly characterised (at least at the species level) and lack suitable genome sequences, thereby restricting the extent to which functional predictions can be made. Using Tax4Fun2 and the datasets created in this thesis, only ~20% of the sequences were able to be used in generating functional predictions. This is not surprising considering that microbial constituents found in the YTK microbiota are largely underrepresented in the available databases. To this end, generating microbial genomes from YTK could be useful to examine the 16S rDNA sequences generated throughout this study, thereby providing a platform for enabling more informative investigations of the involvement of the microbiome in the health and disease of this commercially important fish species.

Concluding remarks

In this thesis, each chapter maintains its own significance and contribution to the field of fish microbiome research. Altogether, this work explores the influence of the microbiome in the health and disease of YTK, in particular in a production context. By understanding how the host and its microbiome interact, by exploring the functional involvement of the fish microbiome, and by identifying how the fish microbiome can be modulated, new strategies can be developed and implemented to support disease treatment or its mitigation. To this end, a more holistic approach that extends to include the elucidation of the involvement and interactions between the host, the microbiome, the pathogen and the environment is needed to further improve our understanding of these unique systems and their capacity to support a stronger and more productive aquaculture industry.

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Appendix 1: Supplementary materials for Chapter 2

Figure S1: Rarefaction plot of all samples used in this study showing sufficient sequencing depth at 16,255 reads/sample.

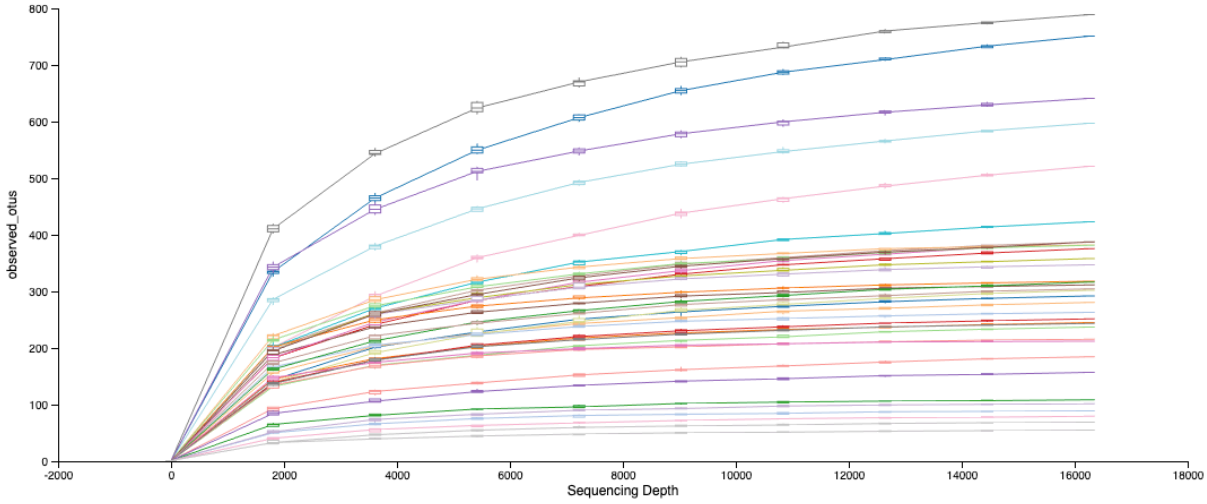


Figure S2: PCoA plot representing the gene expression of both gut and skin samples of all fish used in this study.

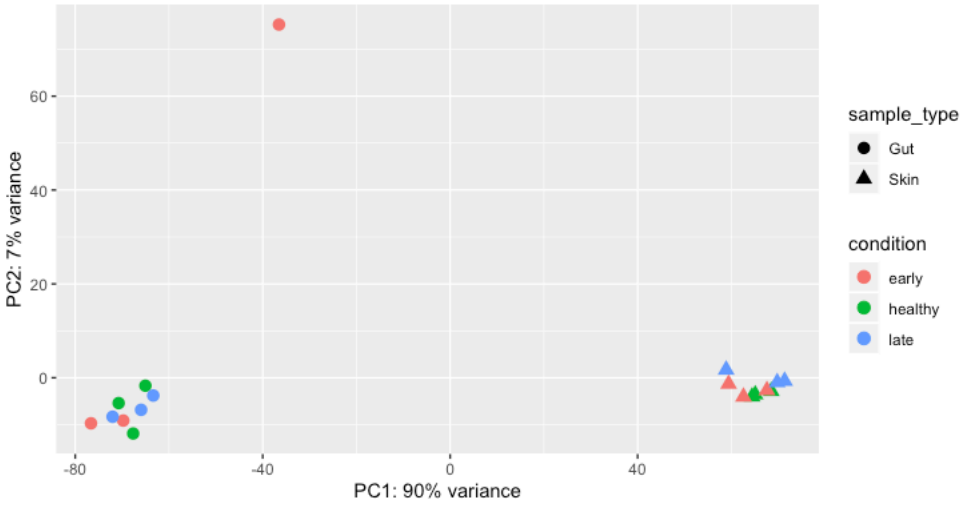


Figure S3: Gut global bacterial community changes associated with gut enteritis. PCoA plot based on the unweighted Unifrac distance matrix.

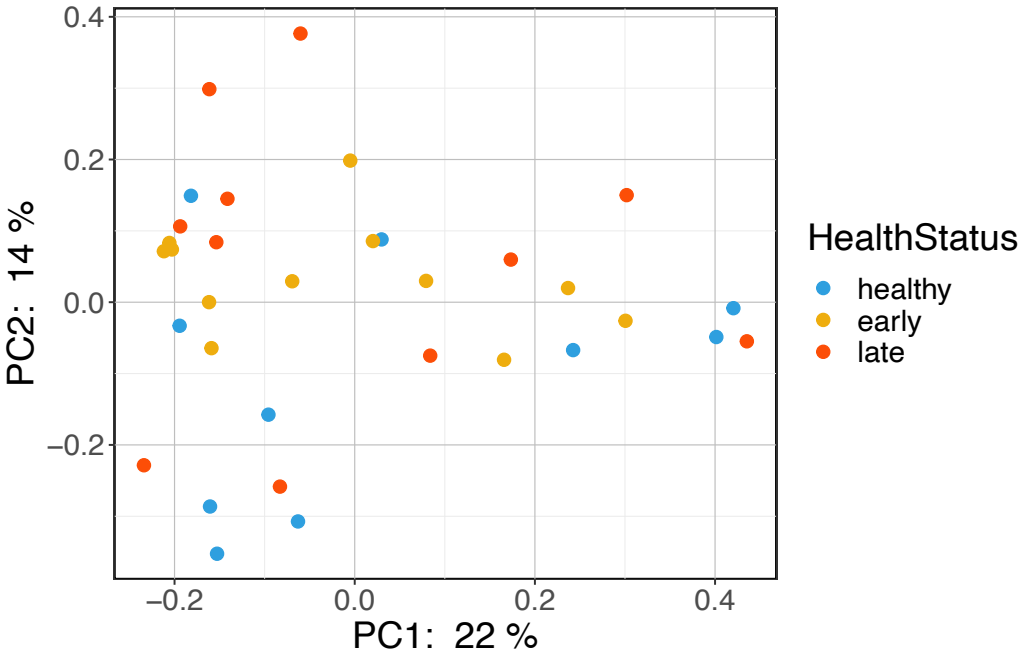


Figure S4: Skin global bacterial community changes associated with gut enteritis. PCoA plot based on the unweighted Unifrac distance matrix.

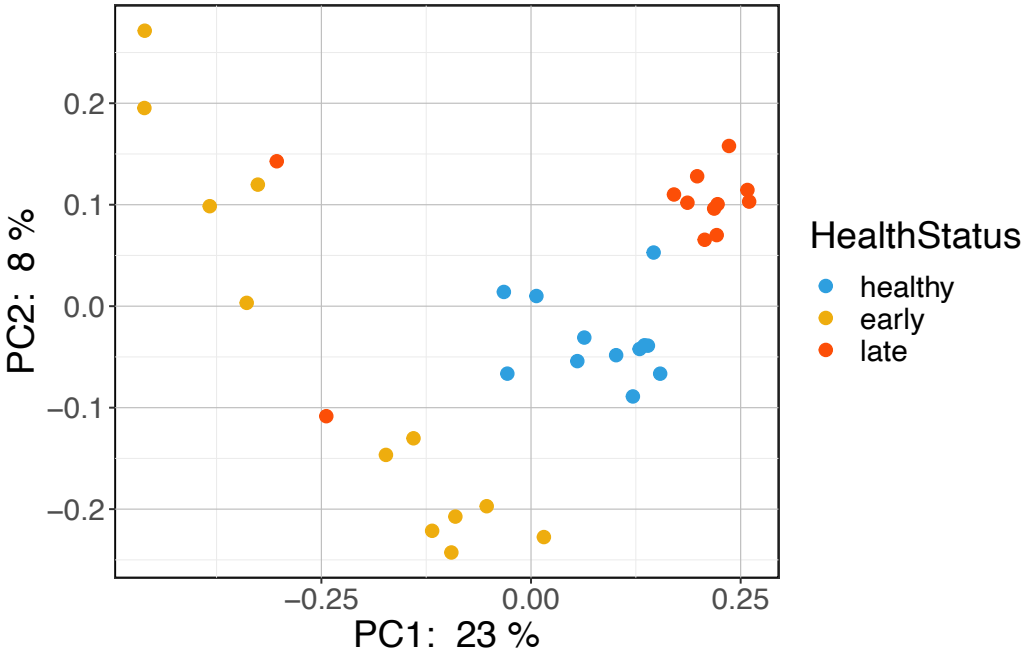


Figure S5: Boxplot representing the Pielou's evenness in the gut microbiota for the different health status. Statistical differences were assessed using a Kruskall-Wallis test.

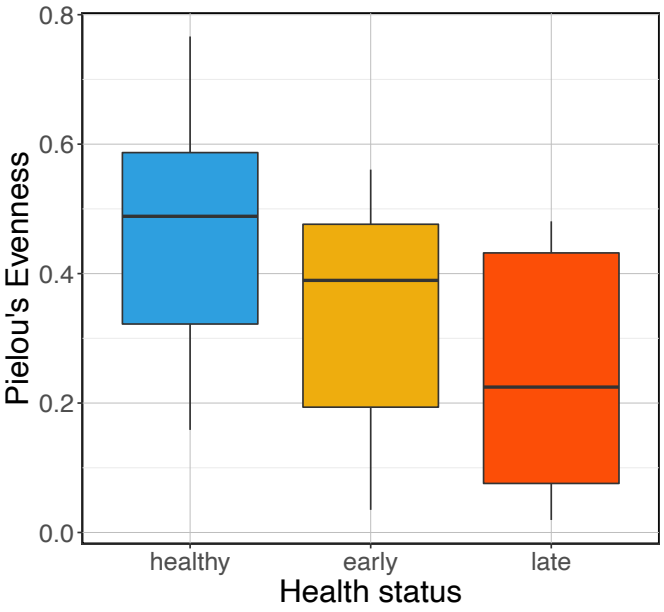


Figure S6: Boxplot representing the Chao1 richness in the gut microbiota for the different health status. Statistical differences were assessed using a Kruskall-Wallis test.

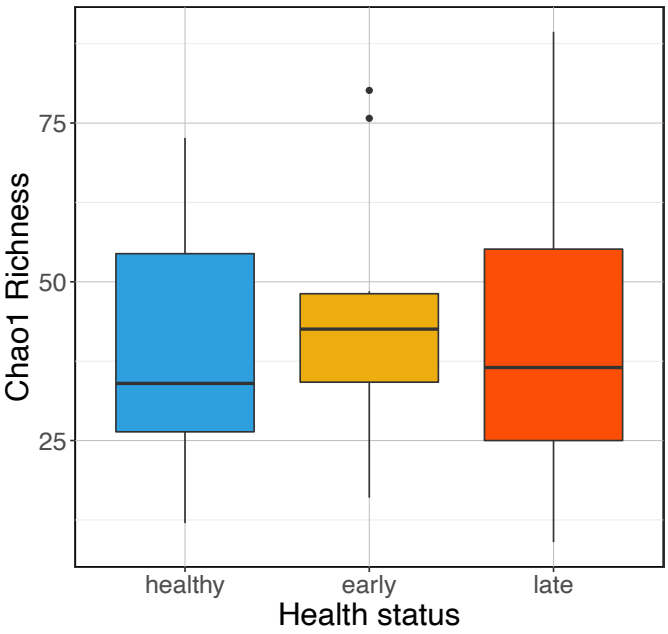


Figure S7: Boxplot representing the Pielou's evenness in the skin microbiota for the different health status. Statistical differences were assessed using a Kruskall-Wallis test.

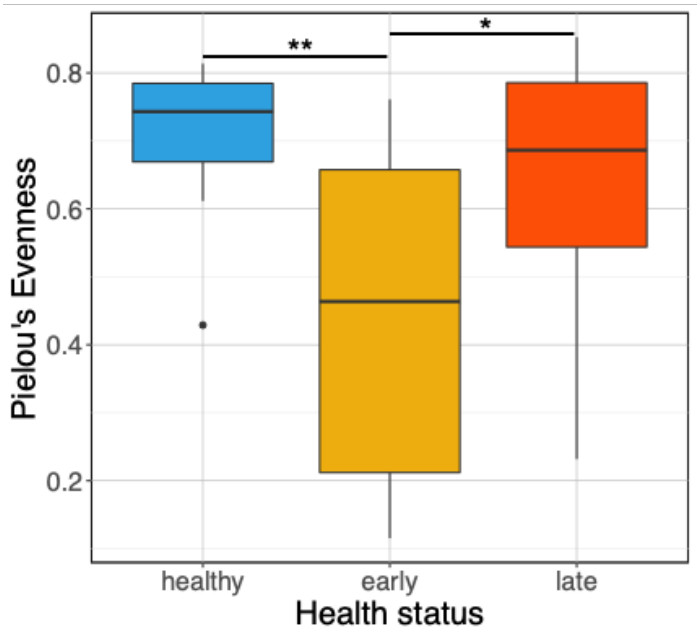


Figure S8: Boxplot representing the Chao1 richness in the skin microbiota for the different health status. Statistical differences were assessed using a Kruskall-Wallis test.

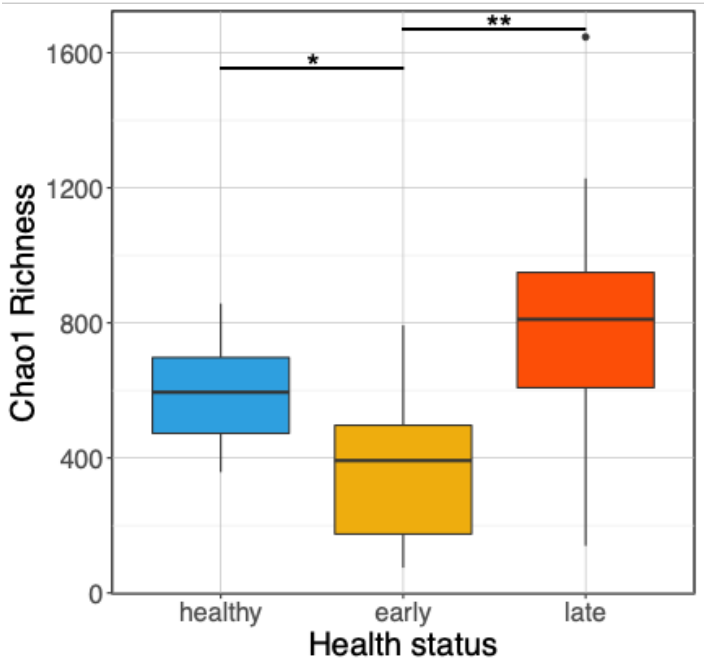
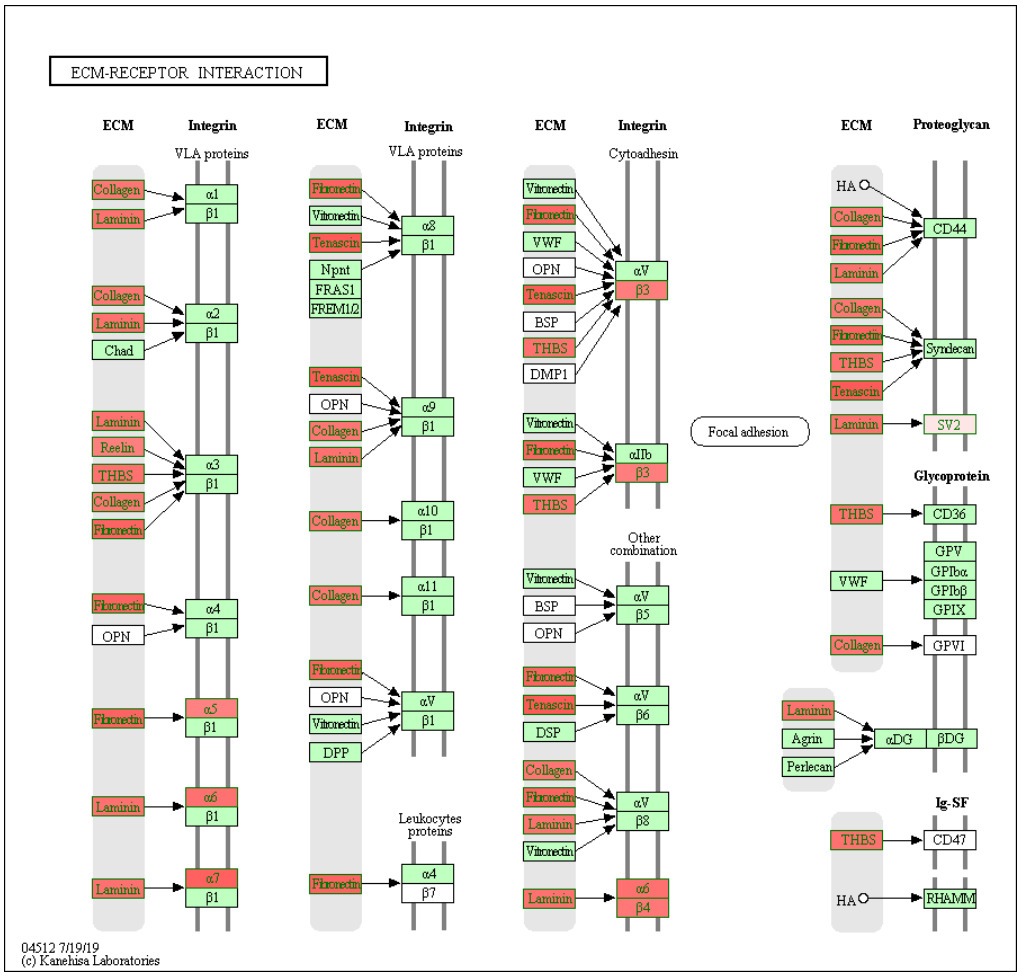


Figure S9: Representation of genes involved in the ECM-receptor interaction pathway in the fish at the late stage of the disease. Genes in red were upregulated, in blue were downregulated and in green were not statistically differentially expressed.



Supplementary tables

Due to the length of the supplementary tables, they were not included in this thesis. However, all supplementary tables are available at https://www.mdpi.com/2076-2607/8/9/1267/s1

Appendix 2: Supplementary materials for Chapter 3

Figure S1: Rarefaction plot of all samples analysed in this study

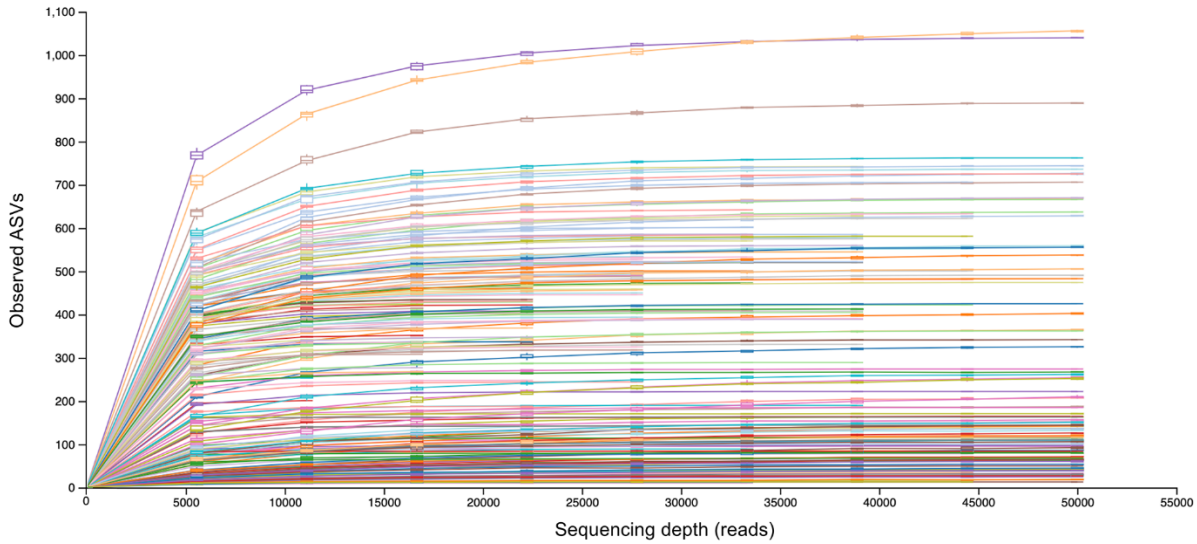


Figure S2: Stacked barplots presenting the mean relative abundance (%) of the top 10 bacterial Phylum found in the seawater (SW) and on the skin of fish, comparing fish housed in seacages and those relocated and acclimatised in tanks.

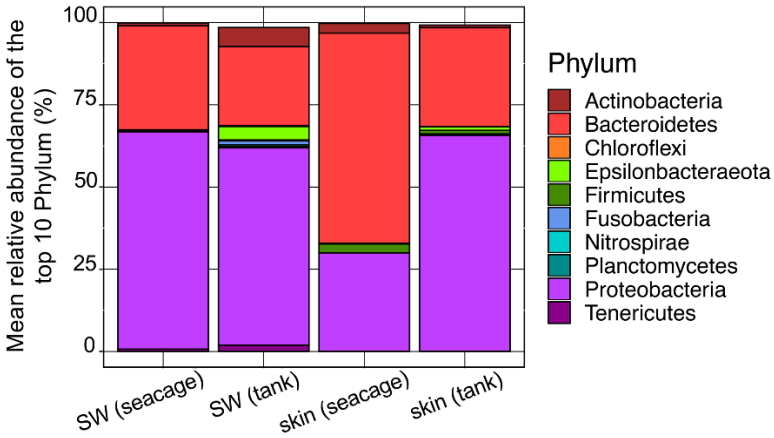


Figure S3: Means plots showing the change in mean value of Pielou's evenness (a) and Faith's phylogenetic diversity (b) in the gut bacterial communities, over the 18-day treatment period (from $n = 4$ fish).

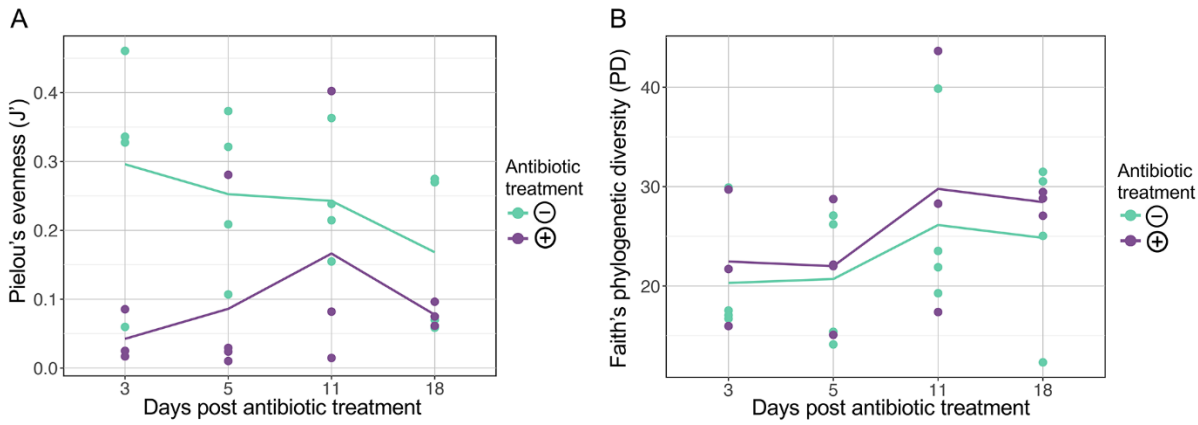


Figure S4: PCoA plot representing Bray-Curtis similarities comparing the change in global skin bacterial assemblages after treatment with antibiotics (+) over 18-days, with those fish that did not receive treatment (−).

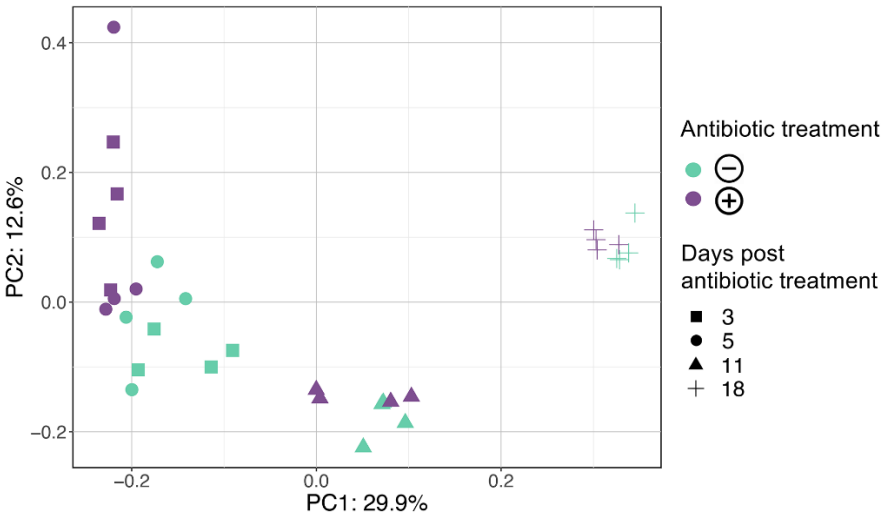


Figure S5: Means plots showing the change in mean value of Shannon's index of diversity (a), Pielou's evenness (b), total observed ASVs (as a measure of richness) (c) and Faith's phylogenetic diversity (d) in the skin bacterial communities, over the 18-day treatment period (from $n = 4$ fish).

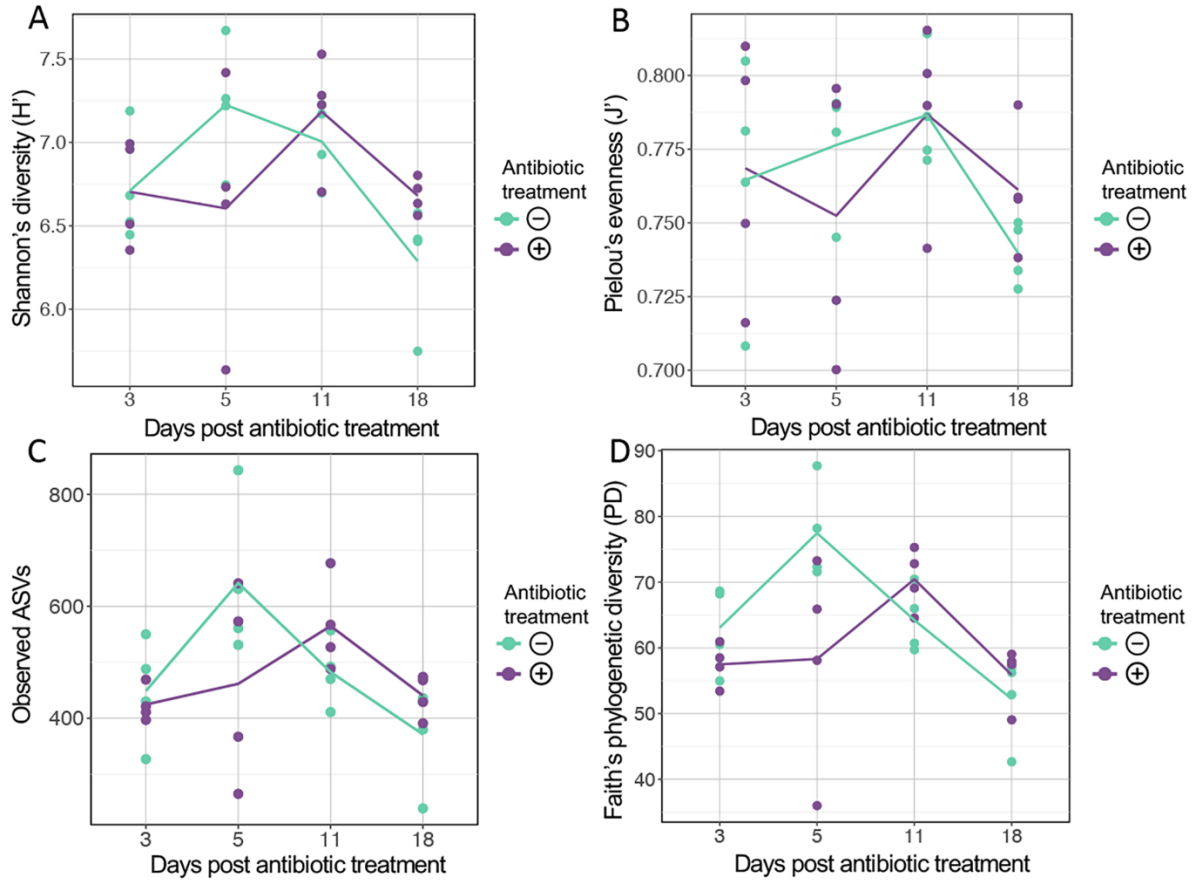


Figure S6: Venn diagram showing the distribution of unique and shared ASVs in the seawater, and the DNA and RNA inoculum samples. The total number of ASVs within each group are denoted in parentheses.

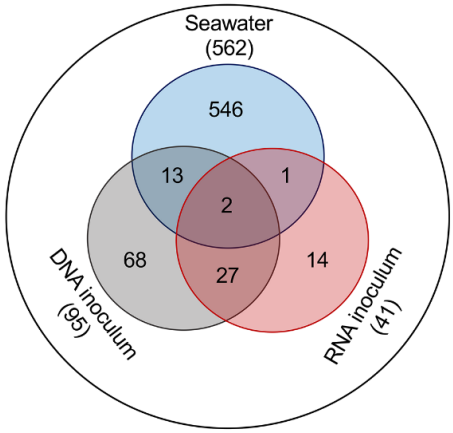
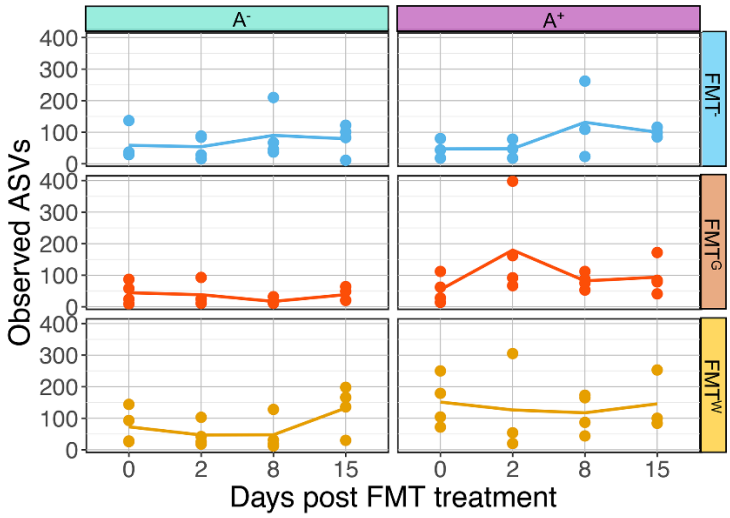


Figure S7: Mean plot presenting the mean number of observed ASVs for the different treatment groups; A^{-}/FMT^{-}, A^{+}/FMT^{-}, A^{-}/FMT^{G}, A^{+}/FMT^{G}, A^{-}/FMT^{W} and A^{+}/FMT^{W} over 15 days post FMT (mean from $n = 4$ fish).



TableS1: Water temperature (°C) recorded during the experiment.

Tank	Day0	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Day9	Day10	Day11	Day12	Day13	Day14	Day15	Day16	Day17	Day18
1	14	13.6	13.5		12.7	13.4	13.4	13	12.9	12.9	13	13.4	13.2		13.3	13.2	12.9	12.9	13.4
2	14	13.6	13.5		12.7	13.4	13.4	13	12.9	12.9	13	13.4	13.2		13.3	13.2	12.9	12.9	13.4
3	14	13.6	13.5		12.7	13.4	13.4	13	12.9	12.9	13	13.4	13.2		13.3	13.2	12.9	12.9	13.4
4	14	13.6	13.5		12.7	13.4	13.4	13	12.9	12.9	13	13.4	13.1	13.1	13.3	13.2	12.9	12.9	13.4
5	14	13.6	13.5		12.7	13.4	13.4	13	12.9	12.9	13	13.4	13.2		13.3	13.2	12.9	12.9	13.4
6	14	13.6	13.5		12.7	13.4	13.4	13	12.9	12.9	13	13.4	13.2		13.3	13.2	12.9	12.9	13.4
7	14	13.6	13.5		12.7	13.4	13.4	13	12.9	12.9	13	13.4	13.2		13.3	13.2	12.9	12.9	13.4
8	14	13.6	13.5		12.7	13.4	13.4	13	12.9	12.9	13	13.4	13.1	13.2	13.3	13.2	12.9	12.9	13.4
9	14	13.6	13.5		12.7	13.4	13.4	13	12.9	12.9	13	13.4	13.2		13.3	13.2	12.9	12.9	13.4
10	14	13.6	13.5		12.7	13.4	13.4	13	12.9	12.9	13	13.4	13.2		13.3	13.2	12.9	12.9	13.4
11	14	13.6	13.5		12.7	13.4	13.4	13	12.9	12.9	13	13.4	13.2		13.3	13.2	12.9	12.9	13.4
12	14	13.6	13.5		12.7	13.4	13.4	13	12.9	12.9	13	13.4	13.2	13.2	13.3	13.2	12.9	12.9	13.4
Average	14.0	13.6	13.5		12.7	13.4	13.4	13.0	12.9	12.9	13.0	13.4	13.2	13.2	13.3	13.2	12.9	12.9	13.4

Table S2: Oxygen concentration (%) recorded during the experiment.

Tank	Day0	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Day9	Day10	Day11	Day12	Day13	Day14	Day15	Day16	Day17	Day18
1	105	111	106		100	99	99	98	92	94	95	96	97	93	95	95	95	95	97
2	109	124	109		97	97	95	101	96	99	97	100	100	96	99	100	97	99	100
3	110	114	107		101	101	101	98	93	95	93	98	97	94	97	98	96	98	99
4	115	125	119		102	101	100	97	91	97	95	97	97	95	95	97	95	98	97
5	116	116	114		106	107	107	100	95	98	97	99	98	95	98	98	95	97	98
6	111	112	107		100	100	100	100	94	96	93	97	98	94	97	97	96	96	98
7	115	115	113		102	101	102	99	93	95	92	97	96	94	96	98	95	96	99
8	112	115	111		101	98	102	100	93	95	91	93	97	92	92	96	94	95	94
9	110	110	104		100	101	103	100	92	93	93	98	98	94	95	97	94	97	98
10	110	113	113		109	107	103	100	92	95	93	95	98	95	94	98	94	97	97
11	104	120	120		91	96	97	101	94	97	92	96	98	95	97	98	96	98	97
12	106	107	104		95	94	97	100	91	93	93	93	95	91	95	96	94	95	97
Average	110	115	111		100	100	101	100	93	96	94	97	97	94	96	97	95	97	98

Table S3: pH recorded during the experiment.

Tank	Day0	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Day9	Day10	Day11	Day12	Day13	Day14	Day15	Day16	Day17	Day18
1		7.63	7.66		7.62				7.6	7.61	7.68				7.73	7.64	7.61	7.63	
2		7.64	7.66		7.63				7.61	7.61	7.67				7.72	7.63	7.61	7.63	
3		7.63	7.66		7.63				7.61	7.61	7.67				7.72	7.65	7.61	7.63	
4		7.63	7.65		7.63		7.65	7.58	7.62	7.61	7.67		7.67	7.69	7.72	7.62	7.61	7.63	
5		7.63	7.65		7.61				7.6	7.61	7.67				7.73	7.64	7.61	7.62	
6		7.62	7.65		7.61				7.6	7.61	7.67				7.72	7.65	7.61	7.63	
7		7.63	7.65		7.62				7.61	7.61	7.67				7.73	7.63	7.61	7.63	
8		7.63	7.65		7.62		7.65	7.59	7.61	7.61	7.66		7.68	7.68	7.72	7.65	7.61	7.63	
9		7.64	7.66		7.61				7.6	7.62	7.67				7.73	7.62	7.63	7.63	
10		7.65	7.66		7.62				7.59	7.61	7.66				7.74	7.64	7.61	7.62	
11		7.63	7.65		7.61				7.6	7.61	7.66				7.74	7.64	7.61	7.63	
12		7.63	7.64		7.61		7.64	7.6	7.6	7.61	7.67		7.7	7.68	7.73	7.63	7.61	7.63	
Average		7.63	7.65		7.62		7.65	7.59	7.60	7.61	7.67		7.68	7.68	7.73	7.64	7.61	7.63	

Table S4: Salinity (‰) recorded during the experiment.

Tank	Day0	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Day9	Day10	Day11	Day12	Day13	Day14	Day15	Day16	Day17	Day18
1		36						36			37		36						36
2		36						36			37		36						36
3		36						36			37		36						36
4		36						36			37		36						36
5		36						36			37		36						36
6		36						36			37		36						36
7		36						36			37		36						36
8		36						36			37		36						36
9		36						36			37		36						36
10		36						36			37		36						36
11		36						36			37		36						36
12		36						36			37		36						36
Average		36						36			37		36						36

Table S5: Ammonia concentration (ppm) recorded during the experiment.

Tank	Day0	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Day9	Day10	Day11	Day12	Day13	Day14	Day15	Day16	Day17	Day18
1																			
2																			
3																			
4					<0.25		0	0	0	0	0		0	0	<0.25	<0.25	<0.25		
5																			
6																			
7																			
8					<0.25		0	0	0	0	0		0	0	<0.25	<0.25	<0.25		
9		<0.25	<0.25																
10		<0.25	<0.25																
11		<0.25	<0.25																
12					<0.25		0	0	0	0	0		0	0	<0.25	<0.25	<0.25		
Average		<0.25	<0.25		<0.25		0	0	0	0	0		0	0	<0.25	<0.25	<0.25		

Table S6: Characteristics (weight and length) of the fish stocked in the 12 experimental tanks prior the start of the experiment.

TANK 1			TANK 2			TANK 3			TANK 4			TANK 5			TANK 6		
	Weight (kg)	Length (cm)		Weight (kg)	Length (cm)		Weight (kg)	Length (cm)		Weight (kg)	Length (cm)		Weight (kg)	Length (cm)		Weight (kg)	Length (cm)
	1.775	50		1.581	47		1.675	50.5		1.545	47		1.73	49		1.676	48.5
	1.853	50		1.62	48.5		1.781	50		1.69	50		1.594	46.5		1.504	47
	1.632	48.5		1.505	47		1.608	47.5		1.381	45.5		1.81	50		1.516	47.5
	1.686	49		1.72	50		1.575	47.5		1.645	48.5		1.723	48.5		1.686	48.5
	1.682	48		1.636	48.5		1.498	46		1.671	49		1.811	51		1.339	45
	1.762	48.5		1.584	48.5		1.625	47.5		1.652	49		1.747	50.5		1.854	50
	1.64	49		1.657	49		1.636	47.5		1.826	50.5		1.81	51		1.508	48
	1.653	48.5		1.249	45.5		1.6	48.5		1.505	47.5		1.773	50		1.821	49.5
	1.761	51.5		1.678	48.5		1.694	50		1.705	47.5		1.294	45		1.644	49
	1.934	49.5		1.485	47		1.807	51		1.771	49.5		1.33	46.5		1.553	49
	1.599	48		1.786	49		1.624	48		1.734	48.5		1.62	49		1.403	47
	1.703	50		1.794	50.5		1.733	49.5		1.801	49		1.684	49.5		1.706	50.5
TOTAL	20.680		TOTAL	19.295		TOTAL	19.856		TOTAL	19.926		TOTAL	19.926		TOTAL	19.21	
AVERAGE	1.72	49.21	AVERAGE	1.61	48.25	AVERAGE	1.65	48.63	AVERAGE	1.66	48.46	AVERAGE	1.66	48.88	AVERAGE	1.60	48.29

TANK 7			TANK 8			TANK 9			TANK 10			TANK 11			TANK 12		
	Weight (kg)	Length (cm)		Weight (kg)	Length (cm)		Weight (kg)	Length (cm)		Weight (kg)	Length (cm)		Weight (kg)	Length (cm)		Weight (kg)	Length (cm)
	1.743	50		1.855	49		1.968	52		1.84	51		1.521	47.5		1.858	50.5
	1.801	51.5		1.644	48		1.612	49		1.897	50.5		1.258	44.5		1.357	45.5
	1.65	50.5		1.772	50		1.669	49.5		1.875	51		1.486	45.5		1.834	50
	1.712	49		1.723	49.5		1.665	48		1.618	50		1.796	50.5		1.828	51
	1.847	50.5		1.887	52		1.653	49.5		1.869	49		1.683	50.5		1.8	50
	1.785	50.5		1.725	50		1.835	50		1.96	52		1.614	48		1.502	47.5
	1.726	48		1.571	48.5		1.586	47.5		1.836	49.5		1.531	46		1.723	50
	1.621	47		1.721	48.5		1.341	46.5		1.856	50.5		1.46	48		1.663	48
	1.634	47.5		1.705	51		1.744	47		1.91	50.5		1.574	47.5		1.713	49
	1.654	51		1.526	48		1.73	48.5		2.001	52		1.135	43		1.759	49
	1.541	47		1.992	52		1.89	50		1.935	52		1.728	48.5		1.517	47.5
	1.709	49.5		1.643	49.5		1.987	52		1.953	51		1.615	47.5		1.704	49
TOTAL	20.423		TOTAL	20.764		TOTAL	20.65		TOTAL	22.55		TOTAL	18.401		TOTAL	20.258	
AVERAGE	1.70	49.33	AVERAGE	1.73	49.67	AVERAGE	1.72	49.13	AVERAGE	1.88	50.75	AVERAGE	1.53	47.25	AVERAGE	1.69	48.92

Table S7: Differential abundant ASVs found in the gut microbiota of fish housed in seacage and tanks.

ASV	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Kingdom	Phylum	Class	Order	Family	Genus	Species
2987622bcea36c5b34bc32ce0c037486	2435.51928	5.73888787	1.75778534	3.26483999	0.00109526	0.03388458	Bacteria	Spirochaetes	Spirochaetia	Brevinematales	Brevinemataceae	Brevinema	uncultured spirochete
ed1a4b07d3435c713e709e90a790564d	14.8856363	-7.68441032	2.37411855	-3.23674246	0.00120902	0.03520395	Bacteria	Bacteroidetes	Bacteroidia	Flavobacteriales	Crocinitomicaceae	Brumimicrobium	Brumimicrobium sp. N62
f863c9cb4d62cb5b3b5b3bf03080c61b	19.9954441	-7.75210553	2.22794121	-3.47949286	0.00050236	0.016578	Bacteria	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Mesonia	Salegentibacter sp. p68
82143ef78eb88d0df71ed343a4628832	33.2414074	-8.84853544	2.27211208	-3.89440975	9.84E-05	0.00487269	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Marinobacteraceae	Marinobacter	unclassified
b5c58e6d56b3a2b418d1e6247fa6020d	11.440587	-7.31121643	2.06199865	-3.5456941	0.00039158	0.01491018	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Marinobacteraceae	Marinobacter	Marinobacter sp. H3(2009)
00f3ff95fa9c8844d36417ddf09f8380	66.9580973	-9.52961843	2.10189995	-4.53381162	5.79E-06	0.00057349	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Cobetia	unclassified
0643d9449aa3d54f3d0f09110537846b	30.9708023	-8.75123439	2.15769865	-4.05581863	5.00E-05	0.00309121	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas	unclassified
bc611b6d2ec009ffb4ee97065d829bd1	25.4846315	-8.45895387	2.42231169	-3.49209967	0.00047924	0.016578	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Psychrobacter	unclassified
5a91d809a445a493a05fb327fca345d5	15.2094582	-7.72883948	2.11509834	-3.65412773	0.00025806	0.0116126	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas	unclassified
9284084eb57b58bb8496f899bb93389	55.1317267	-9.57912858	2.21200706	-4.33051447	1.49E-05	0.00122728	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas	unclassified
ee0a958ad50cfe222fa2fa1b295609d1	181.896101	-11.3020721	2.19276075	-5.15426599	2.55E-07	4.20E-05	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas	unclassified
5b225fd1347c24c1285b33cb462474d7	53.8730525	-26.9555914	2.64338391	-10.1973805	2.04E-24	1.01E-21	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas	unclassified
b25d705eb23d3ed8d83afcc46ff91023	225.44225	-7.87301387	1.62900714	-4.83301374	1.34E-06	0.00016642	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas	unclassified
984a138b195c1546cb4914a57ff4e975	29.7055832	-8.67803533	2.40538841	-3.60774804	0.00030887	0.01274073	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas	unclassified
5c06c95e93beff1f99680b211f743758	280.065488	-8.52780932	2.05380843	-4.15219316	3.29E-05	0.00232865	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas	unclassified
b596d2ce8106bc8e5534e829cf63f03e5	26.8456905	-8.54232034	2.18166844	-3.91549888	9.02E-05	0.00487269	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas	unclassified
96282be5d768c9e60851d391bd698feb	42.4116301	21.8954389	3.14587998	6.96003631	3.40E-12	8.42E-10	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	unclassified

Table S8: Differential abundant ASVs found in the gut microbiota of fish treated with antibiotic and non-treated with antibiotic.

ASV	baseMean	log2FoldCh±	lfcSE	stat	pvalue	padj	Kingdom	Phylum	Class	Order	Family	Genus	Species
2987622bcea36c5b34bc32ce0c037486	3538.44844	-12.0976066	1.66285306	-7.27521082	3.46E-13	3.25E-10	Bacteria	Spirochaetes	Spirochaetia	Brevinematales	Brevinemataceae	Brevinema	uncultured spirochete
524d4e332769bad5d50a40c9c46db145	1879.71492	-7.62165454	1.48615969	-5.12842234	2.92E-07	9.15E-05	Bacteria	Spirochaetes	Spirochaetia	Brevinematales	Brevinemataceae	Brevinema	uncultured spirochete
5174f4aa9aa296ff5e30846f5a665c20	969.175022	-9.90533175	1.70941256	-5.79458229	6.85E-09	3.22E-06	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Allivibrio	unclassified

Table S9: Pair-wise PERMANOVA results investigating the influence of time on the skin microbial communities.

pairs	Df	SumsOfSqs	F.Model	R2	p.value	p.adjusted
T0vsT1	1	0.3255113	2.551344	0.1541472	0.001	0.006
T0vsT2	1	0.5518907	5.94727	0.2981496	0.001	0.006
T0vsT3	1	1.1222352	12.791463	0.4774455	0.001	0.006
T1vsT2	1	0.5927852	5.522193	0.2828675	0.001	0.006
T1vsT3	1	1.16844	11.423734	0.4493334	0.001	0.006
T2vsT3	1	0.629703	9.329643	0.3999051	0.002	0.012

Table S10: Differential abundant ASVs found in the skin microbiota of fish treated with antibiotic and non-treated with antibiotic.

ASV	baseMean	log2FoldChang	lfcSE	stat	pvalue	padj	Kingdom	Phylum	Class	Order	Family	Genus	Species
51aa2da5026604a6976bc6d7c50392a2	1.36562566	19.6466254	2.94077871	6.68075615	2.38E-11	7.56E-09	Bacteria	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Tenacibaculum	unclassified
6784182256 1c63c0acbc97f343ad1571	11.4628156	7.08238194	1.83661038	3.85622451	0.00011515	0.01046236	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oleiphilaceae	Oleiphilus	uncultured Oleiphilus sp.
172d3e413675bc8a3b8e154706663aa6	26.801354	3.9848501	0.76177326	5.23101863	1.69E-07	2.68E-05	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oleiphilaceae	Oleiphilus	uncultured gamma proteobacterium
706afe657aa3a94c4606e4f25e31378	4.9995939	5.5035281	1.26984938	4.33400068	1.46E-05	0.00155209	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oleiphilaceae	Oleiphilus	uncultured bacterium
b070273d0cd7eb55cfce1094428e4931	24.6709529	2.81011745	0.59159776	4.75004748	2.03E-06	0.00025869	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Saccharospirillaceae	uncultured	unclassified
fe0d1d00baef546997e1dcde558b7c00	3.37808197	3.74380204	1.07539481	3.48132797	0.00049893	0.03966527	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Glaciecola	Glaciecola nitratireducens FR1064
e89affe5dc1c4574801472 67abc834ed	8.3395473	5.33218898	0.89675393	5.94610049	2.75E-09	5.82E-07	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Glaciecola	Cylindrotheca closterium
d98e4430aac608f33ade029bd3483400	19.1512744	6.18796174	0.89614994	6.90505179	5.02E-12	3.19E-09	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Paraglaciecola	unclassified

Appendix 3: Supplementary materials for Chapter 4

Figure S1: Rarefaction plot of all samples analysed in this study.

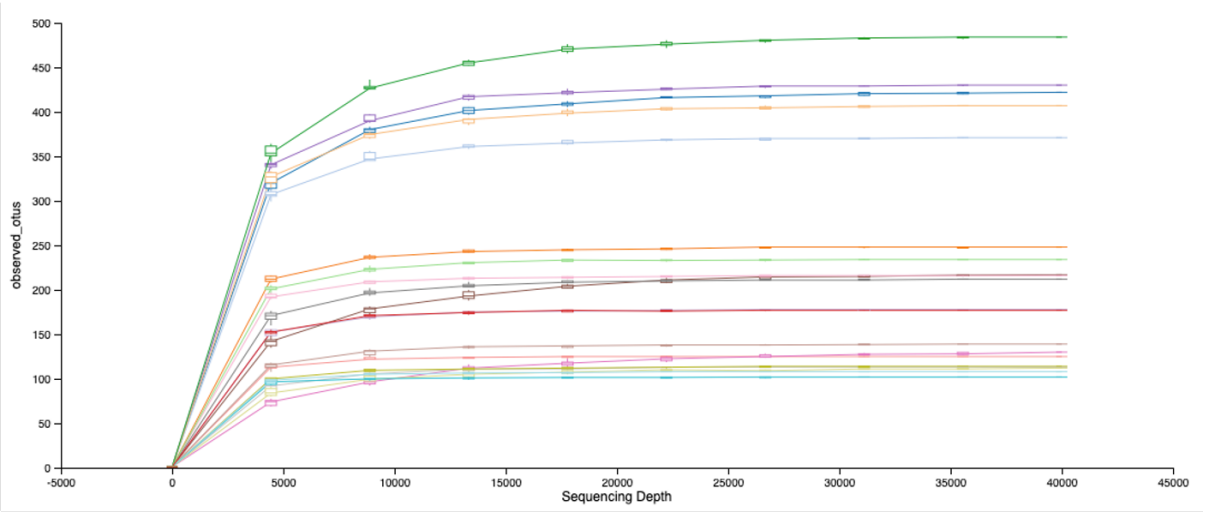


Figure S2: Boxplot presenting the median and IQR of (a) Simpson's diversity and (b) Pielou's evenness in digesta and mucosal samples. The levels of significant difference is denoted by *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001, following the Wilcoxon rank sum test.

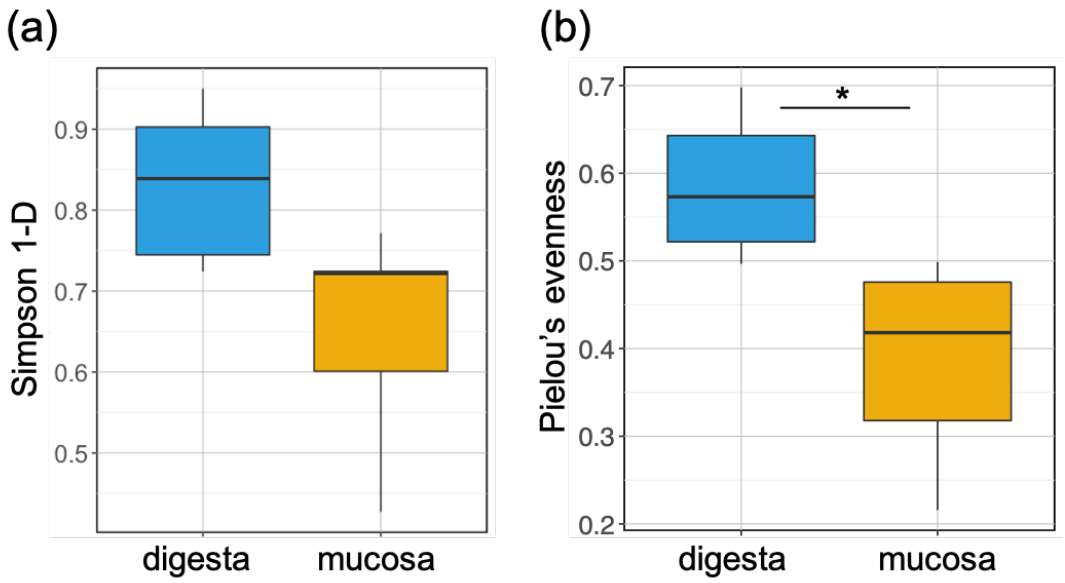


Figure S3: Boxplot presenting the median and IQR of (a) Simpson's diversity and (b) Pielou's evenness in PMA treated and control digesta samples. The levels of significant difference is denoted by *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001, following the Wilcoxon rank sum test.

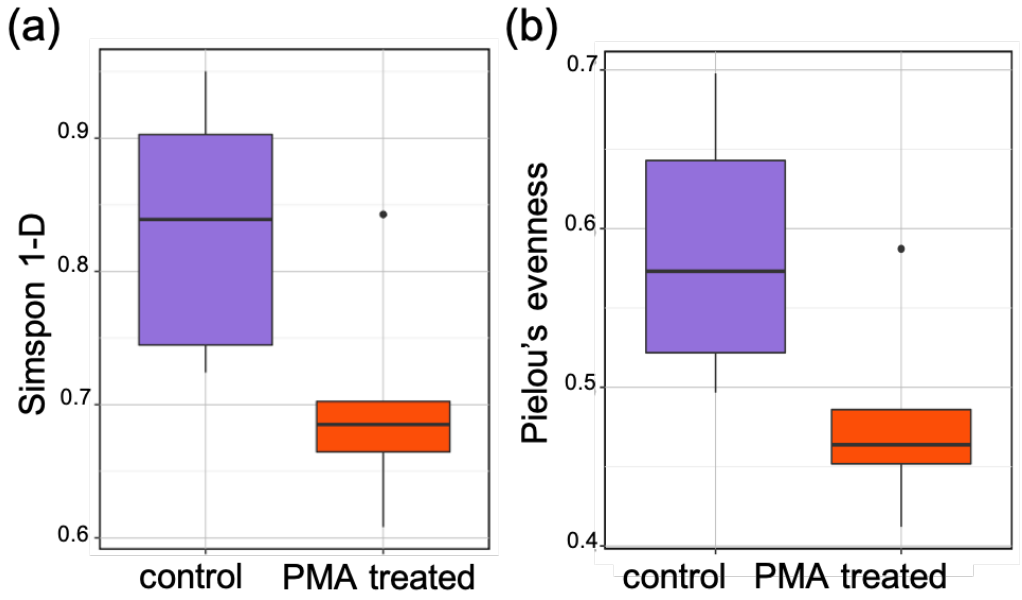


Figure S4: Boxplot presenting the median and IQR of the relative abundances of the summed Lactobacillales associated ASVs found in PMA treated and control digesta samples. The levels of significant difference is denoted by *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001, following the Wilcoxon rank sum test.

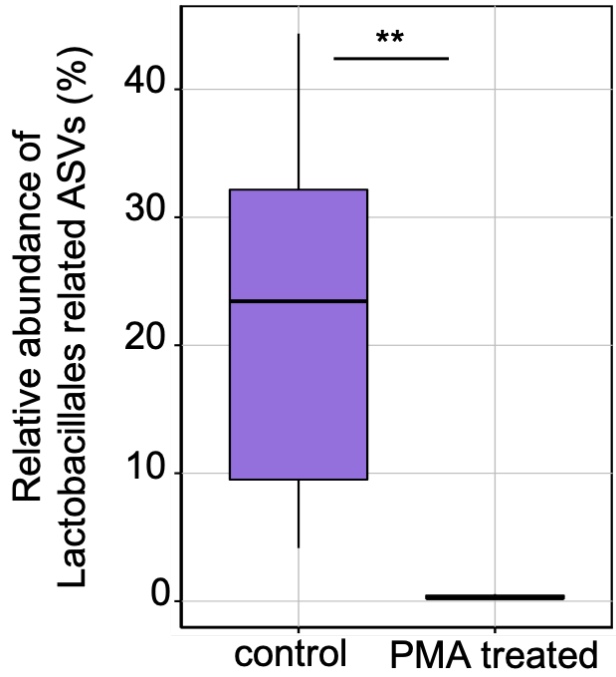


Figure S5: Boxplot presenting the median and IQR of (a) Simpson's diversity and (b) Pielou's evenness in PMA treated and control mucosal samples. The levels of significant difference is denoted by *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001, following the Wilcoxon rank sum test.

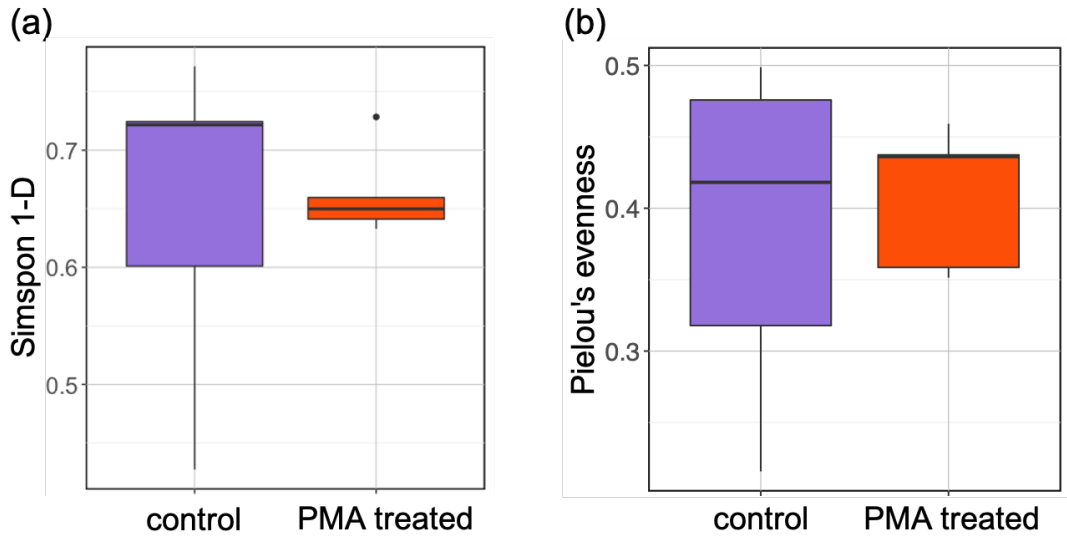


Figure S6: Boxplot presenting the median and IQR of the relative abundances of the summed Lactobacillales associated ASVs found in PMA treated and control mucosal samples. The levels of significant difference is denoted by *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001, following the Wilcoxon rank sum test.

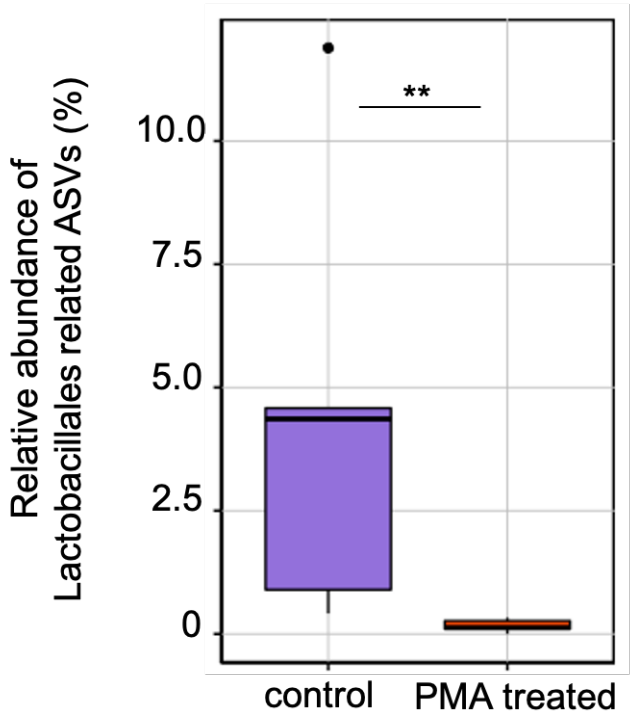


Table S1: Differentially abundant ASVs found in digesta and mucosal samples.

ASV	baseMean	log2FoldChai	lfcSE	stat	pvalue	padj	Domain	Phylum	Class	Order	Family	Genus	Confidence
df058c5dc742aee3188a79190c2860e1	8.96139106	-6.4381861	2.09071674	-3.0794158	0.00207407	0.0343859	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Brevibacteriaceae	Brevibacterium	0.9999954
52f605d9dfe254459307af6a4a66d1b7	89.9303591	10.160434	2.78125804	3.65317919	0.00025901	0.0067991	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Anaplasmataceae	Neorickettsia	0.7249088
86df7218ed8557a0d33483e63be71322	6.97094749	-21.209651	3.03080776	-6.9980192	2.60E-12	1.17E-10	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	NA	0.9999962
37077216b89019c4e293136a2e2b8bc8	521.681565	8.88195091	2.91580268	3.04614265	0.00231798	0.03650816	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	0.7697352
412e7831e6c07de82dfcb70ff9a50d42	195.31081	25.2970534	3.02643982	8.35868376	6.34E-17	9.99E-15	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	0.935329
fb19a46264758f4fc0ed743d266be883	18.18355	21.988128	3.02889417	7.25945732	3.89E-13	2.04E-11	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	0.9133922
40afea1c0f80c98d2f97be0c6d4b83b2	22.648261	20.8763011	3.02836278	6.89359319	5.44E-12	2.14E-10	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	0.946339
8f55eebbcb9fc1eaee5d71b4b64375d2	33.0394246	22.8193827	3.02767985	7.53692061	4.81E-14	3.03E-12	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	0.9804048
d9b148a887a0caa4601c7c7e4846e99e	55.1364368	23.5275985	3.02708467	7.7723622	7.70E-15	6.07E-13	Bacteria	Spirochaetes	Spirochaetia	Brevinematales	Brevinemataceae	Brevinema	0.9716926
7c393bac925a0dc8f34832ecd7c0d1d9	11802.1942	9.48027819	2.88741973	3.28330451	0.00102598	0.0230845	Bacteria	Spirochaetes	Spirochaetia	Brevinematales	Brevinemataceae	Brevinema	0.9765096
5fab83e2ddbb4eaf79fd3b63df3cfba1	691.192075	27.0615491	3.02625825	8.94224711	3.81E-19	1.20E-16	Bacteria	Spirochaetes	Spirochaetia	Brevinematales	Brevinemataceae	Brevinema	0.9582485
dc7e74b2230573eed3a6d8b33eb4b816	20.3056173	-7.6186463	2.42195781	-3.1456561	0.00165715	0.03262509	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Tepidimicrobium	0.9999656
504c43d105d63ebe55959ff5bec90da9	14.7587031	-7.1579545	2.40961799	-2.9705765	0.00297241	0.04070915	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Romboutsia	0.9278561
f67f715ab717f5c217e599a88a29a36b	20.8295418	-7.6547269	2.48001966	-3.086559	0.00202488	0.0343859	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Pygmaiobacter	0.9955584
c7da083319d1cb5e351d16a7b079a1a1	14.6011264	-7.1432012	2.39817667	-2.9785967	0.00289572	0.04070915	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	0.99868
967cb375147177 4d6bcdafd621fc21c1	5.35496776	-20.832813	3.03219927	-6.8705289	6.40E-12	2.24E-10	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Tyzzerella 3	0.9999821
7fcde376afa09566956d5ca0f55d5662	20.7362982	-7.6490436	2.44781983	-3.1248393	0.00177902	0.03296424	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto 15	0.8057801
9a5573e8614d2654dd57bdd4e770a8d5	17.6040632	-7.412235	2.45820365	-3.0153055	0.00256721	0.03850808	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Macrococcus	0.9998405
3fd6c015bac4b7f2eeb01567795bedc4	20.7642826	-7.6517257	2.39115578	-3.2000114	0.00137422	0.02885866	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Brevibacillus	0.9999963
522b62504fb41ab9fce76711483a3a23	42.3055954	-23.648595	3.02695455	-7.8126692	5.60E-15	5.88E-13	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.7452057
fec8a59a5e2636b5690bd66fa4d28ac5	31.0514374	-8.2327464	2.12797476	-3.8688177	0.00010936	0.00344498	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.9999982
9a891705636a19d582ac09fe289337eb	50.2427089	-7.2972315	2.05393591	-3.5528039	0.00038115	0.00923552	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.9986214
7366ec88f0a2b7621b2aa6fb8b08fd60	31.5251678	-8.2538503	2.18949707	-3.7697471	0.00016341	0.00467955	Bacteria	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Weissella	1

Table S2: Amount of sequences used to generate the prediction of microbial functions using Tax4Fun2.

Sample Id	Proportion of sequences (%)
Dig10.16S_V3.V4_CVDWN	72%
Dig13.16S_V3.V4_CVDWN	85%
Dig2.16S_V3.V4_CVDWN	83%
Dig4.16S_V3.V4_CVDWN	65%
Dig5.16S_V3.V4_CVDWN	77%
Digd10.16S_V3.V4_CVDWN	88%
Digd13.16S_V3.V4_CVDWN	88%
Digd2.16S_V3.V4_CVDWN	93%
Digd4.16S_V3.V4_CVDWN	82%
Digd5.16S_V3.V4_CVDWN	90%
M10.16S_V3.V4_CVDWN	35%
M13.16S_V3.V4_CVDWN	62%
M2.16S_V3.V4_CVDWN	20%
M4.16S_V3.V4_CVDWN	86%
M5.16S_V3.V4_CVDWN	92%
Md10.16S_V3.V4_CVDWN	45%
Md13.16S_V3.V4_CVDWN	65%
Md2.16S_V3.V4_CVDWN	38%
Md4.16S_V3.V4_CVDWN	92%
Md5.16S_V3.V4_CVDWN	93%

Table S3: Prediction of the functional profiles of all samples used in this study.

pathway	Dig10.16S_	Dig13.16S_	Dig2.16S_\	Dig4.16S_\	Dig5.16S_\	Digd10.16S	Digd13.16S	Digd2.16S_	Digd4.16S_	Digd5.16S_	M10.16S_V	M13.16S_V	M2.16S_V3	M4.16S_V3	M5.16S_V3	Md10.16S_	Md13.16S_	Md2.16S_V	Md4.16S_V	Md5.16S_V	level1	level2	level3
ko00010	0.008766	0.007873	0.007758	0.01013	0.008367	0.007628	0.007655	0.00766	0.00791	0.007725	0.007821	0.007677	0.007521	0.007873	0.007966	0.007605	0.007753	0.007633	0.007622	0.007741	Glycolysis /	Carbohydra	Metabolism
ko00020	0.005342	0.005364	0.005328	0.00539	0.005395	0.005314	0.005329	0.005346	0.005233	0.005342	0.005391	0.005394	0.005446	0.005336	0.00541	0.005315	0.005382	0.005334	0.005241	0.005331	Citrate cycl	Carbohydra	Metabolism
ko00030	0.00484	0.004583	0.004527	0.005135	0.004682	0.004534	0.004516	0.004518	0.004569	0.004537	0.004569	0.004501	0.004523	0.004537	0.004547	0.004497	0.00453	0.004466	0.004457	0.004507	Pentose ph	Carbohydra	Metabolism
ko00040	0.002415	0.002429	0.00246	0.002488	0.002419	0.002349	0.002456	0.002456	0.002415	0.002429	0.002432	0.00244	0.002377	0.002423	0.002317	0.002389	0.002477	0.002417	0.002409	0.002462	Pentose an	Carbohydra	Metabolism
ko00051	0.004441	0.00356	0.003486	0.005575	0.003997	0.003393	0.003389	0.003363	0.003629	0.003437	0.003525	0.0034	0.003263	0.003557	0.003697	0.003327	0.00348	0.003372	0.003362	0.003435	Fructose ar	Carbohydra	Metabolism
ko00052	0.003261	0.002674	0.002598	0.004082	0.002999	0.002505	0.002564	0.002559	0.002581	0.002577	0.002703	0.002588	0.002514	0.002666	0.002703	0.002487	0.002635	0.002552	0.002475	0.002592	Galactose r	Carbohydra	Metabolism
ko00053	0.002777	0.002903	0.002916	0.002734	0.002889	0.002846	0.002916	0.002956	0.00278	0.002891	0.002942	0.002947	0.002977	0.00288	0.002847	0.002876	0.002931	0.002913	0.002805	0.002867	Ascorbate a	Carbohydra	Metabolism
ko00061	0.007589	0.007903	0.00813	0.007368	0.007829	0.007941	0.008107	0.008126	0.008013	0.008043	0.007974	0.008081	0.008028	0.008123	0.00789	0.008093	0.00806	0.008155	0.008233	0.008195	Fatty acid b	Lipid metab	Metabolism
ko00062	3.56E-08	3.58E-08	2.67E-08	7.67E-08	5.14E-08	1.12E-07	1.86E-08	8.91E-09	1.90E-07	1.83E-07	4.28E-08	8.62E-08	2.35E-08	0	1.11E-08	0	2.64E-07	2.39E-10	3.01E-08	0	Fatty acid e	Lipid metab	Metabolism
ko00071	0.008432	0.009346	0.009774	0.007827	0.009084	0.009425	0.009696	0.009782	0.009537	0.009631	0.0095	0.009819	0.009602	0.009755	0.009467	0.009715	0.009755	0.009934	0.00997	0.009949	Fatty acid d	Lipid metab	Metabolism
ko00072	0.002068	0.00229	0.002391	0.001937	0.002233	0.002281	0.002375	0.002402	0.002288	0.002353	0.002332	0.002404	0.002347	0.002378	0.00227	0.002364	0.002376	0.002427	0.002407	0.002425	Synthesis a	Lipid metab	Metabolism
ko00073	1.28E-07	0	1.56E-08	3.30E-08	6.85E-08	0	0	4.66E-08	0	4.95E-09	5.21E-08	4.92E-08	0	0	0	0	6.91E-08	0	0	0	Cutin, sube	Lipid metab	Metabolism
ko00100	3.85E-06	4.61E-06	5.55E-06	3.72E-06	3.96E-06	4.86E-06	5.14E-06	6.10E-06	7.21E-06	5.79E-06	3.83E-06	5.75E-06	3.13E-06	5.30E-06	4.63E-06	4.76E-06	5.94E-06	6.48E-06	6.18E-06	5.90E-06	Steroid bios	Lipid metab	Metabolism
ko00120	0.000371	0.000379	0.000397	0.00039	0.000384	0.000389	0.000393	0.000392	0.000385	0.000381	0.00039	0.000393	0.000386	0.000406	0.000398	0.000409	0.000398	0.000397	0.000438	0.000411	Primary bile	Lipid metab	Metabolism
ko00121	8.51E-05	4.70E-05	5.51E-05	0.000144	6.86E-05	4.46E-05	4.39E-05	4.31E-05	6.22E-05	4.88E-05	4.59E-05	4.24E-05	3.10E-05	6.19E-05	6.86E-05	5.19E-05	4.61E-05	5.05E-05	6.36E-05	5.98E-05	Secondary	Lipid metab	Metabolism
ko00130	0.002759	0.002761	0.002729	0.002573	0.002728	0.00284	0.002764	0.002754	0.002778	0.00276	0.002769	0.002774	0.002813	0.002733	0.002826	0.002784	0.002797	0.002762	0.002812	0.002743	Ubiquinone	Metabolism	Metabolism
ko00140	0.000482	0.000543	0.000616	0.000454	0.000529	0.000557	0.000606	0.000611	0.000619	0.000598	0.000555	0.000604	0.000549	0.000618	0.000618	0.000602	0.000606	0.000637	0.000672	0.00065	Steroid hor	Lipid metab	Metabolism
ko00190	0.008964	0.009076	0.008867	0.008364	0.008907	0.009127	0.008918	0.008939	0.008923	0.009047	0.008941	0.008941	0.009188	0.008809	0.008914	0.008893	0.008857	0.008865	0.008612	0.008788	Oxidative p	Energy met	Metabolism
ko00195	0.002015	0.001407	0.001183	0.001829	0.001457	0.001586	0.001166	0.001116	0.001739	0.001362	0.001182	0.001109	0.001171	0.001172	0.001177	0.001144	0.001135	0.001106	0.001105	0.001115	Photosynth	Energy met	Metabolism
ko00196	0.000559	0.000194	5.49E-05	0.000352	0.000204	0.000352	5.52E-05	2.12E-05	0.000474	0.000192	4.23E-05	6.14E-06	3.88E-05	4.40E-05	5.04E-06	3.86E-05	2.39E-05	1.21E-05	2.71E-05	1.10E-05	Photosynth	Energy met	Metabolism
ko00220	0.003797	0.003816	0.003778	0.00379	0.00378	0.003759	0.003765	0.003794	0.003751	0.003807	0.003787	0.003787	0.003803	0.003763	0.003782	0.003736	0.003769	0.003795	0.003674	0.00377	Arginine bic	Amino acid	Metabolism
ko00230	0.011958	0.01108	0.010858	0.012713	0.011347	0.011041	0.010797	0.010733	0.011102	0.010876	0.010934	0.010738	0.0109	0.010926	0.011242	0.010845	0.010766	0.010731	0.010788	0.010749	Purine meta	Nucleotide	Metabolism
ko00232	4.42E-06	4.99E-06	6.84E-06	3.59E-06	4.56E-06	6.33E-06	7.07E-06	6.86E-06	9.40E-06	6.70E-06	5.17E-06	6.11E-06	5.13E-06	7.22E-06	6.23E-06	8.81E-06	6.46E-06	8.37E-06	9.59E-06	8.87E-06	Caffeine me	Biosynthesi	Metabolism
ko00240	0.007329	0.006391	0.006179	0.008233	0.006735	0.00633	0.006107	0.006049	0.006462	0.006205	0.00628	0.006112	0.006173	0.006268	0.006525	0.006133	0.006142	0.006098	0.006064	0.00607	Pyrimidine	Nucleotide	Metabolism
ko00250	0.005141	0.005005	0.004947	0.005265	0.005003	0.004926	0.004904	0.004929	0.005076	0.005	0.004899	0.004923	0.004831	0.004939	0.004977	0.004863	0.004943	0.004945	0.004862	0.004951	Alanine, as	Amino acid	Metabolism
ko00253	0.000114	0.000123	0.000127	0.000112	0.000122	0.000124	0.000131	0.000131	0.00012	0.000123	0.000126	0.000127	0.000128	0.000128	0.00012	0.00013	0.00013	0.000128	0.00014	0.000126	Tetracycline	Metabolism	Metabolism
ko00260	0.007441	0.007435	0.007366	0.007378	0.007374	0.00736	0.007348	0.007371	0.007343	0.007371	0.00737	0.007362	0.007373	0.007303	0.007267	0.007333	0.00737	0.007312	0.007232	0.007308	Glycine, ser	Amino acid	Metabolism
ko00261	0.001249	0.001244	0.001249	0.001223	0.001239	0.001263	0.00125	0.001249	0.001284	0.001263	0.001232	0.001249	0.00123	0.001249	0.001324	0.001262	0.001252	0.001265	0.00128	0.001268	Monobacta	Biosynthesi	Metabolism
ko00270	0.006157	0.005697	0.005583	0.006502	0.00582	0.005754	0.005584	0.00554	0.005868	0.005633	0.005613	0.005527	0.005494	0.005623	0.005671	0.005669	0.005566	0.005576	0.005697	0.005583	Cysteine ar	Amino acid	Metabolism
ko00280	0.009928	0.010849	0.011218	0.009324	0.010588	0.01086	0.011129	0.011207	0.010862	0.011034	0.010987	0.011248	0.011104	0.011159	0.010761	0.011113	0.011184	0.011313	0.011295	0.011279	Valine, leuc	Amino acid	Metabolism
ko00281	0.002812	0.003205	0.003312	0.0025	0.003069	0.003182	0.003292	0.00334	0.003116	0.003247	0.003259	0.003343	0.003342	0.003288	0.003191	0.003269	0.003284	0.003351	0.003289	0.003331	Geraniol de	Metabolism	Metabolism
ko00290	0.002433	0.002465	0.002475	0.0023	0.002444	0.002537	0.002485	0.002468	0.002494	0.002462	0.002479	0.002471	0.002492	0.002459	0.002463	0.002523	0.002499	0.002466	0.002553	0.00246	Valine, leuc	Amino acid	Metabolism
ko00300	0.003467	0.003236	0.003127	0.003716	0.003286	0.003135	0.00311	0.003118	0.003146	0.003142	0.003172	0.003117	0.003135	0.003141	0.003144	0.003087	0.003111	0.003096	0.003036	0.003092	Lysine bios	Amino acid	Metabolism
ko00310	0.005121	0.005719	0.005914	0.00468	0.005526	0.005697	0.005878	0.005959	0.005715	0.005845	0.005782	0.005964	0.00586	0.005863	0.005709	0.005859	0.005923	0.005992	0.005933	0.005969	Lysine degr	Amino acid	Metabolism
ko00311	0.000226	0.000214	0.000211	0.000222	0.000219	0.000214	0.000211	0.000213	0.000222	0.000222	0.000211	0.000214	0.000201	0.000208	0.000202	0.000207	0.000215	0.000214	0.000203	0.000218	Penicillin ar	Biosynthesi	Metabolism
ko00330	0.005419	0.005651	0.005723	0.005181	0.005542	0.005661	0.005697	0.005744	0.005735	0.005726	0.005608	0.005714	0.005606	0.00564	0.005528	0.00568	0.005736	0.005734	0.005712	0.005688	Arginine an	Amino acid	Metabolism
ko00331	0	0	0	0	0	0	0	0	1.09E-08	1.53E-07	5.95E-08	1.04E-07	0	7.82E-08	0	7.38E-08	0	0	0	0	Clavulanic	Biosynthesi	Metabolism
ko00332	0.000289	0.000254	0.000247	0.000314	0.000266	0.000257	0.000244	0.000243	0.000268	0.000252	0.00025	0.000245	0.000239	0.000249	0.000248	0.000248	0.000247	0.000246	0.000245	0.000248	Carbapene	Biosynthesi	Metabolism
ko00333	0.003975	0.004327	0.004523	0.003682	0.00421	0.004346	0.004537	0.00455	0.004416	0.004469	0.004382	0.004504	0.004407	0.004487	0.004277	0.0045	0.004502	0.00457	0.004618	0.004583	Prodigiosin	Biosynthesi	Metabolism
ko00340	0.003869	0.004	0.003959	0.003568	0.003892	0.004003	0.003975	0.003997	0.003972	0.004015	0.003968	0.004019	0.003999	0.003915	0.00387	0.003935	0.004011	0.003976	0.003871	0.003953	Histidine m	Amino acid	Metabolism
ko00350	0.005168	0.005518	0.005606	0.004873	0.005408	0.005526	0.005628	0.005662	0.005416	0.005555	0.005613	0.005688	0.005673	0.005553	0.005483	0.005641	0.005715	0.005652	0.005682	0.005626	Tyrosine m	Amino acid	Metabolism
ko00360	0.006875	0.007531	0.007726	0.006342	0.007314	0.007527	0.007724	0.00779	0.007356	0.007583	0.007671	0.00777	0.007829	0.007659	0.007421	0.007709	0.007725	0.007778	0.007754	0.0077	Phenylalan	Amino acid	Metabolism
ko00361	0.001451	0.001591	0.00163	0.001317	0.001547	0.001616	0.001651	0.001647	0.001543	0.001585	0.001647	0.001639	0.001695	0.001634	0.00157	0.001655	0.001645	0.001628	0.001698	0.001632	Chlorocyclo	Xenobiotics	Metabolism
ko00362	0.007904	0.00884	0.00914	0.007185	0.008575	0.008917	0.00917	0.009218	0.008686	0.008932	0.009086	0.009256	0.009288	0.009143	0.00886	0.009185	0.009209	0.009268	0.009409	0.009204	Benzoate d	Xenobiotics	Metabolism
ko00363	0.000126	0.000147	0.000156	0.000109	0.000141	0.000149	0.000154	0.000158	0.000149	0.000156	0.000151	0.00016	0.000156	0.000159	0.000151	0.000154	0.000155	0.000166	0.000155	0.000161	Bisphenol c	Xenobiotics	Metabolism
ko00364	0.000699	0.000782	0.000811	0.0006	0.000754	0.000823	0.000825	0.000818	0.0008	0.000795	0.000803	0.000821	0.000822	0.000813	0.00078	0.00084	0.000831	0.000828	0.000888	0.000818	Fluorobenz	Xenobiotics	Metabolism
ko00365	0.00032	0.000354	0.000377	0.000268	0.000343	0.000403	0.000391	0.000379	0.000377	0.000354	0.000385	0.000374	0.000399	0.000374	0.000367	0.000416	0.000397	0.000375	0.000448	0.00037	Furfural deg	Xenobiotics	Metabolism
ko00380	0.005201	0.005806	0.006094	0.004716	0.005631	0.005927	0.006073	0.006117	0.005943	0.005996	0.005927	0.006113	0.006011	0.006069	0.005868	0.006123	0.006134	0.006189	0.006342	0.006181	Tryptophan	Amino acid	Metabolism
ko00400	0.004248	0.004094	0.003975	0.004279	0.004114	0.004059	0.003963	0.003987	0.00404	0.00405	0.004027	0.004001	0.004011	0.003961	0.003989	0.003945	0.003972	0.003969	0.003818	0.003932	Phenylalan	Amino acid	Metabolism
ko00401	0.001179	0.001183	0.001169	0.001151	0.001173	0.001176	0.001163	0.001173	0.001161	0.001178	0.001171	0.001173	0.001182	0.001151	0.001161	0.001169	0.001156	0.001175	0.001122	0.00115	Novobiocin	Biosynthesi	Metabolism
ko00404	1.21E-05	1.77E-05	2.08E-05	8.95E-06	1.57E-05	1.65E-05	2.48E-05	2.01E-05	1.86E-05	1.98E-05	1.64E-05	1.83E-05	1.28E-05	2.30E-05	1.52E-05	1.96E-05	2.04E-05	1.81E-05	2.78E-05	1.90E-05	Staurospori	Biosynthesi	Metabolism
ko00405	0.00051	0.000499	0.000501	0.000487	0.000501	0.000524	0.000508	0.0005	0.000528	0.000507	0.000503	0.000503	0.000495	0.000503	0.000511	0.000514	0.000515	0.000508	0.000533	0.000511	Phenazine	Biosynthesi	Metabolism
ko00410	0.005005	0.005632	0.005807	0.004479	0.005403	0.005634	0.005797	0.005862	0.005635	0.005747	0.00569	0.005852	0.005783	0.005758	0.005566	0.005776	0.005824	0.005872	0.005845	0.005865	beta-Alanin	Metabolism	Metabolism
ko00430	0.001085	0.001042	0.001036	0.001154	0.001057	0.00103	0.001028	0.001033	0.00105	0.001032	0.001033	0.001024	0.00101	0.001042	0.001044	0.00103	0.001026	0.001025	0.001032	0.001037	Taurine anc	Metabolism	Metabolism
ko00440	0.000754	0.000773	0.000782	0.000746	0.000774	0.000778	0.000783	0.000782	0.000745	0.000753	0.000792	0.000775	0.000805	0.000775	0.000763	0.000793	0.000778	0.00077	0.000792	0.000759	Phosphona	Metabolism	Metabolism
ko00450	0.002387	0.002209	0.002212	0.002468	0.002262	0.002295	0.002223	0.002189	0.002389	0.00223	0.002198	0.002196	0.002145	0.00224	0.002267	0.002275	0.002245	0.002252	0.002367	0.002247	Selenocom	Metabolism	Metabolism
ko00460	0.000734	0.000633	0.000624	0.000833	0.000674	0.00063	0.00062	0.000611	0.000673	0.000631	0.000617	0.000601	0.000572	0.000633	0.000602	0.000611	0.000613	0.000603	0.000627	0.000625	Cyanoamin	Metabolism	Metabolism
ko00471	0.000617	0.000566	0.000564	0.000677	0.000586	0.000568	0.000555	0.000554	0.000598	0.000569	0.000557	0.000558	0.000534	0.000569	0.000566	0.000554	0.000559	0.000567	0.000561	0.000569	D-Glutamin	Metabolism	Metabolism
ko00472	0.000134	0.000127	0.000127	0.000134	0.000126	0.000126	0.000127	0.000125	0.000132	0.000132	0.000123	0.00013	0.000121	0.000127	0.000117	0.000126	0.00013	0.000126	0.000122	0.000124	D-Arginine	Metabolism	Metabolism
ko00473	0.00055	0.00048	0.00047	0.000641	0.00051	0.000467	0.000461	0.000461	0.000488	0.000468	0.000474	0.000466	0.000449	0.000477	0.000465	0.000463	0.000472	0.000471	0.000465	0.000474	D-Alanine r	Metabolism	Metabolism
ko00480	0.003442	0.003552	0.003533	0.003193	0.003478	0.00359	0.00356	0.003557	0.003486	0.003555	0.003537	0.003519	0.00358	0.003505	0.003479	0.003588	0.003504	0.003513	0.003552	0.003514	Glutathione	Metabolism	Metabolism
ko00500	0.006284	0.004366	0.004051	0.008758	0.005366	0.003974	0.00393	0.003879	0.004282	0.004052	0.00432	0.003934	0.003765	0.004357	0.004204	0.003824	0.004005	0.003855	0.003839	0.003991	Starch and	Carbohydra	Metabolism
ko00510	0.000109	8.52E-05	8.21E-05	8.86E-05	8.63E-05	0.000104	8.29E-05	7.89E-05	0.000117	9.14E-05	7.49E-05	7.41E-05	6.58E-05	8.03E-05	6.89E-05	8.96E-05	8.47E-05	7.89E-05	9.92E-05	8.95E-05	N-Glycan b	Glycan bios	Metabolism
ko00511	0.00025	0.000168	0.000152	0.000349	0.000199	0.000152	0.000164	0.000152	0.000177	0.000173	0.000168	0.000162	0.000136	0.000162	0.000188	0.000146	0.000176	0.000154	0.000144	0.000165	Other glyca	Glycan bios	Metabolism
ko00513	1.56E-05	1.01E-05	1.01E-05	2.36E-05	1.26E-05	7.08E-06	1.15E-05	1.16E-05	9.98E-06	1.33E-05	9.28E-06	1.11E-05	4.23E-06	1.04E-05	2.73E-05	8.88E-06	1.35E-05	1.16E-05	1.01E-05	1.35E-05	Various typ	Glycan bios	Metabolism
ko00514	1.21E-05	1.65E-05	2.25E-05	1.02E-05	1.48E-05	1.56E-05	2.09E-05	1.94E-05	2.18E-05	2.07E-05	1.61E-05	1.90E-05	1.25E-05	2.14E-05	1.78E-05	1.84E-05	2.31E-05	2.03E-05	2.60E-05	2.34E-05	Other types	Glycan bios	Metabolism
ko00515	5.16E-06	7.23E-06	9.86E-06	5.32E-06	6.57E-06	6.16E-06	9.63E-06	9.04E-06	1.04E-05	1.02E-05	7.68E-06	9.52E-06	5.89E-06	1.01E-05	8.25E-06	8.00E-06	1.23E-05	9.57E-06	1.14E-05	1.26E-05	Mannose ty	Glycan bios	Metabolism
ko00520	0.008282	0.00682	0.006471	0.009885	0.007451	0.006581	0.00641	0.006318	0.006916	0.006522	0.006623	0.006367	0.006216	0.006623	0.00704	0.00623	0.006434	0.006309	0.006237	0.006338	Amino suga	Carbohydra	Metabolism
ko00521	0.001617	0.001435	0.001426	0.001721	0.001497	0.001438	0.001416	0.001414	0.001508	0.001455	0.001425	0.001413	0.001335	0.001432	0.001404	0.001412	0.001455	0.001425	0.001446	0.001478	Streptomyc	Biosynthesi	Metabolism
ko00522	1.75E-06	2.21E-06	2.20E-06	1.65E-06	2.48E-06	2.11E-06	3.27E-06	2.18E-06	2.54E-06	3.86E-06	1.66E-06	3.84E-06	1.41E-06	2.53E-06	1.84E-06	3.25E-06	2.72E-06	2.45E-06	1.93E-06	2.08E-06	Biosynthesi	Metabolism	Metabolism
ko00523	0.000961	0.000918	0.000901	0.000944	0.000924	0.000925	0.000903	0.000903	0.00091	0.000908	0.000909	0.000897	0.000894	0.000896	0.000865	0.000898	0.000903	0.000894	0.000902	0.000895	Polyketide s	Metabolism	Metabolism
ko00524	0.000304	0.000238	0.00024	0.000364	0.000271	0.000224	0.000238	0.000242	0.000272	0.000257	0.000245	0.000244	0.000193	0.000247	0.000228	0.000225	0.000267	0.00025	0.00024	0.000284	Neomycin,	Biosynthesi	Metabolism
ko00525	0.000382	0.000347	0.000339	0.000381	0.000353	0.000355	0.000337	0.000333	0.00036	0.000342	0.000336	0.000333	0.000327	0.00034	0.000332	0.000335	0.000335	0.000337	0.000347	0.000337	Acarbose a	Biosynthesi	Metabolism
ko00531	0.0002	0.000185	0.000186	0.000216	0.000192	0.000185	0.000192	0.00019	0.000191	0.000197	0.000186	0.000193	0.000172	0.000188	0.000199	0.000185	0.000202	0.000191	0.000192	0.0002	Glycosamin	Glycan bios	Metabolism
ko00532	1.93E-07	3.74E-07	5.00E-07	2.58E-07	4.52E-07	8.20E-08	6.66E-07	3.43E-07	5.42E-07	6.64E-08	2.84E-07	3.53E-07	1.45E-07	5.73E-07	1.71E-07	2.92E-07	9.21E-08	9.23E-08	5.99E-07	6.11E-08	Glycosamin	Glycan bios	Metabolism
ko00534	2.95E-07	4.29E-07	5.00E-07	3.33E-07	5.03E-07	1.66E-07	6.66E-07	3.43E-07	6.96E-07	6.64E-08	2.84E-07	3.53E-07	1.71E-07	6.91E-07	1.71E-07	2.92E-07	9.21E-08	9.23E-08	5.99E-07	6.11E-08	Glycosamin	Glycan bios	Metabolism
ko00540	0.002626	0.002884	0.002812	0.002266	0.002773	0.002884	0.002854	0.002883	0.002679	0.002864	0.002884	0.002886	0.002973	0.002789	0.002904	0.002862	0.002807	0.002836	0.002717	0.002795	Lipopolysa	Glycan bios	Metabolism
ko00550	0.003563	0.002962	0.002824	0.004267	0.00319	0.002879	0.002772	0.002761	0.002999	0.002851	0.002885	0.002776	0.00274	0.002896	0.00281	0.002769	0.002781	0.002761	0.00272	0.002774	Peptidoglyc	Glycan bios	Metabolism
ko00561	0.002818	0.002526	0.002481	0.003163	0.002653	0.00246	0.002444	0.002463	0.002574	0.002503	0.002486	0.00249	0.002418	0.002522	0.002451	0.002409	0.002497	0.002496	0.002416	0.002486	Glycerolipic	Lipid metab	Metabolism
ko00562	0.001526	0.00147	0.001469	0.001625	0.00151	0.00144	0.001458	0.001479	0.001455	0.00147	0.001471	0.001475	0.00144	0.001465	0.001411	0.001454	0.001482	0.001476	0.001445	0.00147	Inositol pho	Carbohydra	Metabolism
ko00563	5.07E-09	1.17E-08	0	0	1.99E-08	0	2.01E-08	3.54E-08	2.37E-08	0	5.58E-09	0	0	5.62E-08	0	3.00E-08	1.44E-08	1.32E-08	0	0	Glycosylph	Glycan bios	Metabolism
ko00564	0.00332	0.003231	0.003171	0.003512	0.003275	0.003151	0.003172	0.003189	0.003125	0.003188	0.003226	0.003203	0.003217	0.00319	0.003265	0.003154	0.003183	0.003186	0.003093	0.003167	Glycerophо	Lipid metab	Metabolism
ko00565	0.000376	0.000423	0.000421	0.000321	0.000404	0.000412	0.000425	0.000435	0.00039	0.00042	0.000427	0.000434	0.000439	0.000412	0.000407	0.000413	0.000426	0.000429	0.0004	0.000417	Ether lipid r	Lipid metab	Metabolism
ko00571	0.000108	0.000118	0.000142	9.24E-05	0.000117	0.000133	0.000138	0.000139	0.000168	0.000149	0.000119	0.000144	0.000115	0.000144	0.000132	0.000138	0.000152	0.000153	0.000157	0.000158	Lipoarabinc	Glycan bios	Metabolism
ko00590	0.00029	0.00029	0.000282	0.000286	0.00029	0.000285	0.000286	0.000284	0.00027	0.000283	0.000293	0.000287	0.000295	0.000284	0.000279	0.00028	0.00028	0.000283	0.00027	0.000276	Arachidonic	Lipid metab	Metabolism
ko00591	6.84E-05	8.03E-05	8.43E-05	5.85E-05	7.79E-05	7.93E-05	8.49E-05	8.62E-05	8.09E-05	8.64E-05	8.38E-05	8.94E-05	8.02E-05	8.67E-05	8.95E-05	8.24E-05	8.68E-05	9.26E-05	8.46E-05	9.21E-05	Linoleic aci	Lipid metab	Metabolism
ko00592	0.000669	0.000749	0.000775	0.000612	0.000726	0.000738	0.00077	0.000785	0.000742	0.000771	0.000762	0.000795	0.000769	0.000775	0.00077	0.000756	0.000775	0.000808	0.000761	0.000793	alpha-Linol	Lipid metab	Metabolism
ko00600	0.000215	0.000175	0.000187	0.000266	0.000192	0.000173	0.00019	0.000177	0.000205	0.000184	0.00018	0.000177	0.00016	0.000191	0.000237	0.000177	0.000189	0.000184	0.000196	0.000196	Sphingolipi	Lipid metab	Metabolism
ko00601	0	0	0	0	0	0	0	0	0	0	3.91E-07	0	0	0	0	0	0	0	0	0	Glycosphin	Glycan bios	Metabolism
ko00603	4.89E-05	2.46E-05	2.22E-05	8.32E-05	3.70E-05	1.38E-05	2.43E-05	2.16E-05	2.24E-05	2.48E-05	2.39E-05	2.07E-05	9.76E-06	2.49E-05	4.55E-05	1.61E-05	2.56E-05	2.19E-05	1.89E-05	2.72E-05	Glycosphin	Glycan bios	Metabolism
ko00604	1.56E-05	1.00E-05	9.91E-06	2.36E-05	1.24E-05	7.06E-06	1.13E-05	1.16E-05	9.91E-06	1.31E-05	9.26E-06	1.10E-05	4.21E-06	1.02E-05	2.72E-05	8.86E-06	1.32E-05	1.16E-05	1.01E-05	1.34E-05	Glycosphin	Glycan bios	Metabolism
ko00620	0.011173	0.010894	0.010926	0.011868	0.011116	0.010723	0.010805	0.010854	0.010773	0.010802	0.010916	0.01089	0.010836	0.010977	0.010857	0.010794	0.01085	0.010853	0.010817	0.010878	Pyruvate m	Carbohydra	Metabolism
ko00621	0.000751	0.000817	0.000834	0.000686	0.000801	0.000852	0.000853	0.000846	0.0008	0.000804	0.000858	0.000856	0.000883	0.000844	0.000815	0.000871	0.000867	0.000846	0.000904	0.000838	Dioxin degr	Xenobiotics	Metabolism
ko00622	0.001621	0.001767	0.001795	0.001508	0.001729	0.001767	0.001813	0.001826	0.001705	0.00176	0.001819	0.001848	0.001855	0.001795	0.001741	0.001803	0.001839	0.001825	0.001826	0.001802	Xylene deg	Xenobiotics	Metabolism
ko00623	0.001197	0.00133	0.001333	0.00105	0.001276	0.001336	0.001353	0.001362	0.001257	0.001314	0.001367	0.001376	0.001417	0.001329	0.001311	0.001342	0.001366	0.001342	0.001344	0.001333	Toluene de	Xenobiotics	Metabolism
ko00624	0.000537	0.000594	0.000646	0.000451	0.000595	0.000709	0.000681	0.000641	0.000645	0.000602	0.000666	0.000664	0.000686	0.000667	0.000652	0.000737	0.000719	0.000656	0.000853	0.000657	Polycyclic a	Xenobiotics	Metabolism
ko00625	0.002779	0.002918	0.002994	0.002707	0.002886	0.002932	0.002997	0.003011	0.002954	0.002969	0.002961	0.002999	0.002958	0.002971	0.0029	0.00301	0.003023	0.003009	0.003044	0.003024	Chloroalkar	Xenobiotics	Metabolism
ko00626	0.001313	0.001313	0.001353	0.001365	0.001339	0.001359	0.001367	0.001346	0.001362	0.001334	0.001359	0.001362	0.001335	0.001368	0.001362	0.00141	0.001403	0.001361	0.001476	0.001396	Naphthalen	Xenobiotics	Metabolism
ko00627	0.003635	0.003979	0.004138	0.003314	0.003889	0.004116	0.004177	0.004167	0.004002	0.004039	0.004124	0.004163	0.004217	0.004145	0.004002	0.004238	0.004211	0.004182	0.004417	0.004167	Aminobenz	Xenobiotics	Metabolism
ko00630	0.009806	0.010377	0.010538	0.0092	0.010143	0.010497	0.010524	0.01055	0.010383	0.010506	0.01043	0.010525	0.010561	0.010426	0.01029	0.010623	0.010524	0.010526	0.01058	0.010551	Glyoxylate a	Carbohydra	Metabolism
ko00633	0.000536	0.000565	0.000571	0.000557	0.000566	0.00055	0.000572	0.000581	0.000557	0.000572	0.000568	0.000574	0.000567	0.000577	0.000598	0.000566	0.000565	0.000577	0.000567	0.00058	Nitrotoluene	Xenobiotics	Metabolism
ko00640	0.009431	0.00978	0.010004	0.009437	0.009737	0.00975	0.009918	0.009961	0.00982	0.009854	0.009871	0.009964	0.009894	0.010036	0.00975	0.009952	0.009953	0.010008	0.010116	0.010078	Propanoate	Carbohydra	Metabolism
ko00642	0.000674	0.000753	0.000779	0.000616	0.000732	0.000746	0.000774	0.000788	0.000747	0.000777	0.000766	0.000799	0.000774	0.000778	0.000766	0.000764	0.00078	0.00081	0.00077	0.000796	Ethylbenze	Xenobiotics	Metabolism
ko00643	0.001361	0.001457	0.001544	0.001292	0.001444	0.001517	0.001539	0.001538	0.001535	0.001511	0.0015	0.00152	0.001508	0.001538	0.001459	0.001578	0.001548	0.001547	0.001643	0.001562	Styrene deg	Xenobiotics	Metabolism
ko00650	0.010199	0.010947	0.011235	0.009812	0.010766	0.01091	0.011205	0.011277	0.010862	0.011032	0.011123	0.011284	0.011205	0.011202	0.010947	0.011199	0.011234	0.011299	0.011348	0.011275	Butanoate r	Carbohydra	Metabolism
ko00660	0.001738	0.001774	0.001834	0.001633	0.001776	0.001917	0.001862	0.001814	0.00187	0.001797	0.001839	0.001835	0.00185	0.001843	0.001853	0.001955	0.00191	0.001835	0.002069	0.001849	C5-Branche	Carbohydra	Metabolism
ko00670	0.001969	0.001819	0.001765	0.002068	0.001857	0.001804	0.00175	0.00175	0.001817	0.001794	0.001778	0.001762	0.001769	0.001771	0.001816	0.001751	0.001762	0.001756	0.001713	0.001745	One carbon	Metabolism	Metabolism
ko00680	0.005166	0.005068	0.005111	0.005374	0.005077	0.005087	0.005075	0.005072	0.005167	0.005096	0.005045	0.005022	0.005014	0.005065	0.005051	0.005144	0.00504	0.005042	0.005085	0.005116	Methane m	Energy met	Metabolism
ko00710	0.002001	0.001744	0.001682	0.00222	0.001827	0.001728	0.001647	0.001644	0.001814	0.001717	0.001667	0.001621	0.00163	0.001694	0.00178	0.001657	0.001624	0.00163	0.001612	0.001654	Carbon fixa	Energy met	Metabolism
ko00720	0.006257	0.006242	0.00629	0.006377	0.006266	0.006254	0.006239	0.00624	0.00629	0.006254	0.006244	0.006277	0.006266	0.0063	0.00633	0.006267	0.00627	0.006272	0.006291	0.006288	Carbon fixa	Energy met	Metabolism
ko00730	0.001965	0.001813	0.001739	0.002046	0.001828	0.001796	0.001725	0.001717	0.001824	0.001764	0.001752	0.001732	0.001755	0.001735	0.00182	0.001729	0.001724	0.00172	0.00168	0.00171	Thiamine m	Metabolism	Metabolism
ko00740	0.001322	0.001107	0.001048	0.001528	0.001177	0.001066	0.001038	0.001026	0.001113	0.001059	0.001067	0.001043	0.001041	0.001071	0.001175	0.001032	0.00105	0.001043	0.001025	0.001037	Riboflavin r	Metabolism	Metabolism
ko00750	0.001055	0.001034	0.001024	0.001063	0.00104	0.001036	0.001034	0.001029	0.001043	0.00104	0.001036	0.001032	0.001028	0.001027	0.001056	0.00103	0.001046	0.001026	0.001029	0.00104	Vitamin B6	Metabolism	Metabolism
ko00760	0.003866	0.003831	0.003839	0.003875	0.003835	0.003878	0.003837	0.00383	0.003884	0.003814	0.003826	0.003823	0.003819	0.003836	0.003826	0.003854	0.003872	0.003814	0.00394	0.003812	Nicotinate a	Metabolism	Metabolism
ko00770	0.002705	0.002591	0.002577	0.002748	0.002626	0.002648	0.002579	0.002557	0.002647	0.002566	0.00259	0.002551	0.00257	0.002581	0.00259	0.002623	0.002593	0.002549	0.00265	0.002561	Pantothena	Metabolism	Metabolism
ko00780	0.005847	0.006257	0.00641	0.005382	0.006095	0.006309	0.006452	0.006463	0.006308	0.006387	0.006301	0.006416	0.006394	0.006362	0.006247	0.006413	0.006403	0.006455	0.006474	0.006457	Biotin meta	Metabolism	Metabolism
ko00785	0.000311	0.000266	0.000247	0.000312	0.000265	0.000271	0.000246	0.000244	0.000283	0.00026	0.000248	0.000246	0.000248	0.000248	0.00026	0.000246	0.00025	0.000247	0.000245	0.000248	Lipoic acid	Metabolism	Metabolism

ko00790	0.003781	0.00372	0.00361	0.003539	0.003657	0.003771	0.003643	0.003632	0.00376	0.003725	0.003643	0.003656	0.003666	0.003593	0.003701	0.003633	0.003636	0.003627	0.003541	0.003618	Folate biosy	Metabolism	Metabolism
ko00791	0.000795	0.000874	0.000901	0.000688	0.000835	0.000894	0.000898	0.000901	0.000888	0.000891	0.000878	0.00089	0.000906	0.000879	0.000887	0.000895	0.000888	0.000903	0.000904	0.000897	Atrazine de	Xenobiotics	Metabolism
ko00830	0.00087	0.000904	0.000931	0.000833	0.000889	0.000921	0.000938	0.000933	0.000934	0.000934	0.000919	0.000938	0.000908	0.000924	0.000917	0.000953	0.000962	0.000939	0.000978	0.000971	Retinol met	Metabolism	Metabolism
ko00860	0.005392	0.005177	0.005039	0.004801	0.005033	0.005435	0.005058	0.005006	0.005578	0.005255	0.004949	0.005013	0.005037	0.004921	0.004937	0.005025	0.005035	0.004977	0.004931	0.004948	Porphyrin a	Metabolism	Metabolism
ko00900	0.00281	0.002608	0.002584	0.002979	0.002678	0.002599	0.00254	0.002544	0.002674	0.002602	0.002573	0.002566	0.002559	0.002611	0.002652	0.002552	0.002568	0.002582	0.002553	0.00259	Terpenoid b	Metabolism	Metabolism
ko00901	6.28E-06	8.29E-06	1.01E-05	5.19E-06	7.15E-06	8.48E-06	1.05E-05	1.02E-05	1.08E-05	8.98E-06	8.38E-06	8.64E-06	6.74E-06	1.01E-05	8.06E-06	1.07E-05	1.22E-05	9.60E-06	1.44E-05	1.17E-05	Indole alkal	Biosynthesi	Metabolism
ko00902	3.41E-06	6.38E-06	8.98E-06	4.21E-06	5.12E-06	4.78E-06	8.04E-06	7.65E-06	7.77E-06	7.09E-06	5.83E-06	5.48E-06	6.06E-06	9.50E-06	6.80E-06	6.75E-06	6.20E-06	6.42E-06	1.02E-05	9.88E-06	Monoterpen	Metabolism	Metabolism
ko00903	0.002504	0.002835	0.002947	0.002246	0.002728	0.002831	0.002933	0.002981	0.002833	0.002914	0.002876	0.002982	0.002936	0.002934	0.002835	0.002919	0.002957	0.002996	0.002972	0.002988	Limonene a	Metabolism	Metabolism
ko00906	0.000239	0.000176	0.000163	0.000205	0.000182	0.000215	0.000159	0.000149	0.000252	0.000187	0.000151	0.000145	0.00014	0.000155	0.000138	0.000167	0.000152	0.000149	0.000166	0.000156	Carotenoid	Metabolism	Metabolism
ko00908	0.000143	0.000124	0.000119	0.000158	0.00013	0.000122	0.000117	0.000116	0.000124	0.000121	0.000121	0.000117	0.00012	0.00012	0.000126	0.000117	0.000117	0.000117	0.000114	0.000117	Zeatin bios	Metabolism	Metabolism
ko00909	9.45E-06	1.06E-05	1.29E-05	7.97E-06	8.93E-06	1.52E-05	1.29E-05	1.32E-05	1.90E-05	1.27E-05	9.81E-06	9.57E-06	8.05E-06	1.13E-05	9.70E-06	1.67E-05	1.13E-05	1.15E-05	1.81E-05	1.37E-05	Sesquiterpe	Metabolism	Metabolism
ko00910	0.004329	0.004569	0.004544	0.004034	0.004472	0.00459	0.004573	0.004586	0.00445	0.004584	0.004584	0.004603	0.004658	0.004544	0.004494	0.004569	0.004554	0.004578	0.004499	0.004531	Nitrogen m	Energy met	Metabolism
ko00920	0.006896	0.00722	0.007354	0.006381	0.007107	0.007501	0.007429	0.007368	0.007392	0.007319	0.007332	0.007365	0.00741	0.007333	0.007236	0.00758	0.007426	0.007426	0.007715	0.00744	Sulfur meta	Energy met	Metabolism
ko00930	0.002716	0.00309	0.003205	0.00241	0.002967	0.003103	0.0032	0.003237	0.003051	0.00316	0.00316	0.003239	0.003253	0.003196	0.003088	0.003198	0.003198	0.003255	0.003227	0.003244	Caprolacta	Xenobiotics	Metabolism
ko00940	0.000372	0.000309	0.000302	0.000444	0.000338	0.00029	0.000299	0.000299	0.000318	0.000306	0.000299	0.000295	0.000263	0.000308	0.00028	0.000278	0.000299	0.000294	0.000287	0.000301	Phenylprop	Biosynthesi	Metabolism
ko00941	6.82E-06	3.33E-06	1.05E-06	1.35E-05	4.20E-06	2.87E-06	1.49E-06	7.88E-07	6.33E-06	1.18E-06	2.13E-06	7.09E-07	4.27E-07	2.31E-06	8.18E-07	1.05E-06	7.22E-07	8.35E-07	8.87E-07	3.05E-07	Flavonoid b	Biosynthesi	Metabolism
ko00943	3.06E-06	4.27E-06	6.09E-06	2.39E-06	3.97E-06	3.94E-06	5.37E-06	5.02E-06	6.03E-06	6.02E-06	3.93E-06	5.23E-06	2.93E-06	6.07E-06	5.06E-06	4.39E-06	5.30E-06	6.54E-06	6.11E-06	6.76E-06	Isoflavonoi	Biosynthesi	Metabolism
ko00944	8.56E-06	4.50E-06	3.93E-06	1.53E-05	4.93E-06	2.92E-06	4.71E-06	4.57E-06	4.29E-06	5.85E-06	4.17E-06	5.12E-06	2.65E-06	4.57E-06	3.02E-06	3.46E-06	6.62E-06	4.77E-06	3.98E-06	5.94E-06	Flavone an	Biosynthesi	Metabolism
ko00945	6.82E-06	3.33E-06	1.05E-06	1.35E-05	4.20E-06	2.87E-06	1.49E-06	7.88E-07	6.33E-06	1.18E-06	2.13E-06	7.09E-07	4.27E-07	2.31E-06	8.18E-07	1.05E-06	7.22E-07	8.35E-07	8.87E-07	3.05E-07	Stilbenoid,	Biosynthesi	Metabolism
ko00950	0.000834	0.000857	0.00085	0.000812	0.000846	0.000841	0.000844	0.000855	0.000816	0.000846	0.000854	0.000852	0.000867	0.000837	0.000849	0.000844	0.000841	0.000849	0.000815	0.000834	Isoquinolin	Biosynthesi	Metabolism
ko00960	0.000995	0.000991	0.000979	0.000976	0.00098	0.000983	0.000974	0.000984	0.000984	0.000987	0.000973	0.000975	0.000976	0.000963	0.00097	0.000972	0.000966	0.000981	0.000946	0.00096	Tropane, pi	Biosynthesi	Metabolism
ko00965	0.000157	0.000181	0.000181	0.000135	0.000174	0.000179	0.000185	0.000186	0.000167	0.000183	0.000184	0.000187	0.000188	0.00018	0.000174	0.000182	0.000185	0.000184	0.000178	0.000181	Betalain bio	Biosynthesi	Metabolism
ko00966	0.000189	0.00019	0.000187	0.000177	0.000186	0.000191	0.000188	0.000187	0.000185	0.000187	0.00019	0.00019	0.000191	0.000186	0.000185	0.000189	0.000192	0.000186	0.000188	0.000185	Glucosinola	Biosynthesi	Metabolism
ko00970	0.004225	0.003709	0.003589	0.004681	0.003876	0.003675	0.003541	0.003518	0.003753	0.003626	0.00362	0.003538	0.003597	0.003638	0.003748	0.003563	0.003557	0.003536	0.003521	0.003545	Aminoacyl-	Translation	Genetic Info
ko00980	0.001888	0.002057	0.002117	0.001698	0.001983	0.002108	0.002147	0.002127	0.002072	0.002105	0.002083	0.002093	0.002101	0.002097	0.002043	0.00218	0.002123	0.002101	0.002228	0.002151	Metabolism	Xenobiotics	Metabolism
ko00981	0.000943	0.001046	0.001061	0.000858	0.00101	0.001026	0.001058	0.001081	0.001032	0.001063	0.001043	0.00108	0.001054	0.001044	0.001007	0.00104	0.001075	0.001074	0.001035	0.001061	Insect horm	Metabolism	Metabolism
ko00982	0.002163	0.002356	0.00241	0.001932	0.002275	0.002415	0.00244	0.002424	0.002345	0.002396	0.002393	0.002406	0.002428	0.002387	0.00234	0.002478	0.002431	0.0024	0.002513	0.002437	Drug metab	Xenobiotics	Metabolism
ko00983	0.002384	0.002333	0.002334	0.002394	0.002348	0.002381	0.00235	0.00232	0.002354	0.002333	0.00234	0.002306	0.002342	0.002339	0.002349	0.002381	0.002331	0.00231	0.0024	0.002316	Drug metab	Xenobiotics	Metabolism
ko00984	0.000386	0.000448	0.000547	0.000343	0.000447	0.000505	0.000526	0.000529	0.00058	0.000534	0.000469	0.000555	0.000464	0.000564	0.000524	0.000551	0.000562	0.000606	0.000652	0.000597	Steroid deg	Xenobiotics	Metabolism
ko01040	0.004151	0.00458	0.004798	0.003792	0.004436	0.004615	0.004818	0.004839	0.004684	0.004747	0.00465	0.004798	0.004676	0.004771	0.004602	0.004783	0.004797	0.004869	0.004929	0.004886	Biosynthesi	Lipid metab	Metabolism
ko01051	0.000246	0.000222	0.000225	0.000269	0.000235	0.000222	0.000222	0.000221	0.000236	0.000225	0.000219	0.000216	0.000211	0.000224	0.000232	0.000221	0.000219	0.00022	0.000215	0.000222	Biosynthesi	Metabolism	Metabolism
ko01052	0.000744	0.000829	0.000827	0.00064	0.000803	0.000851	0.000858	0.000855	0.000804	0.000855	0.000852	0.00088	0.000891	0.00082	0.000808	0.000861	0.000855	0.000856	0.000806	0.000828	Type I polyk	Metabolism	Metabolism
ko01053	0.002071	0.002248	0.002377	0.001886	0.002221	0.002416	0.00238	0.002401	0.002493	0.002412	0.002306	0.002449	0.00234	0.002345	0.0023	0.002426	0.002429	0.002508	0.002467	0.002451	Biosynthesi	Metabolism	Metabolism
ko01054	0.002845	0.003125	0.003164	0.002536	0.003001	0.003314	0.003208	0.00322	0.003338	0.003194	0.003151	0.003252	0.003253	0.003078	0.003078	0.003249	0.003201	0.003273	0.003196	0.003184	Nonribosom	Metabolism	Metabolism
ko01055	0.000408	0.000382	0.000408	0.000376	0.000384	0.000443	0.000412	0.000399	0.000481	0.000415	0.000387	0.000402	0.000372	0.000405	0.0004	0.000437	0.000416	0.000426	0.000474	0.000427	Biosynthesi	Metabolism	Metabolism
ko01056	0.000104	0.000111	0.00011	0.000104	0.00011	0.000106	0.000112	0.000115	9.97E-05	0.000109	0.000112	0.000112	0.000115	0.00011	0.000103	0.000108	0.000109	0.000111	0.000107	0.000107	Biosynthesi	Metabolism	Metabolism
ko01057	7.32E-05	8.39E-05	0.000104	6.12E-05	8.27E-05	0.000107	0.000111	0.000101	0.000117	9.58E-05	9.29E-05	9.97E-05	8.83E-05	0.000104	9.63E-05	0.00012	0.000117	0.000106	0.000153	0.000111	Biosynthesi	Metabolism	Metabolism
ko01059	0.000229	0.000261	0.000282	0.000194	0.000247	0.000273	0.000286	0.000283	0.000292	0.000284	0.000264	0.000277	0.000263	0.000276	0.000266	0.000285	0.000282	0.000284	0.000292	0.000289	Biosynthesi	Metabolism	Metabolism
ko01062	0.000116	0.000117	0.000116	0.000104	0.000116	0.00012	0.000115	0.000116	0.000119	0.000119	0.000116	0.000115	0.000115	0.000114	0.000107	0.000118	0.000114	0.000114	0.000113	0.000117	Biosynthesi	Chemical s	Metabolism
ko01100	0.134106	0.131097	0.13015	0.134251	0.131519	0.131791	0.130003	0.129843	0.13234	0.130861	0.13019	0.130055	0.130132	0.130022	0.130052	0.130044	0.13035	0.129788	0.129792	0.129846	Metabolic p	Global and	Metabolism
ko01110	0.053146	0.051519	0.050942	0.053477	0.051746	0.051722	0.050841	0.050793	0.052298	0.05146	0.050997	0.050936	0.050789	0.05096	0.051314	0.050862	0.051112	0.050853	0.050699	0.050975	Biosynthesi	Global and	Metabolism
ko01120	0.056859	0.058647	0.059559	0.05567	0.058238	0.059219	0.059626	0.059667	0.058782	0.058995	0.059371	0.059724	0.059713	0.059463	0.059018	0.059978	0.060004	0.0597	0.060745	0.059808	Microbial m	Global and	Metabolism
ko01130	0.046041	0.045724	0.045776	0.046049	0.045773	0.045756	0.045689	0.045751	0.046102	0.045932	0.04561	0.045804	0.045492	0.045743	0.045441	0.045653	0.045897	0.045843	0.04574	0.045928	Biosynthesi	Global and	Metabolism
ko01200	0.023258	0.023336	0.023453	0.023543	0.023363	0.023251	0.023341	0.023389	0.023389	0.023413	0.023338	0.023387	0.023321	0.023426	0.023337	0.023355	0.023357	0.023367	0.023253	0.02349	Carbon me	Global and	Metabolism
ko01210	0.005531	0.005609	0.005543	0.005316	0.005536	0.005631	0.005568	0.00558	0.0055	0.005563	0.005613	0.005587	0.005686	0.00551	0.005578	0.005591	0.005593	0.005542	0.005518	0.005518	2-Oxocarbo	Global and	Metabolism
ko01212	0.012396	0.01341	0.01389	0.011626	0.013106	0.013495	0.013836	0.013909	0.013565	0.013707	0.013571	0.013882	0.013742	0.013861	0.013456	0.013813	0.01378	0.014025	0.014085	0.014029	Fatty acid m	Global and	Metabolism
ko01220	0.006998	0.007605	0.007813	0.006515	0.007455	0.007739	0.007896	0.007884	0.007478	0.007634	0.007877	0.007936	0.00804	0.00783	0.007625	0.00795	0.007989	0.007878	0.008139	0.007866	Degradatio	Global and	Metabolism
ko01230	0.021455	0.020424	0.019951	0.022014	0.020621	0.020361	0.01992	0.019883	0.020361	0.02015	0.020184	0.019961	0.020057	0.019983	0.020096	0.019964	0.020005	0.019844	0.019701	0.019819	Biosynthesi	Global and	Metabolism
ko01501	0.005528	0.005645	0.005595	0.005542	0.005592	0.005574	0.005625	0.005643	0.005481	0.005573	0.00564	0.005591	0.005658	0.005556	0.005625	0.005637	0.005559	0.005596	0.005531	0.005556	beta-Lactar	Drug resista	Human Dis
ko01502	0.001066	0.000834	0.000768	0.001287	0.000907	0.000807	0.000758	0.000741	0.000882	0.000781	0.000788	0.000744	0.000731	0.000788	0.00076	0.000738	0.000752	0.000741	0.00073	0.000745	Vancomyci	Drug resista	Human Dis
ko01503	0.003969	0.004011	0.003955	0.003843	0.003973	0.004029	0.003988	0.003983	0.003952	0.003984	0.003985	0.00396	0.004011	0.003928	0.004065	0.003979	0.003916	0.003955	0.003915	0.00391	Cationic an	Drug resista	Human Dis
ko01521	7.91E-07	0	2.06E-07	2.25E-06	3.83E-07	0	0	0	3.83E-08	0	2.23E-08	0	0	0	0	0	1.96E-07	0	0	0	EGFR tyros	Drug resista	Human Dis
ko01522	0	0	0	7.69E-08	0	0	0	0	0	0	0	0	0	1.48E-07	0	0	0	0	0	0	Endocrine r	Drug resista	Human Dis
ko01523	0.000681	0.000618	0.000602	0.000741	0.00064	0.00061	0.000598	0.000596	0.000616	0.000609	0.00061	0.000602	0.000599	0.000609	0.000621	0.000603	0.000605	0.000599	0.000594	0.000601	Antifolate re	Drug resista	Human Dis
ko01524	0.002174	0.002293	0.002289	0.00206	0.002251	0.002294	0.002303	0.002303	0.002157	0.002265	0.002324	0.002284	0.002372	0.002286	0.002218	0.00233	0.002258	0.002254	0.00229	0.002267	Platinum dr	Drug resista	Human Dis
ko02010	0.046034	0.043838	0.044267	0.049513	0.044966	0.043677	0.043908	0.043679	0.044699	0.043402	0.044026	0.043199	0.042973	0.044495	0.04349	0.044386	0.044106	0.043815	0.045467	0.044488	ABC transp	Membrane	Environmer
ko02020	0.049131	0.052075	0.051552	0.046767	0.050941	0.051508	0.052447	0.052375	0.050297	0.052191	0.052199	0.05226	0.052537	0.051491	0.05224	0.051735	0.051303	0.052168	0.050595	0.05138	Two-compo	Signal trans	Environmer
ko02024	0.02723	0.028255	0.028701	0.026742	0.027829	0.028388	0.028764	0.028655	0.02838	0.028249	0.028488	0.028229	0.028549	0.028583	0.028327	0.029025	0.028473	0.028545	0.029338	0.028932	Quorum se	Cellular cor	Cellular Pro
ko02025	0.008721	0.009791	0.009797	0.007641	0.009377	0.009733	0.010035	0.010026	0.009385	0.00999	0.009837	0.009962	0.009961	0.009768	0.010046	0.009881	0.009772	0.009977	0.009692	0.009829	Biofilm form	Cellular cor	Cellular Pro
ko02026	0.007093	0.007593	0.007497	0.006493	0.007384	0.007501	0.007627	0.007648	0.007247	0.007588	0.007586	0.007623	0.007711	0.007448	0.007635	0.007512	0.007474	0.007605	0.007297	0.007417	Biofilm form	Cellular cor	Cellular Pro
ko02030	0.008846	0.010038	0.009937	0.007726	0.009586	0.00987	0.010156	0.010224	0.009428	0.010109	0.010075	0.010193	0.010241	0.009805	0.010087	0.010004	0.009918	0.010161	0.009623	0.009843	Bacterial ch	Cell motility	Cellular Pro
ko02040	0.004568	0.005053	0.005002	0.004207	0.004924	0.004945	0.005058	0.005095	0.004747	0.005043	0.005089	0.005089	0.005149	0.004939	0.005164	0.004998	0.004981	0.005029	0.004779	0.004978	Flagellar as	Cell motility	Cellular Pro
ko02060	0.00625	0.003436	0.002902	0.010496	0.005005	0.002637	0.002612	0.002541	0.00292	0.002617	0.00334	0.002713	0.002663	0.003348	0.003899	0.002485	0.002688	0.002516	0.002381	0.002513	Phosphotra	Membrane	Environmer
ko03008	0.000275	0.000264	0.000258	0.000293	0.000268	0.000256	0.000257	0.00026	0.000249	0.000263	0.000261	0.000263	0.000264	0.000256	0.000275	0.000251	0.000261	0.000259	0.000243	0.000256	Ribosome b	Translation	Genetic Info
ko03010	0.007846	0.006761	0.006443	0.0088	0.007118	0.006624	0.00635	0.00632	0.006732	0.006551	0.006576	0.006394	0.006518	0.00655	0.006813	0.006361	0.006402	0.006347	0.006192	0.006348	Ribosome	Translation	Genetic Info
ko03013	0.000363	0.000296	0.000283	0.000394	0.000316	0.000319	0.000284	0.000273	0.000317	0.000284	0.000292	0.000278	0.000286	0.000288	0.000287	0.000295	0.000289	0.000276	0.000308	0.000275	RNA transp	Translation	Genetic Info
ko03015	1.58E-06	0	4.12E-07	4.49E-06	7.66E-07	0	0	0	8.76E-08	5.20E-08	4.46E-08	1.04E-07	0	1.02E-07	0	7.38E-08	3.92E-07	0	0	0	mRNA surv	Translation	Genetic Info
ko03018	0.003426	0.003261	0.003168	0.003556	0.003314	0.003212	0.003165	0.003169	0.003215	0.003219	0.003227	0.003194	0.003208	0.003192	0.0034	0.003137	0.003179	0.003167	0.003078	0.00316	RNA degra	Folding, so	Genetic Info
ko03020	0.000677	0.000537	0.000505	0.000795	0.000578	0.000526	0.000491	0.000484	0.000551	0.000513	0.000508	0.000489	0.000507	0.000513	0.000555	0.000491	0.000496	0.000488	0.000481	0.000489	RNA polym	Transcriptio	Genetic Info
ko03022	2.44E-05	1.59E-05	1.65E-05	4.00E-05	1.80E-05	1.18E-05	1.48E-05	1.52E-05	1.87E-05	1.71E-05	1.36E-05	1.63E-05	8.00E-06	1.71E-05	1.29E-05	1.22E-05	2.01E-05	1.73E-05	1.61E-05	1.85E-05	Basal trans	Transcriptio	Genetic Info
ko03030	0.002504	0.002208	0.002114	0.002793	0.002303	0.002154	0.002089	0.002085	0.002186	0.002147	0.002155	0.002102	0.002132	0.002149	0.002252	0.002096	0.002094	0.002089	0.002034	0.002098	DNA replica	Replication	Genetic Info
ko03040	5.50E-08	5.73E-09	0	1.11E-07	3.27E-08	7.55E-08	0	5.24E-09	1.71E-07	3.47E-08	1.24E-07	0	0	1.88E-08	4.36E-08	0	5.76E-08	0	0	0	Spliceosom	Transcriptio	Genetic Info
ko03050	1.56E-05	1.32E-05	1.83E-05	2.51E-05	1.44E-05	1.47E-05	1.75E-05	1.65E-05	2.53E-05	2.05E-05	1.31E-05	2.05E-05	9.75E-06	1.81E-05	1.53E-05	1.51E-05	2.58E-05	2.04E-05	2.03E-05	2.18E-05	Proteasome	Folding, so	Genetic Info
ko03060	0.002458	0.002269	0.002203	0.002618	0.002327	0.002249	0.002183	0.002174	0.002281	0.002237	0.002228	0.002195	0.002236	0.002228	0.002366	0.002193	0.002195	0.002188	0.002147	0.002201	Protein exp	Folding, so	Genetic Info
ko03070	0.00611	0.006424	0.00626	0.005765	0.006316	0.006281	0.006322	0.006392	0.005984	0.006381	0.006391	0.006402	0.006588	0.006219	0.006647	0.006254	0.006264	0.006341	0.005962	0.006213	Bacterial se	Membrane	Environmer
ko03320	0.002112	0.002356	0.002524	0.001929	0.00228	0.002433	0.002488	0.002493	0.002514	0.002467	0.002394	0.002499	0.00242	0.002532	0.002427	0.002507	0.0025	0.002559	0.002664	0.002586	PPAR signa	Endocrine s	Organismal
ko03410	0.002026	0.001892	0.001847	0.002128	0.001933	0.001876	0.001844	0.001841	0.001895	0.001883	0.001868	0.00185	0.001835	0.001863	0.001861	0.001848	0.001853	0.001845	0.001829	0.001857	Base excisi	Replication	Genetic Info
ko03420	0.001735	0.001595	0.001535	0.001868	0.001628	0.001546	0.001529	0.00153	0.00156	0.001561	0.001566	0.001551	0.001543	0.00155	0.001563	0.001507	0.001548	0.001535	0.001477	0.001533	Nucleotide	Replication	Genetic Info
ko03430	0.003178	0.002871	0.002739	0.003484	0.002972	0.002786	0.002722	0.00272	0.002772	0.002771	0.002825	0.002747	0.00282	0.002777	0.002912	0.002716	0.002715	0.002711	0.002607	0.002705	Mismatch re	Replication	Genetic Info
ko03440	0.003327	0.002928	0.002795	0.003651	0.00305	0.002879	0.002765	0.002761	0.002926	0.002858	0.002844	0.002781	0.002817	0.002837	0.003001	0.002768	0.002769	0.002767	0.00269	0.002765	Homologou	Replication	Genetic Info
ko03450	0.000151	0.000178	0.000196	0.000132	0.000171	0.000182	0.000199	0.000198	0.000196	0.000192	0.000185	0.00019	0.000183	0.000199	0.000182	0.000198	0.000198	0.000195	0.000217	0.000209	Non-homol	Replication	Genetic Info
ko03460	1.60E-06	0	4.12E-07	4.49E-06	7.73E-07	2.16E-08	0	0	1.02E-07	3.15E-08	4.46E-08	0	0	0	0	0	9.79E-07	0	0	0	Fanconi an	Replication	Genetic Info
ko04011	7.16E-05	7.74E-05	7.93E-05	7.30E-05	7.22E-05	7.31E-05	8.07E-05	8.12E-05	8.84E-05	8.26E-05	7.19E-05	7.65E-05	6.50E-05	7.92E-05	7.89E-05	7.83E-05	7.78E-05	8.12E-05	8.39E-05	8.45E-05	MAPK signa	Signal trans	Environmer
ko04013	0.000155	0.000155	0.000155	0.000169	0.00016	0.000154	0.000156	0.000155	0.000153	0.000155	0.000158	0.000157	0.00016	0.000159	0.000164	0.000158	0.000156	0.000156	0.000156	0.000159	MAPK signa	Signal trans	Environmer
ko04014	5.03E-06	6.94E-06	8.34E-06	4.27E-06	6.34E-06	5.95E-06	7.75E-06	8.31E-06	8.92E-06	8.10E-06	5.90E-06	7.50E-06	4.90E-06	7.71E-06	6.51E-06	6.91E-06	7.23E-06	8.84E-06	8.51E-06	8.00E-06	Ras signali	Signal trans	Environmer
ko04016	0.001053	0.001013	0.000977	0.00114	0.001033	0.000967	0.000969	0.000985	0.000929	0.00098	0.001016	0.000994	0.001015	0.000993	0.000986	0.000967	0.000971	0.000968	0.000921	0.000975	MAPK signa	Signal trans	Environmer
ko04020	1.42E-05	5.09E-06	1.48E-06	8.79E-06	5.25E-06	8.70E-06	1.40E-06	4.98E-07	1.14E-05	4.56E-06	1.11E-06	2.07E-07	4.57E-06	1.61E-06	6.86E-06	1.38E-06	6.78E-07	9.29E-07	8.81E-07	3.61E-07	Calcium sig	Signal trans	Environmer
ko04022	9.96E-06	4.83E-06	3.76E-06	1.73E-05	6.49E-06	2.39E-06	2.87E-06	2.60E-06	3.49E-06	3.64E-06	3.88E-06	3.24E-06	5.25E-06	5.95E-06	9.74E-06	3.06E-06	2.95E-06	4.83E-06	3.70E-06	3.58E-06	cGMP-PKG	Signal trans	Environmer
ko04024	1.86E-05	1.64E-05	1.81E-05	2.45E-05	1.73E-05	1.28E-05	1.63E-05	1.75E-05	2.05E-05	1.87E-05	1.49E-05	1.75E-05	9.97E-06	1.96E-05	1.50E-05	1.51E-05	1.82E-05	2.10E-05	1.99E-05	2.08E-05	cAMP signa	Signal trans	Environmer
ko04064	0	0	0	0	0	0	0	0	0	4.55E-08	0	0	0	0	0	0	8.93E-08	0	0	9.29E-08	NF-kappa B	Signal trans	Environmer
ko04066	0.000676	0.00055	0.000535	0.000792	0.000604	0.000544	0.00052	0.000513	0.000581	0.000537	0.000538	0.000518	0.000513	0.000546	0.000556	0.000523	0.000528	0.000517	0.000525	0.000534	HIF-1 signa	Signal trans	Environmer
ko04068	0.000319	0.000338	0.00034	0.000321	0.000333	0.00033	0.000342	0.000347	0.000338	0.000346	0.000337	0.000344	0.000334	0.000342	0.000343	0.000339	0.000341	0.000348	0.000339	0.000349	FoxO signa	Signal trans	Environmer
ko04070	0.000444	0.00039	0.000381	0.000466	0.000404	0.000397	0.000376	0.000368	0.00041	0.000383	0.000379	0.000368	0.000373	0.000381	0.000391	0.000382	0.000371	0.00037	0.00038	0.000375	Phosphatid	Signal trans	Environmer
ko04071	1.05E-05	1.40E-05	1.96E-05	9.06E-06	1.37E-05	1.47E-05	1.78E-05	1.98E-05	2.28E-05	1.97E-05	1.38E-05	1.89E-05	1.05E-05	1.92E-05	1.64E-05	1.69E-05	2.07E-05	2.31E-05	2.26E-05	2.34E-05	Sphingolipi	Signal trans	Environmer
ko04072	0.000124	0.000117	0.000112	0.000121	0.000119	0.000118	0.000113	0.000113	0.000117	0.000113	0.000114	0.000111	0.000115	0.000111	0.000115	0.00011	0.000109	0.000111	0.000107	0.000107	Phospholip	Signal trans	Environmer
ko04080	1.21E-05	8.45E-06	1.26E-05	1.49E-05	1.12E-05	1.57E-05	1.19E-05	8.34E-06	1.96E-05	1.09E-05	9.68E-06	9.06E-06	6.56E-06	1.10E-05	1.07E-05	1.76E-05	1.18E-05	1.03E-05	2.04E-05	1.23E-05	Neuroactive	Signaling m	Environmer
ko04110	7.91E-07	0	2.06E-07	2.25E-06	3.83E-07	0	0	0	3.83E-08	0	2.23E-08	0	0	0	0	0	1.96E-07	0	0	0	Cell cycle	Cell growth	Cellular Pro
ko04112	0.004965	0.005162	0.005198	0.004714	0.005078	0.00527	0.005273	0.005209	0.005278	0.005277	0.005167	0.00517	0.005155	0.005224	0.005468	0.005352	0.005136	0.005224	0.005321	0.005263	Cell cycle -	Cell growth	Cellular Pro
ko04113	0.000185	0.000223	0.000274	0.000168	0.000203	0.000249	0.000274	0.000257	0.000283	0.000243	0.000228	0.000211	0.000236	0.000277	0.000237	0.00029	0.000242	0.000237	0.000359	0.000285	Meiosis - ye	Cell growth	Cellular Pro
ko04115	0.000296	0.000344	0.000352	0.000256	0.000327	0.000345	0.000355	0.000353	0.000317	0.000342	0.000355	0.000351	0.000369	0.000348	0.000339	0.000358	0.000344	0.000346	0.00035	0.000347	p53 signali	Cell growth	Cellular Pro
ko04122	0.001852	0.001812	0.001753	0.001829	0.001792	0.001804	0.001757	0.001762	0.00181	0.001802	0.001771	0.001774	0.001779	0.00175	0.001863	0.001759	0.001762	0.001762	0.001701	0.00177	Sulfur relay	Folding, so	Genetic Info
ko04138	1.56E-05	1.23E-05	1.30E-05	2.32E-05	1.22E-05	9.14E-06	1.18E-05	1.15E-05	1.52E-05	1.31E-05	1.06E-05	1.08E-05	7.50E-06	1.42E-05	9.79E-06	1.18E-05	1.31E-05	1.19E-05	1.42E-05	1.72E-05	Autophagy	Transport a	Cellular Pro
ko04140	7.91E-07	5.76E-08	2.06E-07	2.30E-06	3.83E-07	5.89E-08	0	4.66E-08	1.53E-07	5.63E-08	2.23E-08	0	0	0	0	0	1.96E-07	0	0	0	Autophagy	Transport a	Cellular Pro
ko04141	0.000832	0.000867	0.000844	0.000753	0.000841	0.000862	0.000852	0.00086	0.00082	0.000855	0.000867	0.000866	0.000895	0.000835	0.00083	0.000837	0.000851	0.000851	0.000804	0.000834	Protein pro	Folding, so	Genetic Info
ko04142	0.000142	0.00013	0.00013	0.000168	0.000137	0.000123	0.000141	0.000136	0.000127	0.00014	0.000132	0.000139	0.000123	0.000132	0.000148	0.000127	0.000143	0.000137	0.000128	0.000137	Lysosome	Transport a	Cellular Pro
ko04144	5.03E-06	6.95E-06	8.36E-06	4.27E-06	6.37E-06	5.95E-06	7.80E-06	8.31E-06	8.93E-06	8.19E-06	5.91E-06	7.50E-06	4.92E-06	7.76E-06	6.51E-06	7.14E-06	7.33E-06	8.98E-06	8.51E-06	8.00E-06	Endocytosis	Transport a	Cellular Pro
ko04145	7.22E-07	0	2.06E-07	2.14E-06	3.74E-07	0	0	0	3.83E-08	0	2.23E-08	0	0	0	0	0	3.92E-07	0	0	0	Phagosome	Transport a	Cellular Pro
ko04146	0.002004	0.002146	0.002238	0.001904	0.002095	0.002198	0.002216	0.002221	0.002247	0.002209	0.002161	0.002211	0.002175	0.002242	0.002189	0.002242	0.002219	0.00224	0.002332	0.002286	Peroxisome	Transport a	Cellular Pro
ko04150	7.91E-07	0	2.06E-07	2.25E-06	3.83E-07	0	0	0	3.83E-08	0	2.23E-08	0	0	0	0	0	1.96E-07	0	0	0	mTOR sign	Signal trans	Environmer
ko04151	0.000215	0.00022	0.000215	0.000204	0.000219	0.000219	0.000217	0.00022	0.000211	0.00022	0.000219	0.000221	0.000225	0.000215	0.00022	0.000212	0.000215	0.000221	0.000205	0.000212	PI3K-Akt sig	Signal trans	Environmer
ko04152	0.000367	0.000379	0.000373	0.000351	0.000375	0.000378	0.000376	0.000381	0.000364	0.000384	0.000378	0.00038	0.000381	0.000373	0.000389	0.000374	0.000375	0.000379	0.000363	0.000376	AMPK signa	Signal trans	Environmer
ko04210	0.000382	0.00044	0.000447	0.000331	0.00042	0.000439	0.000449	0.00045	0.000403	0.000435	0.000452	0.000448	0.000471	0.000438	0.000431	0.000451	0.000438	0.00044	0.000436	0.000436	Apoptosis	Cell growth	Cellular Pro
ko04211	0.000227	0.000232	0.000235	0.000242	0.000232	0.000227	0.000236	0.000237	0.000242	0.000238	0.00023	0.000234	0.000225	0.000238	0.000243	0.000236	0.000234	0.000238	0.00024	0.000243	Longevity r	Aging	Organismal
ko04212	0.001187	0.001133	0.0011	0.001159	0.001133	0.001153	0.001109	0.001105	0.001165	0.001136	0.00111	0.001106	0.001124	0.001107	0.001135	0.001105	0.001112	0.001105	0.001099	0.001106	Longevity r	Aging	Organismal
ko04213	0.000951	0.000954	0.000986	0.000975	0.000943	0.000962	0.000984	0.000969	0.001005	0.000961	0.000949	0.000926	0.000955	0.000995	0.000976	0.000994	0.000951	0.00095	0.001053	0.000998	Longevity r	Aging	Organismal
ko04214	0.000708	0.000786	0.000792	0.000632	0.000757	0.000788	0.000796	0.000797	0.000746	0.000784	0.000797	0.000794	0.00082	0.00078	0.000776	0.000802	0.00078	0.000783	0.000776	0.000781	Apoptosis -	Cell growth	Cellular Pro
ko04215	0.000296	0.000343	0.000352	0.000256	0.000327	0.000344	0.000354	0.000352	0.000317	0.000341	0.000354	0.000351	0.000369	0.000347	0.000338	0.000357	0.000344	0.000345	0.000349	0.000346	Apoptosis -	Cell growth	Cellular Pro
ko04216	0.000972	0.001048	0.001113	0.000944	0.001033	0.001067	0.001095	0.001101	0.001099	0.001088	0.001073	0.001102	0.001079	0.001124	0.001097	0.001102	0.001104	0.001124	0.00116	0.001146	Ferroptosis	Cell growth	Cellular Pro
ko04217	0.000735	0.000684	0.000676	0.000774	0.000702	0.000682	0.000668	0.000666	0.0007	0.000685	0.000671	0.000669	0.000661	0.000681	0.000692	0.000663	0.000672	0.000669	0.000665	0.000669	Necroptosis	Cell growth	Cellular Pro
ko04260	0.000312	0.000354	0.000355	0.000275	0.00034	0.00035	0.000358	0.000361	0.000324	0.000355	0.000358	0.000361	0.000377	0.000352	0.000383	0.000361	0.000352	0.000358	0.000348	0.000352	Cardiac mu	Circulatory	Organismal

ko04261	9.96E-06	4.83E-06	3.76E-06	1.73E-05	6.49E-06	2.39E-06	2.87E-06	2.60E-06	3.49E-06	3.64E-06	3.88E-06	3.24E-06	1.65E-06	5.35E-06	3.01E-06	2.58E-06	2.95E-06	4.20E-06	3.48E-06	3.48E-06	Adrenergic	Circulatory	Organismal
ko04320	0	0	0	7.69E-08	0	0	0	0	0	0	0	0	0	1.48E-07	0	0	0	0	0	0	Dorso-vent	Developme	Organismal
ko04330	0	0	0	7.69E-08	0	0	0	0	0	0	0	0	0	1.48E-07	0	0	0	0	0	0	Notch signa	Signal trans	Environmer
ko04370	0	0	0	0	0	0	0	0	0	4.55E-08	0	0	0	0	0	0	8.93E-08	0	0	9.29E-08	VEGF signa	Signal trans	Environmer
ko04371	7.91E-07	0	2.06E-07	2.25E-06	3.83E-07	0	0	0	3.83E-08	0	2.23E-08	0	0	0	0	0	1.96E-07	0	0	0	Apelin sign	Signal trans	Environmer
ko04380	0	1.67E-08	0	1.98E-08	5.96E-09	0	1.72E-08	0	1.52E-08	1.04E-07	0	1.79E-08	0	0	2.65E-08	1.80E-07	1.21E-07	0	0	3.53E-08	Osteoclast	Developme	Organismal
ko04391	0	0	0	0	4.09E-08	0	0	0	0	0	0	0	0	0	2.35E-05	0	0	0	0	9.64E-07	Hippo signa	Signal trans	Environmer
ko04392	0	0	0	0	4.09E-08	0	0	0	0	0	0	0	0	0	2.35E-05	0	0	0	0	9.64E-07	Hippo signa	Signal trans	Environmer
ko04530	7.91E-07	0	2.06E-07	2.25E-06	3.83E-07	0	0	0	3.83E-08	0	2.23E-08	0	0	0	0	0	1.96E-07	0	0	0	Tight junctio	Cellular cor	Cellular Pro
ko04612	0.000122	0.000115	0.00011	0.000123	0.000118	0.000117	0.00011	0.00011	0.000116	0.000112	0.000113	0.000111	0.000116	0.000111	0.00012	0.000109	0.000108	0.00011	0.000107	0.000106	Antigen pro	Immune sys	Organismal
ko04614	1.57E-05	2.12E-05	2.49E-05	1.14E-05	1.84E-05	1.98E-05	2.54E-05	2.34E-05	2.79E-05	2.67E-05	1.83E-05	2.11E-05	1.20E-05	2.38E-05	2.93E-05	2.15E-05	2.02E-05	2.54E-05	2.44E-05	2.49E-05	Renin-angi	Endocrine s	Organismal
ko04621	0.000796	0.000807	0.000784	0.000765	0.000798	0.000787	0.000791	0.000792	0.000773	0.000794	0.000805	0.000799	0.000816	0.000782	0.000845	0.000771	0.000783	0.00079	0.000743	0.000775	NOD-like re	Immune sys	Organismal
ko04622	5.20E-05	1.85E-05	1.82E-05	0.000106	3.98E-05	1.42E-05	1.63E-05	1.47E-05	2.30E-05	1.62E-05	2.08E-05	1.61E-05	1.09E-05	2.50E-05	1.69E-05	1.51E-05	2.01E-05	1.67E-05	1.95E-05	1.91E-05	RIG-I-like re	Immune sys	Organismal
ko04623	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.96E-07	0	0	0	Cytosolic D	Immune sys	Organismal
ko04625	0	0	0	0	0	0	0	0	0	4.55E-08	0	0	0	0	0	0	8.93E-08	0	0	9.29E-08	C-type lecti	Immune sys	Organismal
ko04626	0.000696	0.000711	0.000707	0.000682	0.000701	0.000697	0.000707	0.000701	0.00071	0.000716	0.000693	0.000698	0.000681	0.000697	0.000742	0.000691	0.000686	0.000702	0.000682	0.000691	Plant-patho	Environmer	Organismal
ko04640	0	0	0	0	0	3.62E-08	0	0	0	9.10E-08	3.35E-08	0	0	0	0	0	1.79E-07	0	0	0	Hematopoi	Immune sys	Organismal
ko04657	0.000122	0.000115	0.00011	0.000123	0.000118	0.000117	0.00011	0.00011	0.000116	0.000112	0.000113	0.000111	0.000116	0.000111	0.00012	0.000109	0.000108	0.00011	0.000107	0.000106	IL-17 signa	Immune sys	Organismal
ko04658	0	0	0	7.69E-08	0	0	0	0	0	0	0	0	0	1.48E-07	0	0	0	0	0	0	Th1 and Th	Immune sys	Organismal
ko04659	0.000122	0.000115	0.00011	0.000123	0.000118	0.000117	0.00011	0.00011	0.000116	0.000112	0.000113	0.000111	0.000116	0.000111	0.00012	0.000109	0.000108	0.00011	0.000107	0.000106	Th17 cell di	Immune sys	Organismal
ko04666	5.03E-06	6.94E-06	8.34E-06	4.27E-06	6.34E-06	5.95E-06	7.75E-06	8.31E-06	8.92E-06	8.10E-06	5.90E-06	7.50E-06	4.90E-06	7.71E-06	6.51E-06	6.91E-06	7.23E-06	8.84E-06	8.51E-06	8.00E-06	Fc gamma I	Immune sys	Organismal
ko04668	0	0	0	0	0	0	0	0	0	4.55E-08	0	0	0	0	0	0	8.93E-08	0	0	9.29E-08	TNF signali	Signal trans	Environmer
ko04714	0.001406	0.00157	0.00164	0.001259	0.001511	0.001606	0.001629	0.001635	0.001613	0.001623	0.001591	0.001633	0.001637	0.00164	0.001648	0.00165	0.00163	0.001652	0.001694	0.001676	Thermogen	Environmer	Organismal
ko04721	1.57E-07	1.61E-07	1.02E-07	2.40E-07	8.17E-09	1.99E-07	7.29E-08	1.16E-07	6.21E-07	7.37E-08	5.16E-08	0	0	4.89E-09	0	0	0	0	1.76E-08	0	Synaptic ve	Nervous sy	Organismal
ko04723	3.37E-05	3.88E-05	3.93E-05	2.88E-05	3.71E-05	3.87E-05	4.02E-05	4.01E-05	3.72E-05	3.99E-05	3.94E-05	3.89E-05	4.05E-05	3.89E-05	3.75E-05	4.10E-05	3.76E-05	4.03E-05	3.84E-05	3.98E-05	Retrograde	Nervous sy	Organismal
ko04724	0.000258	0.000253	0.000265	0.000267	0.000253	0.00026	0.000259	0.000256	0.000287	0.000266	0.000246	0.000254	0.000244	0.000265	0.00027	0.000261	0.000265	0.000261	0.00028	0.000265	Glutamater	Nervous sy	Organismal
ko04725	2.41E-07	2.86E-07	4.45E-08	2.75E-07	6.35E-08	3.24E-07	5.49E-08	5.77E-08	5.22E-07	5.85E-08	0	0	3.18E-08	0	2.04E-08	0	0	0	5.25E-08	0	Cholinergic	Nervous sy	Organismal
ko04726	0.000296	0.000331	0.000332	0.000253	0.000315	0.000333	0.000333	0.000334	0.00031	0.000325	0.000338	0.000339	0.000353	0.000328	0.000324	0.000333	0.00034	0.000329	0.000329	0.000327	Serotonerg	Nervous sy	Organismal
ko04727	0.000285	0.000257	0.000267	0.00032	0.000269	0.000268	0.00026	0.000256	0.000301	0.00027	0.000253	0.000256	0.000245	0.000273	0.000295	0.000265	0.00027	0.000263	0.000287	0.00027	GABAergic	Nervous sy	Organismal
ko04728	0.000296	0.000331	0.000332	0.000253	0.000315	0.000333	0.000333	0.000334	0.00031	0.000325	0.000338	0.000339	0.000353	0.000328	0.000324	0.000333	0.00034	0.000329	0.000329	0.000327	Dopaminer	Nervous sy	Organismal
ko04910	0.000265	0.000253	0.000249	0.000256	0.000254	0.000258	0.000247	0.000251	0.000269	0.000263	0.000245	0.000249	0.00024	0.000248	0.000245	0.000243	0.000245	0.00025	0.000238	0.000248	Insulin sign	Endocrine s	Organismal
ko04911	9.96E-06	4.83E-06	3.76E-06	1.73E-05	6.49E-06	2.39E-06	2.87E-06	2.60E-06	3.49E-06	3.64E-06	3.88E-06	3.24E-06	1.65E-06	5.35E-06	3.01E-06	2.58E-06	2.95E-06	4.20E-06	3.48E-06	3.48E-06	Insulin secr	Endocrine s	Organismal
ko04912	5.03E-06	6.94E-06	8.34E-06	4.27E-06	6.34E-06	5.95E-06	7.75E-06	8.31E-06	8.92E-06	8.10E-06	5.90E-06	7.50E-06	4.90E-06	7.71E-06	6.51E-06	6.91E-06	7.23E-06	8.84E-06	8.51E-06	8.00E-06	GnRH signa	Endocrine s	Organismal
ko04913	0	0	0	0	0	0	0	0	0	4.55E-08	0	0	0	0	0	0	8.93E-08	0	0	9.29E-08	Ovarian ste	Endocrine s	Organismal
ko04914	0.000122	0.000115	0.00011	0.000123	0.000118	0.000117	0.00011	0.00011	0.000116	0.000112	0.000113	0.000111	0.000116	0.000111	0.00012	0.000109	0.000108	0.00011	0.000107	0.000106	Progestero	Endocrine s	Organismal
ko04915	0.000122	0.000115	0.00011	0.000123	0.000118	0.000117	0.00011	0.00011	0.000116	0.000112	0.000113	0.000111	0.000116	0.000111	0.00012	0.000109	0.000108	0.00011	0.000107	0.000106	Estrogen si	Endocrine s	Organismal
ko04916	5.95E-05	6.75E-05	6.54E-05	5.15E-05	6.56E-05	6.62E-05	6.67E-05	6.80E-05	5.93E-05	6.78E-05	6.82E-05	6.90E-05	7.16E-05	6.42E-05	6.39E-05	6.62E-05	6.65E-05	6.67E-05	6.03E-05	6.46E-05	Melanogen	Endocrine s	Organismal
ko04917	4.26E-05	1.32E-05	9.99E-06	7.82E-05	2.73E-05	6.78E-06	7.17E-06	6.92E-06	1.24E-05	8.10E-06	1.32E-05	8.14E-06	4.88E-06	1.52E-05	1.82E-05	6.57E-06	9.06E-06	8.69E-06	8.35E-06	1.06E-05	Prolactin si	Endocrine s	Organismal
ko04918	0.000497	0.00045	0.00043	0.000533	0.000467	0.000438	0.000427	0.000426	0.000418	0.000429	0.000446	0.00043	0.000448	0.000435	0.000419	0.000426	0.000422	0.000423	0.000406	0.000417	Thyroid hor	Endocrine s	Organismal
ko04919	0.000305	0.000339	0.000333	0.00027	0.000325	0.000326	0.000338	0.000349	0.000302	0.000334	0.000345	0.000347	0.000357	0.000328	0.000318	0.000328	0.000341	0.000342	0.000313	0.00033	Thyroid hor	Endocrine s	Organismal
ko04920	0.000878	0.00098	0.00105	0.000806	0.000949	0.001006	0.001033	0.001041	0.00104	0.00103	0.001	0.00104	0.001011	0.001056	0.001024	0.001041	0.001042	0.001067	0.001104	0.001087	Adipocytoki	Endocrine s	Organismal
ko04921	8.52E-07	7.87E-08	2.33E-07	2.26E-06	3.88E-07	7.31E-08	1.93E-07	7.52E-08	8.99E-08	7.77E-08	9.94E-08	4.92E-08	2.71E-08	5.15E-08	8.10E-08	4.25E-08	3.92E-07	4.66E-10	6.56E-08	1.50E-07	Oxytocin si	Endocrine s	Organismal
ko04922	0.000993	0.00082	0.000789	0.001182	0.000892	0.000786	0.000774	0.000773	0.000815	0.000794	0.000808	0.000781	0.000768	0.000809	0.000775	0.000771	0.000786	0.000772	0.000764	0.000784	Glucagon s	Endocrine s	Organismal
ko04923	0	0	0	0	0	0	0	0	0	4.55E-08	0	0	0	0	0	0	8.93E-08	0	0	9.29E-08	Regulation	Endocrine s	Organismal
ko04924	1.94E-07	3.12E-07	2.62E-07	2.59E-07	2.18E-07	1.85E-07	6.39E-07	2.27E-07	7.30E-07	1.67E-07	5.95E-07	6.29E-07	5.70E-08	3.89E-07	1.44E-07	3.63E-07	2.13E-07	1.98E-08	2.55E-07	2.03E-07	Renin secre	Endocrine s	Organismal
ko04925	9.96E-06	4.83E-06	3.76E-06	1.73E-05	6.49E-06	2.39E-06	2.87E-06	2.60E-06	3.49E-06	3.64E-06	3.88E-06	3.24E-06	1.65E-06	5.35E-06	3.01E-06	2.58E-06	2.95E-06	4.20E-06	3.48E-06	3.48E-06	Aldosterone	Endocrine s	Organismal
ko04928	5.82E-06	7.02E-06	8.59E-06	6.65E-06	6.77E-06	5.97E-06	7.76E-06	8.31E-06	9.09E-06	8.10E-06	5.92E-06	7.51E-06	4.90E-06	7.74E-06	1.54E-05	6.96E-06	7.42E-06	8.98E-06	8.63E-06	8.53E-06	Parathyroid	Endocrine s	Organismal
ko04930	0.000256	0.00025	0.000241	0.000257	0.000251	0.000246	0.000242	0.000245	0.000236	0.000246	0.00025	0.000246	0.000253	0.00024	0.000247	0.000242	0.000241	0.00024	0.000228	0.000239	Type II diab	Endocrine a	Human Dis
ko04931	0.000332	0.000313	0.000315	0.000348	0.000319	0.000311	0.000311	0.000314	0.000325	0.000319	0.000304	0.00031	0.000294	0.000313	0.000303	0.000305	0.000312	0.000313	0.000311	0.000313	Insulin resis	Endocrine a	Human Dis
ko04932	0.000599	0.000693	0.000703	0.000516	0.000661	0.000692	0.00071	0.000711	0.000638	0.000693	0.000709	0.000708	0.000744	0.000694	0.000718	0.000716	0.000693	0.000699	0.000694	0.000695	Non-alcoho	Endocrine a	Human Dis
ko04934	0.000138	0.000132	0.000127	0.000131	0.000126	0.00013	0.000127	0.000126	0.000135	0.000132	0.000125	0.000126	0.000126	0.000125	0.000124	0.000127	0.000129	0.000126	0.000126	0.00013	Cushing's s	Endocrine a	Human Dis
ko04940	0.000358	0.000333	0.000327	0.000369	0.000344	0.000348	0.00033	0.000324	0.000342	0.000328	0.000339	0.000329	0.000345	0.000336	0.000353	0.000337	0.000337	0.000324	0.000344	0.000327	Type I diab	Endocrine a	Human Dis
ko04960	9.96E-06	4.83E-06	3.76E-06	1.73E-05	6.49E-06	2.39E-06	2.87E-06	2.60E-06	3.49E-06	3.64E-06	3.88E-06	3.24E-06	1.65E-06	5.35E-06	3.01E-06	2.58E-06	2.95E-06	4.20E-06	3.48E-06	3.48E-06	Aldosterone	Excretory s	Organismal
ko04961	9.96E-06	4.84E-06	3.76E-06	1.73E-05	6.50E-06	2.39E-06	2.87E-06	2.60E-06	3.51E-06	3.64E-06	3.88E-06	3.24E-06	1.66E-06	5.35E-06	3.01E-06	2.58E-06	2.95E-06	4.20E-06	3.48E-06	3.48E-06	Endocrine a	Excretory s	Organismal
ko04962	1.57E-07	1.61E-07	1.02E-07	2.40E-07	8.17E-09	1.99E-07	7.29E-08	1.16E-07	6.29E-07	7.37E-08	5.16E-08	0	0	1.22E-07	0	0	1.96E-07	0	1.76E-08	0	Vasopressi	Excretory s	Organismal
ko04964	0.00024	0.000267	0.000266	0.00022	0.000256	0.00026	0.000268	0.000273	0.000257	0.00027	0.000267	0.000271	0.000267	0.000265	0.000259	0.000262	0.000267	0.000273	0.000258	0.00027	Proximal tu	Excretory s	Organismal
ko04970	9.96E-06	4.83E-06	3.76E-06	1.73E-05	6.49E-06	2.39E-06	2.87E-06	2.60E-06	3.49E-06	3.64E-06	3.88E-06	3.24E-06	1.65E-06	5.35E-06	3.01E-06	2.58E-06	2.95E-06	4.20E-06	3.48E-06	3.48E-06	Salivary se	Digestive s	Organismal
ko04971	9.96E-06	4.83E-06	3.76E-06	1.73E-05	6.49E-06	2.39E-06	2.87E-06	2.60E-06	3.49E-06	3.64E-06	3.88E-06	3.24E-06	1.65E-06	5.35E-06	3.01E-06	2.58E-06	2.95E-06	4.20E-06	3.48E-06	3.48E-06	Gastric acid	Digestive s	Organismal
ko04972	1.00E-05	4.92E-06	3.87E-06	1.74E-05	6.57E-06	2.43E-06	3.13E-06	2.68E-06	3.61E-06	3.67E-06	3.92E-06	3.28E-06	1.78E-06	5.44E-06	3.10E-06	2.74E-06	2.95E-06	4.34E-06	3.84E-06	3.54E-06	Pancreatic	Digestive s	Organismal
ko04973	5.75E-05	2.32E-05	2.12E-05	0.000109	3.95E-05	1.68E-05	2.11E-05	1.88E-05	2.89E-05	2.53E-05	2.41E-05	1.98E-05	1.11E-05	2.86E-05	3.40E-05	1.61E-05	2.26E-05	2.12E-05	1.94E-05	2.59E-05	Carbohydra	Digestive s	Organismal
ko04974	2.30E-05	1.88E-05	2.05E-05	2.93E-05	2.07E-05	1.65E-05	2.17E-05	1.99E-05	2.18E-05	2.36E-05	1.75E-05	1.86E-05	1.04E-05	2.18E-05	1.57E-05	1.76E-05	1.95E-05	2.27E-05	2.31E-05	2.21E-05	Protein dige	Digestive s	Organismal
ko04976	2.57E-05	2.74E-05	3.28E-05	3.34E-05	2.34E-05	1.95E-05	3.31E-05	2.97E-05	2.92E-05	2.84E-05	2.42E-05	2.03E-05	2.10E-05	3.36E-05	3.09E-05	2.63E-05	2.31E-05	2.40E-05	3.37E-05	3.33E-05	Bile secreti	Digestive s	Organismal
ko04978	0.000128	0.000124	0.000121	0.000146	0.000127	0.000118	0.000118	0.000121	0.00012	0.000122	0.000122	0.000122	0.000116	0.000123	0.000111	0.000118	0.000119	0.000123	0.000113	0.00012	Mineral abs	Digestive s	Organismal
ko04979	1.20E-05	8.36E-06	1.25E-05	1.48E-05	1.11E-05	1.57E-05	1.16E-05	8.26E-06	1.95E-05	1.09E-05	9.64E-06	9.01E-06	6.44E-06	1.10E-05	1.06E-05	1.75E-05	1.18E-05	1.01E-05	2.01E-05	1.23E-05	Cholesterol	Digestive s	Organismal
ko05010	0.000768	0.000832	0.000832	0.000699	0.000805	0.000831	0.000835	0.000835	0.000788	0.000829	0.000837	0.000833	0.000869	0.000823	0.000876	0.000841	0.00082	0.000823	0.000815	0.000823	Alzheimer's	Neurodege	Human Dis
ko05012	0.000598	0.000692	0.000703	0.000513	0.00066	0.000692	0.00071	0.000711	0.000638	0.000693	0.000709	0.000708	0.000748	0.000695	0.000725	0.000716	0.000693	0.0007	0.000694	0.000695	Parkinson's	Neurodege	Human Dis
ko05014	0.000464	0.000526	0.000534	0.000411	0.0005	0.000526	0.000541	0.000539	0.000512	0.00053	0.000533	0.000535	0.000544	0.000529	0.000522	0.000541	0.000527	0.000532	0.000535	0.000533	Amyotrophi	Neurodege	Human Dis
ko05016	0.000851	0.000953	0.000962	0.000768	0.000922	0.000955	0.000972	0.000972	0.000897	0.000955	0.000973	0.000973	0.001018	0.000957	0.000993	0.00098	0.000956	0.000962	0.000952	0.000956	Huntington'	Neurodege	Human Dis
ko05020	9.62E-05	0.000105	0.000103	8.29E-05	0.000101	0.000109	0.000106	0.000106	0.000107	0.000106	0.000106	0.000108	0.00011	0.000103	0.000104	0.000106	0.000105	0.000106	0.000102	0.000102	Prion disea	Neurodege	Human Dis
ko05030	0.000296	0.000331	0.000332	0.000253	0.000315	0.000333	0.000333	0.000334	0.00031	0.000325	0.000338	0.000339	0.000353	0.000328	0.000324	0.000333	0.00034	0.000329	0.000329	0.000327	Cocaine ad	Substance	Human Dis
ko05031	0.000296	0.000331	0.000332	0.000253	0.000315	0.000333	0.000333	0.000334	0.00031	0.000325	0.000338	0.000339	0.000353	0.000328	0.000324	0.000333	0.00034	0.000329	0.000329	0.000327	Amphetami	Substance	Human Dis
ko05034	0.000296	0.000331	0.000332	0.000253	0.000315	0.000333	0.000333	0.000334	0.00031	0.000325	0.000338	0.000339	0.000353	0.000328	0.000324	0.000333	0.00034	0.000329	0.000329	0.000327	Alcoholism	Substance	Human Dis
ko05100	1.68E-05	3.82E-06	2.11E-06	4.02E-05	9.84E-06	1.62E-06	1.39E-06	1.22E-06	4.50E-06	1.50E-06	3.66E-06	1.76E-06	1.82E-06	4.03E-06	1.96E-06	1.27E-06	1.58E-06	2.39E-06	8.36E-07	9.72E-07	Bacterial in	Infectious d	Human Dis
ko05110	9.72E-05	9.93E-05	9.39E-05	0.000106	9.97E-05	9.28E-05	9.49E-05	9.55E-05	8.32E-05	9.30E-05	0.0001	9.75E-05	0.000103	9.50E-05	0.000117	9.18E-05	9.48E-05	9.37E-05	8.64E-05	9.17E-05	Vibrio chole	Infectious d	Human Dis
ko05111	0.007484	0.007997	0.00782	0.007002	0.007814	0.007856	0.007957	0.007998	0.007541	0.007956	0.008	0.008016	0.008099	0.007808	0.008424	0.007822	0.007824	0.007928	0.007546	0.007773	Biofilm form	Cellular cor	Cellular Pro
ko05120	0.000552	0.000575	0.000557	0.000524	0.000564	0.000569	0.000562	0.000567	0.000535	0.000562	0.000576	0.000569	0.000601	0.000554	0.000586	0.000554	0.000553	0.000557	0.000526	0.000545	Epithelial c	Infectious d	Human Dis
ko05131	4.49E-09	3.05E-07	8.85E-09	6.81E-07	5.15E-07	3.87E-07	1.52E-07	2.37E-07	3.42E-07	2.85E-09	1.58E-08	2.64E-07	1.04E-07	1.51E-07	8.48E-08	3.03E-09	6.19E-07	2.01E-07	0	8.61E-09	Shigellosis	Infectious d	Human Dis
ko05132	0.001004	0.001134	0.001117	0.000891	0.001084	0.001112	0.001135	0.001144	0.00106	0.001123	0.00114	0.001145	0.001177	0.001098	0.001201	0.001108	0.001112	0.001127	0.001052	0.001098	Salmonella	Infectious d	Human Dis
ko05133	0.002228	0.002502	0.002461	0.001922	0.002407	0.002478	0.002517	0.002538	0.00227	0.002502	0.002544	0.002551	0.002646	0.002438	0.002481	0.002495	0.002481	0.00251	0.00237	0.002437	Pertussis	Infectious d	Human Dis
ko05134	0.00146	0.001529	0.001523	0.001349	0.001493	0.001537	0.001532	0.001521	0.001488	0.001517	0.001538	0.001532	0.001582	0.001518	0.001572	0.001528	0.001523	0.001512	0.001507	0.001509	Legionellos	Infectious d	Human Dis
ko05140	0	0	0	0	0	0	0	0	0	4.55E-08	0	0	0	0	0	0	8.93E-08	0	0	9.29E-08	Leishmania	Infectious d	Human Dis
ko05142	1.08E-05	1.37E-05	1.95E-05	9.12E-06	1.36E-05	1.64E-05	1.96E-05	1.79E-05	2.46E-05	1.90E-05	1.59E-05	1.83E-05	1.17E-05	2.01E-05	2.55E-05	2.03E-05	2.09E-05	2.10E-05	2.75E-05	2.43E-05	Chagas dis	Infectious d	Human Dis
ko05143	1.39E-05	1.70E-05	2.23E-05	1.24E-05	1.65E-05	1.93E-05	2.24E-05	2.03E-05	2.85E-05	2.31E-05	1.80E-05	2.12E-05	1.37E-05	2.38E-05	2.84E-05	2.27E-05	2.41E-05	2.48E-05	3.08E-05	2.81E-05	African tryp	Infectious d	Human Dis
ko05145	0.000296	0.000343	0.000352	0.000256	0.000327	0.000344	0.000354	0.000352	0.000317	0.000341	0.000354	0.000351	0.000372	0.000348	0.000345	0.000358	0.000344	0.000346	0.000349	0.000346	Toxoplasm	Infectious d	Human Dis
ko05146	0.000101	0.000111	0.000109	8.80E-05	0.000105	0.00011	0.000112	0.000114	0.00011	0.000114	0.00011	0.000112	0.000111	0.000107	0.000102	0.000108	0.00011	0.000112	0.000104	0.000112	Amoebiasis	Infectious d	Human Dis
ko05150	0.000343	0.000234	0.000219	0.000503	0.000284	0.000209	0.000209	0.000211	0.000219	0.00021	0.000232	0.000216	0.000209	0.000235	0.000216	0.000211	0.000215	0.000219	0.000213	0.000212	Staphyloco	Infectious d	Human Dis
ko05152	0.001273	0.001256	0.001231	0.001215	0.00125	0.001274	0.001238	0.00123	0.001226	0.001237	0.001255	0.00124	0.001289	0.001232	0.001262	0.001243	0.001231	0.001223	0.001221	0.001213	Tuberculos	Infectious d	Human Dis
ko05160	7.91E-07	5.76E-08	2.06E-07	2.30E-06	3.83E-07	5.89E-08	0	4.66E-08	1.53E-07	5.63E-08	2.23E-08	0	0	0	0	0	1.96E-07	0	0	0	Hepatitis C	Infectious d	Human Dis
ko05161	0.000297	0.000343	0.000352	0.000258	0.000327	0.000344	0.000354	0.000352	0.000317	0.000341	0.000354	0.000351	0.000369	0.000347	0.000338	0.000357	0.000344	0.000345	0.000349	0.000346	Hepatitis B	Infectious d	Human Dis
ko05162	7.91E-07	5.76E-08	2.06E-07	2.30E-06	3.83E-07	5.89E-08	0	4.66E-08	1.53E-07	5.63E-08	2.23E-08	0	0	0	0	0	1.96E-07	0	0	0	Measles	Infectious d	Human Dis
ko05163	0.000296	0.000343	0.000352	0.000256	0.000327	0.000344	0.000354	0.000352	0.000317	0.000341	0.000354	0.000351	0.000369	0.000347	0.000338	0.000357	0.000344	0.000345	0.000349	0.000346	Human cyto	Infectious d	Human Dis
ko05164	0.000297	0.000343	0.000353	0.000258	0.000328	0.000344	0.000354	0.000352	0.000317	0.000341	0.000354	0.000351	0.000369	0.000347	0.000338	0.000357	0.000344	0.000345	0.000349	0.000346	Influenza A	Infectious d	Human Dis
ko05165	0.000257	0.00025	0.000241	0.000259	0.000252	0.000246	0.000242	0.000245	0.000236	0.000246	0.00025	0.000246	0.000253	0.00024	0.000247	0.000242	0.000242	0.00024	0.000228	0.000239	Human pap	Infectious d	Human Dis
ko05166	1.36E-05	8.36E-06	1.29E-05	1.93E-05	1.18E-05	1.57E-05	1.16E-05	8.26E-06	1.96E-05	1.09E-05	9.69E-06	9.01E-06	6.44E-06	1.10E-05	1.06E-05	1.75E-05	1.26E-05	1.01E-05	2.01E-05	1.23E-05	HTLV-I infe	Infectious d	Human Dis
ko05167	0.000296	0.000343	0.000352	0.000256	0.000327	0.000344	0.000354	0.000352	0.000317	0.000341	0.000354	0.000351	0.000369	0.000347	0.000338	0.000357	0.000344	0.000345	0.000349	0.000346	Kaposi's sa	Infectious d	Human Dis
ko05168	0.000298	0.000343	0.000352	0.00026	0.000328	0.000344	0.000354	0.000352	0.000317	0.000341	0.000354	0.000351	0.000369	0.000347	0.000338	0.000357	0.000345	0.000345	0.000349	0.000346	Herpes sim	Infectious d	Human Dis
ko05169	3.10E-06	1.67E-08	8.90E-07	8.89E-06	1.60E-06	5.17E-10	9.37E-09	2.58E-08	1.62E-07	3.82E-09	1.03E-07	3.40E-08	0	1.45E-07	5.89E-10	1.06E-08	2.07E-06	2.33E-09	9.60E-08	0	Epstein-Ba	Infectious d	Human Dis
ko05200	0.001693	0.001834	0.001847	0.001493	0.001764	0.001868	0.001872	0.001857	0.001785	0.001833	0.001844	0.001827	0.001893	0.00183	0.001803	0.001895	0.001823	0.001824	0.001895	0.001831	Pathways ir	Cancers: O	Human Dis
ko05203	0.000259	0.00025	0.000241	0.000265	0.000253	0.000246	0.000242	0.000245	0.000236	0.000246	0.00025	0.000246	0.000253	0.00024	0.000247	0.000242	0.000243	0.00024	0.000228	0.000239	Viral carcin	Cancers: O	Human Dis
ko05204	0.001222	0.00136	0.001405	0.001055	0.001297	0.001396	0.001424	0.001408	0.001371	0.001393	0.001366	0.001368	0.001384	0.001389	0.001338	0.001442	0.001376	0.001382	0.001475	0.001412	Chemical c	Cancers: O	Human Dis
ko05205	0.000145	0.000125	0.000119	0.000162	0.000131	0.000122	0.000118	0.000118	0.000124	0.000122	0.000121	0.000118	0.00012	0.000121	0.000126	0.000117	0.000118	0.000118	0.000114	0.000117	Proteoglyca	Cancers: O	Human Dis
ko05206	0.000142	0.000134	0.000138	0.000169	0.000143	0.000135	0.000134	0.000135	0.000141	0.000136	0.000135	0.000135	0.000131	0.00014	0.000138	0.000139	0.000135	0.000137	0.000142	0.00014	MicroRNAs	Cancers: O	Human Dis
ko05210	0.000296	0.000343	0.000352	0.000256	0.000327	0.000344	0.000354	0.000352	0.000317	0.000341	0.000354	0.000351	0.000369	0.000347	0.000338	0.000357	0.000344	0.000345	0.000349	0.000346	Colorectal	Cancers: S	Human Dis
ko05211	0.000138	0.000132	0.000127	0.000131	0.000126	0.00013	0.000127	0.000126	0.000135	0.000132	0.000125	0.000126	0.000126	0.000125	0.000124	0.000127	0.000129	0.000126	0.000126	0.00013	Renal cell c	Cancers: S	Human Dis
ko05212	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.96E-07	0	0	0	Pancreatic	Cancers: S	Human Dis
ko05215	0.000122	0.000115	0.00011	0.000123	0.000118	0.000117	0.00011	0.00011	0.000116	0.000112	0.000113	0.000111	0.000116	0.000111	0.00012	0.000109	0.000108	0.00011	0.000107	0.000106	Prostate ca	Cancers: S	Human Dis
ko05219	0.00017	0.000166	0.000166	0.00018	0.000173	0.000167	0.000167	0.000167	0.000157	0.000166	0.000174	0.000171	0.000176	0.000168	0.000171	0.000168	0.00017	0.000169	0.000163	0.000164	Bladder car	Cancers: S	Human Dis
ko05222	0.000296	0.000343	0.000352	0.000256	0.000327	0.000344	0.000354	0.000352	0.000317	0.000341	0.000354	0.000351	0.000369	0.000347	0.000338	0.000357	0.000344	0.000345	0.000349	0.000346	Small cell lu	Cancers: S	Human Dis
ko05224	0	0	0	7.69E-08	0	0	0	0	0	0	0	0	0	1.48E-07	0	0	0	0	0	0	Breast canc	Cancers: S	Human Dis
ko05225	0.001137	0.001244	0.001258	0.000983	0.001193	0.001277	0.001281	0.001268	0.001217	0.001248	0.001252	0.00124	0.001282	0.001245	0.001221	0.001302	0.001242	0.001242	0.001313	0.00125	Hepatocellu	Cancers: S	Human Dis
ko05230	0.000983	0.000852	0.00083	0.001134	0.000912	0.000825	0.00082	0.00082	0.000857	0.000835	0.000847	0.000824	0.00081	0.000847	0.000835	0.00082	0.000833	0.000818	0.000814	0.000834	Central carl	Cancers: O	Human Dis
ko05231	0.000125	0.000118	0.000112	0.000123	0.000119	0.000118	0.000113	0.000113	0.000118	0.000114	0.000114	0.000111	0.000115	0.000111	0.000115	0.00011	0.000109	0.000111	0.000107	0.000107	Choline me	Cancers: O	Human Dis
ko05322	2.10E-06	1.63E-06	2.17E-06	5.92E-07	1.60E-06	2.93E-06	2.95E-06	2.16E-06	4.15E-06	3.18E-06	2.05E-06	2.07E-06	1.71E-06	2.01E-06	2.29E-06	3.77E-06	2.20E-06	3.23E-06	2.79E-06	3.03E-06	Systemic lu	Immune dis	Human Dis
ko05323	0	1.67E-08	0	1.98E-08	5.96E-09	0	1.72E-08	0	1.52E-08	1.04E-07	0	1.79E-08	0	0	2.65E-08	1.80E-07	1.21E-07	0	0	3.53E-08	Rheumatoi	Immune dis	Human Dis
ko05340	0.000241	0.000225	0.000218	0.000276	0.000236	0.00021	0.000217	0.00022	0.000206	0.000218	0.000228	0.000227	0.000222	0.000221	0.000226	0.000212	0.000225	0.000222	0.000205	0.000219	Primary imr	Immune dis	Human Dis
ko05410	1.49E-06	2.45E-06	2.15E-06	1.15E-06	1.62E-06	1.43E-06	2.99E-06	2.46E-06	3.03E-06	2.61E-06	2.62E-06	2.89E-06	8.75E-07	2.26E-06	1.61E-06	2.18E-06	3.71E-06	2.34E-06	2.49E-06	3.88E-06	Hypertroph	Cardiovasc	Human Dis
ko05414	1.30E-06	2.13E-06	1.89E-06	8.88E-07	1.40E-06	1.25E-06	2.35E-06	2.24E-06	2.30E-06	2.44E-06	2.02E-06	2.26E-06	8.18E-07	1.87E-06	1.46E-06	1.82E-06	3.49E-06	2.32E-06	2.24E-06	3.67E-06	Dilated car	Cardiovasc	Human Dis
ko05416	0.000296	0.000343	0.000352	0.000256	0.000327	0.000344	0.000354	0.000352	0.000317	0.000341	0.000354	0.000351	0.000369	0.000347	0.000338	0.000357	0.000344	0.000345	0.000349	0.000346	Viral myoca	Cardiovasc	Human Dis
ko05418	0.001863	0.001954	0.001942	0.001692	0.001898	0.001973	0.001969	0.001959	0.001902	0.001946	0.001957	0.001937	0.001995	0.001932	0.001927	0.001978	0.00193	0.001932	0.001969	0.001932	Fluid shear	Cardiovasc	Human Dis

Table S4: Differentially abundant ASVs found in PMA treated and control digesta samples.

ASV	baseMean	log2FoldChai	lfcSE	stat	pvalue	padj	Domain	Phylum	Class	Order	Family	Genus	Confidence
0ca506f169d4395ad012fef6d0024778	13.1037487	-7.6237776	2.7492197	-2.7730696	0.00555302	0.02423566	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Eggerthellaceae	Paraeggerthella	0.7550793
975687db9563a26522f7df6ae4db9672	31.3558868	-8.8848287	2.49988151	-3.5540999	0.00037928	0.00361716	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriales Incertae	uncultured	0.8708957
16e45dbd865c5c34ea700db9a7de65e9	11.4944948	-7.4337983	3.03086596	-2.4526978	0.01417894	0.04868102	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Trueperella	0.9971748
df058c5dc742aee3188a79190c2860e1	14.7885923	-7.80003	2.1399552	-3.6449501	0.00026744	0.00281089	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Brevibacteriaceae	Brevibacterium	0.9999954
86df7218ed8557a0d33483e63be71322	10.0814765	-22.089091	3.03147395	-7.2865844	3.18E-13	2.73E-11	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	NA	0.9999962
c1db7c310d313b1ae3a019223850a144	99.4562679	-25.172543	3.02672836	-8.3167499	9.04E-17	4.66E-14	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Albimonas	0.9379054
14442ca78c5c727cf8aa4926d3d2d5c7	6.43498498	-21.47159	3.03443575	-7.0759747	1.48E-12	8.49E-11	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Rhodoplanes	0.9984895
e3bc742d6770dd8c80bcd6e56435c04d	5.36226889	-6.3441247	2.18443219	-2.9042443	0.00368141	0.01755486	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter	0.9999993
7c393bac925a0dc8f34832ecd7c0d1d9	23.8586152	-23.249081	3.02843159	-7.6769379	1.63E-14	1.68E-12	Bacteria	Spirochaetes	Spirochaetia	Brevinematales	Brevinemataceae	Brevinema	0.9765096
9c15036b8150b1dbcfa1ace82d2def35	21.6340921	-8.3483321	2.78555202	-2.9970117	0.0027264	0.0141828	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	0.9996774
46c02f4d6969285e6823dd4666774a95	30.9048957	-8.8642552	2.4883521	-3.5622994	0.00036762	0.00361716	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	0.9942426
858e4909591a29f819926fb9faf4db4d	12.6054904	-7.5665986	2.76547081	-2.7360978	0.00621725	0.02598175	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	0.9953513
d8f74b52d2f1e3d2c0c5f1ceae3e7c83	6.00598598	-21.393035	3.03501789	-7.0487344	1.81E-12	9.30E-11	Bacteria	Bacteroidetes	Bacteroidia	Sphingobacteriales	env.OPS 17	NA	0.9999998
18f18bfa4e2441e7bf46630690d35e5f	21.0883467	-8.3115056	2.77617414	-2.9938704	0.00275463	0.01418634	Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Eubacterium	0.9758201
7ecea2d7a278082fc52febaf40c9ef99	9.75832677	-7.1948141	2.76669348	-2.60051	0.00930853	0.03551032	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Erysipelothrix	0.9987451
f264b3ad15018a33374aa61bf945bac7	7.13588118	-6.7490112	2.43858246	-2.767596	0.00564714	0.02434996	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Erysipelothrix	0.9998857
39fdf06bc6d7b01063a1deabc2cdbf5a	27.4150466	-8.6919328	2.45852542	-3.5354252	0.00040712	0.00374404	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Erysipelothrix	0.8827366
6e632ad1b53c0d9bbc0d8842022f2530	8.6946346	-7.0298494	2.18441138	-3.2181893	0.00129003	0.00851748	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Erysipelothrix	0.9140102
f90f46c55d16c20f280c2c94b4b75e4c	10.7132363	-7.3349023	2.73524862	-2.6816218	0.00732662	0.0292497	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Erysipelothrix	0.8854352
d9acd004709b47a2e7d9aa2a525ed751	10.6674354	-7.3257576	2.74301239	-2.670698	0.00756937	0.02975745	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Erysipelatoclostridium	0.999999
b8fbe7e4d29d90cf5ab22b3b4985ec70	40.606123	-9.2590678	2.80294301	-3.3033379	0.00095541	0.00682338	Bacteria	RsaHF231	unclassified_RsaHF231	unclassified_RsaHF232	unclassified_RsaHF233	unclassified_RsaHF234	0.999996
8ad9357b761d4286600695bf4444a174	62.9639142	-9.8925478	2.82488429	-3.5019303	0.0004619	0.00399555	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	unclassified_Clostridiales	0.9943005
17e0668aa4e50466322115ead8f46498	124.553048	-7.843372	2.21295394	-3.5442997	0.00039366	0.00368607	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	W5053	0.8284118
4968b315804462a5abe3b8459f4520dd	34.178837	-9.0089957	2.22241416	-4.0536979	5.04E-05	0.00064908	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	W5053	0.8018581
327efc0bd4d55cd58638bd050aa9cece	11.1447769	-7.3869098	3.03102003	-2.4371036	0.01480544	0.0498353	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	W5053	0.99575
cb857c88f91d39c519bdf505b7eaf8b4	36.8522805	-9.1187292	2.49585046	-3.6535559	0.00025863	0.00277492	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	unclassified_Clostridiales	0.9954919
9ddb10fd3868997d9a87b34b47379029	11.2928207	-7.407179	2.76052444	-2.6832507	0.00729103	0.0292497	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Gallicola	0.9984116
7e289929b4fabb1c1d04204b5b23ee88	20.3255303	-8.2589725	2.45979224	-3.3575895	0.00078625	0.00604359	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Gallicola	0.8453969
190711d0cb576b79afb5d41a0348487e	142.534967	-11.071652	2.25180286	-4.9167943	8.80E-07	3.02E-05	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Gallicola	0.9501732
b240d8cb574c762139902755f99e1eed	14.0702271	-7.7256965	2.76371972	-2.795398	0.00518359	0.02341708	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Peptoniphilus	0.964601
fdd6a01bc8004b738c5434138f72e89c	14.6262198	-7.7819536	2.46515908	-3.1567754	0.00159524	0.00989818	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Peptoniphilus	0.9990592
c6972512b70f3cab04ac837d5d4a4489	273.232587	-12.010997	2.20626347	-5.4440448	5.21E-08	2.44E-06	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Peptoniphilus	0.9436652
c6d297b81168ed0df494a7d2af93c5b6	89.6939133	-10.403201	2.52818247	-4.1148935	3.87E-05	0.00053916	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Peptoniphilus	0.8379867
496d3c279b3f103096002e400a433491	33.8821389	-8.9971237	2.49078423	-3.612165	0.00030365	0.00312761	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Peptoniphilus	0.9999993
4857183803c4b5ac8d1f6bd31e3d633d	259.712977	-7.1334264	2.26845095	-3.1446245	0.001663	0.01019578	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Peptoniphilus	0.9999995
1793c262030940ef8d7de4599d906d45	72.2660567	-10.090947	2.2435548	-4.4977494	6.87E-06	0.00016842	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Peptoniphilus	0.9480701
6e92179702bc6544462503dfd79d022b	11.3857007	-7.4183026	3.03091632	-2.4475445	0.01438334	0.04905575	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Peptoniphilus	0.99892
4c99fef5d87efdb7206062e1bc247124	13.1840792	-7.6321906	3.03026649	-2.5186533	0.01178046	0.04184095	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Peptoniphilus	0.9997668
d93ea61c4a629ab942b4abdc4b5b1a70	26.8235197	-8.6594732	2.79122164	-3.1023954	0.00191961	0.01110788	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Peptoniphilus	0.9999992
83de68d0f4859b59a54ba066ac671d95	125.042985	-10.882887	2.54665588	-4.2734031	1.93E-05	0.00031982	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	unclassified_Peptostreptoco	0.9985041
699ba94858442a6ebca7bd8ddd1c5aa0	10.473318	-7.3036949	2.45386345	-2.9764064	0.00291648	0.01487117	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	NA	0.9982317
b6da58068d60acf36920270cd80cba5c	20.4073363	-8.2641394	2.47315466	-3.3415376	0.00083316	0.00630994	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	NA	0.974902
a0221024cf9db20d52c1ecb9c6e1d3c0	6.8143037	-6.6826662	2.4159497	-2.7660618	0.00567378	0.02434996	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	0.9999691
21a1896f566183d727a6e33ca7023a8d	1210.70188	-10.37741	2.19684213	-4.7237849	2.31E-06	7.01E-05	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	0.9999998
19f99c68c6abb4cb981924fbf60d75a5	186.225778	-7.2832979	2.209686	-3.2960782	0.00098045	0.00682338	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	0.9999999
5f7a083649c0a1155dc1b3398d91edd1	21.8195371	-8.361409	2.7685504	-3.0201397	0.00252658	0.01341432	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	0.9999996
3ed90c9b9f849540869fc261936cd973	29.2688167	-8.7864151	2.77545448	-3.1657572	0.0015468	0.00983458	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	0.9999996
c3df3aa9f2705257ec8d005bc57476ab	13.3201623	-7.6468631	3.03022532	-2.5235295	0.01161833	0.04174255	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	0.9999998
908377fa4ac1e33ebe757fca3a96a002	30.250793	-8.8330209	2.81680997	-3.1358242	0.00171372	0.01038312	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	0.9999998
ed0d4d27bee5335048cebc1224574d91	27.4004904	-8.6906998	2.78480621	-3.1207557	0.00180388	0.01080228	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	0.903261
5130a910dcaa85b075bea22bfe3ac4d8	22.9444753	-8.4332572	2.79545243	-3.0167772	0.00255477	0.0134256	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII	Mogibacterium	0.9767563
d4ba9ed1250f8bc3470b7daed843245d	15.3422883	-7.8498231	2.81838597	-2.7852193	0.00534915	0.0239549	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII	S5-A14a	0.7093122
f6f1ed607bf5f999da427b3bd010af4d	15.8288448	-6.7266431	2.63273573	-2.5550013	0.01061874	0.0390618	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII	[Eubacterium] nodatum gro	0.7453587
da4937671d7eb6da3511874d953b2d6e	21.7553218	-8.3561725	2.56661187	-3.2557211	0.00113105	0.00776653	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII	[Eubacterium] nodatum gro	0.7376313
b86eec312daa887b1c5355f00da8153b	31.8609159	-8.9085811	2.78968025	-3.1934058	0.00140605	0.00905146	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII	[Eubacterium] nodatum gro	0.8053724
dcf46733bc298c5f061c82fa7f960bcb	16.8900741	-7.9902774	2.78190177	-2.8722356	0.00407579	0.01891019	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII	unclassified_Clostridiales	0.9997477
aad4d4914c18cb51296cd236d7e321f5	65.3877151	-9.9468084	2.57134713	-3.8683258	0.00010959	0.00134372	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII	[Eubacterium] nodatum gro	0.738382
ff817999cbdfa330acaab0f7346b5941	7.96983535	-6.9017809	2.80444222	-2.4610173	0.01385437	0.0478859	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII	[Eubacterium] nodatum gro	0.7401633
025df0fea5e47da1e48840f8d4c72cc8	22.3485041	-8.3949033	2.21854668	-3.7839651	0.00015435	0.0018486	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII	Family XIII UCG-001	0.9480112
d19e6fe3dcb8ae3c0ec784f547a1d02d	9.06173134	-7.0886582	2.74299176	-2.5842798	0.00975826	0.03695223	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	[Eubacterium] coprostanolig	0.9287059
61c27952b659f928173da12e0ca4d4a3	5.02632677	-6.2359514	2.45674182	-2.5383015	0.0111392	0.04068572	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Intestinimonas	0.9448495
6704d55f23649d01763f5b078e61191f	10.124289	-7.2542783	2.7447512	-2.6429639	0.00821838	0.03206413	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	0.9999099
5d03476bf1ecc5c6a0fa9ed1bd9ad2c6	13.9009742	-7.708166	2.47475905	-3.1147137	0.00184123	0.01089926	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	0.9998747
c591f2cfdc76a144d9176ae20465e106	52.5768419	-9.632144	2.82425686	-3.4105057	0.00064843	0.00513752	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiracea	0.9998311
637cc14b4442251e542a81d440f5bb4c	19.8119696	-8.2210183	2.47127934	-3.3266245	0.00087905	0.00656101	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Hespellia	0.8884485
c7da083319d1cb5e351d16a7b079a1a1	24.6214912	-8.5367211	2.45770071	-3.4734584	0.0005138	0.00433258	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	0.99868
6014fb8522a813b40657f81b4b322949	37.1576159	-9.1307781	2.48494734	-3.6744353	0.00023838	0.00261199	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiracea	0.9973802
967cb3751471774d6bcdafd621fc21c1	8.22452075	-21.794985	3.03266654	-7.1867396	6.64E-13	4.88E-11	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Tyzzerella 3	0.9999821
e39cc8c4c9c9f23e8392193c79dbed8b	18.0559919	-8.0876703	2.76142003	-2.9288084	0.00340264	0.01668914	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto 1	0.9930479
59b1c8a4d94df30bf6b75b9781979a1f	127.925644	-8.6509688	2.47180336	-3.4998612	0.0004655	0.00399555	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Peptococcus	0.8086646
153a20d1e26ebb20ecd5264bfeaed8c6	128.406316	-10.921151	2.58756139	-4.2206345	2.44E-05	0.00035846	Bacteria	Firmicutes	Bacilli	Bacillales	Family XI	Gemella	0.9988221
9a5573e8614d2654dd57bdd4e770a8d5	30.2722845	-8.8341358	2.52029251	-3.5052026	0.00045626	0.00399555	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Macrococcus	0.9998405
66b056ab12d28bbd4b1389f2399507ed	26.8921078	-8.6628211	2.81273837	-3.0798531	0.00207103	0.01185088	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	0.9997952
871823399cd7e6090ecf02bd1dc41cdc	13.4587168	6.74105461	2.43023624	2.77382689	0.00554012	0.02423566	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	0.9999966
29994379322018f8581fcf166f49a1c5	9.46155913	-7.1517308	2.47887827	-2.8850674	0.0039133	0.01848944	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Bhargavaea	0.9810562
4059d37ae35a1befc66a6510f84b3e86	12.4747245	6.63090997	2.44241864	2.71489493	0.00662968	0.0270975	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Brevibacillus	0.9999982
dadde746de9a00ef38a1386d10287ad8	7.17254716	-21.628371	3.03360829	-7.129586	1.01E-12	6.48E-11	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	0.9986699
bb593b074d63577d87da32f808a68ced	14.3187907	-7.7497856	3.02994801	-2.5577289	0.01053582	0.0390618	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	0.9768594
bf6fd68e41ec09706d1bd81da77f483a	12.3090366	-7.5332967	2.4487432	-3.0763931	0.00209521	0.01185753	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Kurthia	0.751452
f82cc98d9b21d9e4aa182abe16475a43	37.7126514	-9.1529791	2.46892114	-3.7072788	0.0002095	0.00239759	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Kurthia	0.8226753
b7e52067bfc060ff546c72841f26ba3e	9.35587926	-7.1395574	2.72489073	-2.6201261	0.00878973	0.0340354	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	0.7820848
292fbe99d1a557b8032e9936ace25693	6.42706973	-6.5984056	2.17353429	-3.0357955	0.00239902	0.01300521	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Savagea	0.9998179
f2900f1b3e4767702c55206db69533ca	6.55398396	-6.6276973	2.41498247	-2.744408	0.00606201	0.02580113	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	0.9987536
e88525da9b48c3b7853711783ed2fcd8	16.3428408	-7.9440249	2.45076133	-3.2414519	0.00118923	0.00795391	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Savagea	0.9394795
22e97829644aef214c9ff443fe7b72cb	11.3733651	-7.4161872	2.77544531	-2.6720711	0.00753847	0.02975745	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	ZOR0006	0.8564104
fc5817026f13c76066be766c009afb6f	88.1788926	-10.378852	2.53319884	-4.0971327	4.18E-05	0.00056691	Bacteria	Firmicutes	Bacilli	Lactobacillales	unclassified_Lactobacillal	unclassified_Lactobacillales	0.9900712
9211dc5c88a30d0cd9fb8f5b125e9f10	6.28974398	-6.5781201	2.67023063	-2.4635026	0.01375869	0.04787704	Bacteria	Firmicutes	Bacilli	Lactobacillales	NA	NA	0.990939
f008eeee750e241b4303d391cb827292	408.237762	-6.3711647	1.86475808	-3.4166173	0.00063404	0.00510207	Bacteria	Firmicutes	Bacilli	Lactobacillales	unclassified_Lactobacillal	unclassified_Lactobacillales	0.9900401
7381d039da5e74ce9ceeb3b1a54df41f	13.2637203	-7.6420128	3.03023889	-2.5219176	0.0116717	0.04174255	Bacteria	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Aerosphaera	0.766088
724be21be867e73cd40562041cfa4c88	17.6868674	-8.0580641	2.77071336	-2.9082994	0.003634	0.01749076	Bacteria	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Aerosphaera	0.9192254
1690633623b1eaf16b5f7159e320abde	27.7733778	-8.7085066	2.21191767	-3.9370844	8.25E-05	0.001036	Bacteria	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	uncultured	0.9355587
5dc1187602180d1d1a39bd188fa4ee8d	294.734579	-12.120345	2.34154871	-5.1762086	2.26E-07	8.97E-06	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.9999674
42a4394680dd09f2b31288ce334552a5	23.4686072	-8.4662263	2.51113415	-3.3714751	0.00074767	0.00583408	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.9999539
2c96e598721d7212af28e5a03030fd14	154.233189	-11.185623	2.26315238	-4.9424967	7.71E-07	2.84E-05	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.7320687
8709c8058fe13c658f81978f89a69d2d	44.7593585	-24.106996	3.02738865	-7.9629671	1.68E-15	2.88E-13	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.7005234
8faaac9fceafa5b5746b952a355da29c	69.9260843	-10.044081	2.82362634	-3.5571566	0.00037489	0.00361716	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.7444515
522b62504fb41ab9fce76711483a3a23	74.1103771	-24.787522	3.02691355	-8.1890418	2.63E-16	6.78E-14	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.7452057
e0922853e9f829bc24e142e4d7f97299	4.24864746	-5.9891996	2.45234195	-2.4422367	0.01459657	0.04945549	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.999995
797f2689d133ffa53e523f53510686a4	12.4562635	-7.5533484	2.43404566	-3.1032074	0.00191435	0.01110788	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.8396119
ca75e7982e5a1527fd8989ff065e2be8	15.1123093	-7.8299884	2.75227108	-2.844919	0.00444227	0.0203217	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Pediococcus	0.8237103
d2e650a496a452b8634a864c32729f6e	16.5379269	-7.9618946	2.52134553	-3.1577959	0.00158967	0.00989818	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.7921263
fec8a59a5e2636b5690bd66fa4d28ac5	51.6186888	-9.6059384	2.18488497	-4.396542	1.10E-05	0.00022658	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.9999982
136743c3e440dafe529820bddecac6ee	36.7799947	-9.1184211	2.14610003	-4.2488332	2.15E-05	0.00033535	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.9999549
9a891705636a19d582ac09fe289337eb	84.4453285	-10.316234	2.22401312	-4.6385671	3.51E-06	9.03E-05	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.9986214
3f248bf8acd2db8d1df8d1354b2e4564	34.3374291	-9.0174638	2.45344369	-3.6754313	0.00023745	0.00261199	Bacteria	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Weissella	1
7366ec88f0a2b7621b2aa6fb8b08fd60	53.7326549	-9.663037	2.24919316	-4.2962237	1.74E-05	0.00031954	Bacteria	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Weissella	1
6c105a94fc00421b0a46a19d8d79e8c1	9.47949161	-7.1577088	2.44786544	-2.9240614	0.00345497	0.01678592	Bacteria	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Leuconostoc	0.9999776
9f2798d9ad8a4ebf69d89d641a1abae2	21.3718814	-8.3301434	2.51706877	-3.309462	0.00093475	0.00682338	Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Trichococcus	0.951824
ada2fe787c9abdd697ea94ff66db9abc	1142.58502	-10.622727	2.1824417	-4.8673588	1.13E-06	3.64E-05	Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Carnobacterium	0.9995522
260bc5249ef3254024dc6889312180cf	11.9684844	-7.4929636	2.74325073	-2.7314177	0.00630625	0.02598175	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Vagococcus	0.953075
ceac3908d974405676fcdc7b33321d23	202.572221	-11.579229	2.18741952	-5.2935564	1.20E-07	5.15E-06	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Vagococcus	0.9435546
7e47bf3f2b44d3c6257dee626847d6c8	34.4251535	-9.019656	2.50876076	-3.5952635	0.00032406	0.0032724	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Vagococcus	0.9707185
904bfa407d99ab103dfd2e073f5fb2f9	13.4515539	-7.6600725	3.03018862	-2.5279194	0.01147407	0.04161371	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Vagococcus	0.9918009
ff0afb2bc0253d2593687845af8e68cc	9.04699945	-7.0945196	2.7196817	-2.6085845	0.00909176	0.03494219	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Vagococcus	0.9913665
b57f3b429c5430f42b54c8afdd67f057	1009.43561	-7.3493212	2.11831679	-3.4694155	0.00052159	0.00433258	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Vagococcus	0.9955289
e585101e6532641e7dfee3188b1a71b3	23.3297266	-8.4579724	2.77300127	-3.0501149	0.00228754	0.01266755	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Vagococcus	0.9811852
91498ffb72ad0ea433902ecf90153e67	20.2964155	-8.2561937	2.77743658	-2.9725949	0.00295294	0.01490945	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Vagococcus	0.9911226
41f4046a0e9aa52ccbdfbe60060dcf58	49.7875777	-9.5525562	2.2351164	-4.2738518	1.92E-05	0.00031982	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.905288
563ef7bf0750eb6f42f1d182438b9e9d	1529.77199	-8.2142007	1.9220531	-4.2736596	1.92E-05	0.00031982	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Anthococcus	0.9833737
3425f45224524ba338dd02e51bae862a	86.2557271	-10.34666	2.21382699	-4.6736534	2.96E-06	8.02E-05	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	0.9999989
666cb8015f46e7200eebe9079df6541a	19.8086834	-8.2215684	2.76932377	-2.9688	0.00298965	0.01494826	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	0.7204718
247100cf411e1f928662cdf314d9d83e	220.437001	-7.359172	2.29011344	-3.213453	0.00131149	0.0085496	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	0.7691583
1363a0bcf0aba5c5a24dbf3164135cad	171.303326	-6.3218063	2.27383866	-2.7802352	0.00543195	0.024116	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	0.9999779
264c57e2d0c321eb391238dc5118b35b	125.870467	-6.4282769	2.26051193	-2.8437262	0.00445894	0.0203217	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	0.9998157
d0729897b3d78e838d5e586446d9e630	24.2853427	-23.273602	3.02840161	-7.6851109	1.53E-14	1.68E-12	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.9997554
70ac1b0517a99686ff342d18beb48705	276.024898	-8.2346566	2.49746676	-3.2972037	0.00097653	0.00682338	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.9678453
e425485d6bf3d277950ae02bf562f6a9	13.588569	-7.6741776	2.80487579	-2.7360133	0.00621885	0.02598175	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.9995549
e178344644f160878185d6a2a1b9a796	25.86116	-8.6067424	2.84465896	-3.0255797	0.00248157	0.01331259	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.9283919
666cdd1315e66506c3eb3d0661809419	24.2060849	-8.5108988	2.79462792	-3.0454497	0.00232333	0.01272886	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.8487424
3397061d8eb22e1a6f6601040296fa35	506.057594	-10.463844	2.50648212	-4.1747131	2.98E-05	0.00042682	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.7050089
d827a1500678e0bb1e189b4964395318	19.6959727	-8.2123525	2.48811132	-3.3006371	0.00096466	0.00682338	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.9273195
ec63929d89e0b4b65a77539d99305870	146.89977	-11.115451	2.55920011	-4.3433301	1.40E-05	0.00026768	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.9552409
2c595a2f852c6c4b428e63e2eb16d6ed	855.033928	-9.3686816	2.09516626	-4.4715695	7.76E-06	0.00018177	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.8996151
e88aa42af2623de81a1d2adfe40ce97d	175.62698	-11.373219	2.59561345	-4.3817077	1.18E-05	0.00023324	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.9353917
03684874a215ab7f37c8e49a33d107e9	111.3377	-10.715189	2.28514254	-4.6890679	2.74E-06	7.85E-05	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.9601651
f8572087e63f1182a98f1d12362c9c00	8.44008552	-6.9882136	2.73321522	-2.556774	0.01056478	0.0390618	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.8524926
abe5e885327da25efdd707804f580d81	12.3292023	-7.5358948	2.75790176	-2.732474	0.00628606	0.02598175	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.788357
0a93e4c1c5da4e83419083d1f3497b5b	11.5083352	-7.4372978	2.74235338	-2.7120129	0.0066876	0.027119	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.9999993
dff44a84f65b25cb944a2212970ace15	6.78331	-6.6786053	2.71102446	-2.4634987	0.01375884	0.04787704	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.8351762
076aa69088cd3bb4cef4f79e9838c167	35.6706698	-9.0707783	2.22605164	-4.0748283	4.60E-05	0.00060807	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.9999992
70fb48995feaf5e8c01cd6d47241b95c	16.7605378	-7.9789175	2.7740111	-2.8763106	0.00402354	0.01883746	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.9999995
261ac1b7cb08b76a2760cd9ea663c765	82.2805151	-6.4837696	2.12455804	-3.0518204	0.00227458	0.01266755	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.7758578
d7d0e3769808620bd1c7ce0206d5f86f	21.705434	-8.3524095	2.57635114	-3.2419531	0.00118714	0.00795391	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.9979253
5c06b34c3ce81d3ed79e1ea5f450f97e	27.1436333	-8.6758144	2.50414203	-3.4645856	0.00053105	0.00434112	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.9967558
6ecbb0f43c6a404b5d11668d28fac09c	83.7275195	-7.8802445	2.12355232	-3.7108784	0.00020654	0.00239759	Bacteria	Firmicutes	Bacilli	Bacillales	Listeriaceae	Brochothrix	0.7604109
235016cc3f38b40648baf5d8578dba00	8.81595081	-7.0512423	2.73564659	-2.5775414	0.0099506	0.03740553	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.9966583
224c8907fe02083e93b1a528dd5d4a6a	11.8815497	-7.4790711	3.03072185	-2.4677524	0.01359643	0.04787704	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.9744694
808ca464e39159546f971a5d9a2f70cc	29.7458685	-8.8085602	2.50316026	-3.5189757	0.00043322	0.00391415	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.9129886
e54c15f5bf09acf28d4d472050b5712e	43.7024627	-9.3646916	2.19672279	-4.2630284	2.02E-05	0.00032457	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.9965381
3d0ad79566c0b9fe0fb896e4d4e8c2f2	49.5424322	-9.5463061	2.15606165	-4.4276591	9.53E-06	0.00020441	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.9819841
7d8e8a7c64a5dc733b7eed561ece7050	69.8801962	-10.042433	2.25137184	-4.4605837	8.17E-06	0.00018302	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.997642
fc281204b7ef0a6f75be499497f0aa99	185.879398	-8.8713784	2.09516714	-4.2342104	2.29E-05	0.00034741	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.9987112
40697a5109cd22aa076d57fa6b54519a	19.2820913	-8.1827535	2.76337543	-2.9611443	0.00306498	0.01517756	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.998355

Table S5: Differentially abundant ASVs found in PMA treated and control mucosal samples.

ASV	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Domain	Phylum	Class	Order	Family	Genus	Confidence
abedeacac6b0ef8a19a2bc2dbe3026b1	5.54513016	-21.22928183	3.03580312	-6.9929705	2.69E-12	1.26E-10	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter	0.9961402
fb19a46264758f4fc0ed743d266be883	18.8857399	-22.63352765	3.02904612	-7.4721634	7.89E-14	4.94E-12	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	0.9133922
40afea1c0f80c98d2f97be0c6d4b83b2	23.5228635	-23.23003819	3.0284849	-7.6705148	1.71E-14	1.61E-12	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	0.946339
8f55eebbcb9fc1eaee5d71b4b6437 5d2	34.5561843	-23.75138962	3.02775372	-7.8445558	4.34E-15	8.17E-13	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	0.9804048
c6972512b70f3cab04ac837d5d4a4489	14.43252	-7.681411075	2.7168185	-2.8273553	0.00469342	0.04411817	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Peptoniphilus	0.9436652
4857183803c4b5ac8d1f6bd31e3d633d	25.208419	-8.485823465	2.15927543	-3.9299403	8.50E-05	0.00145216	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XI	Peptoniphilus	0.9999995
21a1896f566183d727a6e33ca7023a8d	116.042131	-10.68791648	2.21413785	-4.8271233	1.39E-06	3.72E-05	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	0.9999998
19f99c68c6abb4cb9819 24fbf60d75a5	22.8259745	-8.3433266	2.71635145	-3.0715196	0.00212972	0.02355222	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	0.9999999
59b1c8a4d94df30bf6b75b9781979a1f	12.7520863	-7.503688193	2.70580474	-2.7731817	0.00555111	0.04969567	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Peptococcus	0.8086646
fc5817026f13c76066be766c009afb6f	18.3973044	-8.033360557	2.72277998	-2.9504259	0.00317336	0.03139958	Bacteria	Firmicutes	Bacilli	Lactobacillales	unclassified_Lactobacillales	unclassified_Lactobacillales	0.9900712
f008eeee750e241b4303d391cb827292	46.2344595	-9.360556993	2.13270043	-4.3890632	1.14E-05	0.0002378	Bacteria	Firmicutes	Bacilli	Lactobacillales	unclassified_Lactobacillales	unclassified_Lactobacillales	0.9900401
ada2fe787c9abdd697ea94ff66db9abc	104.634594	-10.53864053	2.24497573	-4.6943227	2.67E-06	6.29E-05	Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Carnobacterium	0.9995522
ceac3908d9744056 76fcdc7b33321d23	25.4789016	-8.500094253	2.45900848	-3.4567161	0.0005468	0.00788131	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Vagococcus	0.9435546
b57f3b429c5430f42b54c8afdd67f057	123.927084	-10.78284003	2.18296674	-4.9395347	7.83E-07	2.45E-05	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Vagococcus	0.9955289
563ef7bf0750eb6f42f1d182438b9e9d	179.758293	-11.31927799	2.18762789	-5.1742246	2.29E-07	8.61E-06	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Anthococcus	0.9833737
1363a0bcf0aba5c5a24dbf3164135cad	20.4212701	-8.18154094	2.44527378	-3.3458589	0.00082028	0.01028086	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	0.9999779
264c57e2d0c321eb391238dc5118b35b	21.6487824	-8.266434771	2.74018902	-3.0167389	0.0025551	0.02668658	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	0.9998157
70ac1b0517a99686ff342d18beb48705	24.9182953	-8.469252463	2.46371491	-3.4375944	0.00058691	0.00788131	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.9678453
3397061d8eb22e1a6f66010 40296fa35	24.3933332	-8.43903773	2.16709655	-3.8941679	9.85E-05	0.00154374	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.7050089
2c595a2f852c6c4b428e63e2eb16d6ed	82.3070843	-10.19244968	2.51396654	-4.0543299	5.03E-05	0.00094523	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.8996151
fc281204b7ef0a6f75be499497f0aa99	16.8617159	-7.907743437	2.39454541	-3.3023986	0.00095862	0.01126375	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.9987112