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The Impact of Diet & Temperature on Yellowtail Kingfish Health & Microbiome

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Ethics & Biosafety Statement

All experimental protocols and procedures involving animals were carried out in accordance with the requirements of the Department of Primary Industries (Fisheries) Animal Care and Ethics Committee (NSW DPI ACEC Authority 93/5). The animal ethics committee, Macquarie University, Australia, noted the collaborative report (reference no: 520180534618).

All experimental protocols and procedures were carried out in accordance with the approved biosafety application for this project (reference no: 52017008997).

Statement of Originality

This is to certify that to the best of my knowledge; the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

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Abstract

Negative health outcomes related to plant-based proteins remain a barrier to effective fishmeal replacement in farmed carnivorous fish such as Yellowtail Kingfish (*Seriola lalandi*). Here, farmed Yellowtail Kingfish housed at optimal and non-optimal temperatures (22 and 26°C) were fed a fishmeal diet (FM) or a FM diet partially replaced with soy-protein concentrate (SPC) to investigate impacts on host health and microbial community composition within skin mucosa, gut mucosa and digesta. The combination of SPC and elevated temperature significantly reduced weight gain and measured digesta myeloperoxidase and increased plasma lysozyme levels. Skin microbial communities were distinct from and more diverse than the gut and digesta microbiomes, which both had low diversity. The overall microbial composition and relative abundance of specific OTUs were significantly impacted by SPC and elevated temperature. The SPC diet and elevated temperature were both associated with significantly increased levels of an OTU identified as *Photobacterium* in the digesta and skin. Increased relative abundance of *Photobacterium* was also significantly correlated with reduced levels of digesta peroxidase, an innate immunity defence mechanism. The shifts in the microbial communities and the increase in *Photobacterium* reveal the importance of considering the microbiome in future efforts to replace fishmeal in Yellowtail Kingfish diets.

1. Introduction

1.1 Aquaculture & Sustainability

Aquaculture, with its roots in ancient China over 4,000 years ago, is now a vast global industry which provides over half of all fish for human consumption (77m tonnes in 2015) [1]. Per capita fish consumption has doubled in the last 50 years, with aquaculture providing the bulk of the fish to fuel this rise [2]. Heightened demand together with the development of improved technologies has led to the diversification of aquaculture species and the successful farming of several highly desirable pelagic carnivorous species, such as Bluefin tuna (*Thunnus thynnus*) and Yellowtail Kingfish (*Seriola lalandi*).

The Yellowtail Kingfish is a large carnivorous pelagic bony fish of the genus *Seriola* found in subtropical and temperate waters of the Pacific and Indian Oceans [3, 4]. Historically, farming of *Seriola* species has occurred in Japan due to the desirability of their strong flavoured and firm flesh [5]. Commercial Yellowtail Kingfish aquaculture operations have recently commenced in Australia, Chile, and New Zealand due to targeting of the species for aquaculture at a national level [6]. Yellowtail Kingfish are good aquaculture candidates due to high growth rates and good cage adaptability; nonetheless, farming routinely exposes Yellowtail Kingfish to stocking densities, temperatures, and diets that are at odds with their pelagic and carnivorous lifestyle [7, 8].

Farmed fish often experience conditions that are drastically different from those of their natural environment [9]. These differences can arise due to the inclusion of plant-based raw feed materials in their diets, farming in water temperatures outside their normal range, and stocking at high densities [10, 11]. These changes can have negative impacts on health, immunity and growth, thus reducing farm production and fish well-being [12, 13]. Understanding what drives these negative outcomes is a key priority for the aquaculture industry. There are likely numerous causative factors contributing to these issues; however, recent evidence suggests the commensal microbiome plays a key role [14].

1.2 The Fish Microbiome

Microorganisms inhabit virtually every mucosal surface of vertebrates, forming complex communities termed the ‘microbiome’. The microbiomes of mucosal surfaces are mostly comprised of bacteria, but also include other microorganisms such as viruses, archaea, and fungi [15]. The microbiome can consist of incredibly dense populations of microorganisms (e.g. greater than

$10^{12}/cm^3$ in the human gut) and often outnumber all other cells in the host body [16]. In recent years it has become clear that these microorganisms are not simply opportunistic residents, rather they appear to form organised commensal populations that have a long evolutionary history with their host [17, 18]. The microbiota of vertebrates is both diverse and distinct based on factors such as diet, phylogeny, and host morphology [19, 20]. Whilst much consideration has been given to the importance of these microbial communities in humans and economically important terrestrial vertebrates, it is becoming increasingly clear that the microbiome influences the health of a wide range of species, including fish [21, 22].

The mucosal microbiota of fish comprises a diverse community of microbial species that are rapidly established during the larval stage and, once fish are fully developed, appear to remain stable and species dependent throughout life [20, 23]. During the early developmental stages, the composition of the microbes in the rearing water heavily impacts fish microbial populations; however, as maturity is reached these populations stabilise, and the influence of the surrounding environment is greatly reduced [24, 25]. Distinct populations appear to be formed through host manipulation of the microbiota, resulting in complex communities, especially in the skin, gill, and gut mucosa [26, 27]. Despite significant research efforts in a variety of vertebrates, the mechanisms utilised by host organisms to shape their resident microbial populations remain uncertain [23, 26, 28]. It is likely that the adaptive immune system of vertebrates provides a memory-based system to select for microbes that are innocuous yet beneficial for the host [28, 29]. This mechanism is potentially relevant for bony fish as they share homologs of many immunoglobulins with other vertebrates, including humans [30, 31]. The composition of the mucosa, based upon the glycosylation of the mucins present, also determines the structure of the mucosal environment and thereby influences the composition of the microbiota present [32, 33]. Whilst it is not entirely clear how hosts manage their complex microbiomes, the resulting microbiota has significant commensal advantages for fish, influencing nutrient availability, nutrient assimilation, the immune system, gut health, and susceptibility to infection [21, 23, 34, 35].

1.2.1 Protection from Infection

The mucosal surfaces of fish (skin and gut), are the primary line of defence against pathogenic invasion and colonisation [23]. These surfaces prevent infection through providing a physical barrier that is difficult to breach and by housing a variety of adaptive and innate immune molecules that the gut epithelium secretes [26, 28, 31]. The commensal microbiota that inhabits these mucosal surfaces also plays a key role in protection from infection [26]. Well established microbial

communities form biofilms and strongly linked nutrient networks that competitively exclude new microorganisms through utilisation of key resources and space [36]. Commensal bacterial species also provide defence through the production of antimicrobials such as organic acids, antifungals, and antibiotics [21, 37]. As such, maintenance of a healthy microbiome across all mucosal surfaces provides a microbial 'buffer' that plays a key role in ensuring protection from pathogens.

1.2.2 The Immune System

The commensal microbiome has an intimate relationship with the fish immune system, facilitating its development and continuing to influence immune status throughout adulthood. Gnotobiotic studies involving zebrafish (*Danio rerio*) highlight the role the microbiome plays in stimulation of the immune response and lymphoid tissue growth. Germ-free zebrafish have severely impaired immune system development which is likely due to the lack of a commensal microbiome [23]. The microbiome of mature fish can influence the levels of antibodies, complement, cytokines, and stimulate leucocyte phagocytosis and B cell proliferation, leading to increased effectiveness of the fish immune response to potential infection [23, 38, 39]. The specific molecular mechanisms through which the microbiota stimulate fish immune response and development are not fully characterised; however, bacterial amino acids, lipids, and carbohydrates are thought to be central [38, 40]. This intricate commensal association is the product of a long evolutionary history and has wide-ranging impacts on maintenance and development of a healthy immune system [41].

1.2.3 Digestion and Nutrient Uptake

The microbiota of the gut of vertebrates, including fish, plays a role in assisting with digestion of complex indigestible carbohydrates and in the production of substances such as vitamins and short-chain fatty acids that are absorbed through the intestine and utilised by the host [21, 42]. In this way, the microbiome extends the hosts digestive ability and provides valuable nutrient production capacity [43, 44]. For example, in mice, microbial degradation of indigestible dietary fibre can produce short-chain fatty acids that have valuable calorific content and can be immunomodulatory [45]. Short-chain fatty acids are relevant for fish health and nutrition, and supplementation with these nutrients has been shown to positively impact growth and immunity [46].

Aside from assisting with breaking down food, the microbiome also impacts the host's ability to absorb the nutrients present. Gnotobiotic studies in zebrafish have shown that the composition of the microbiome influences the uptake of fatty acids and the formation of epithelial fat droplets [47]. Efficient absorption and retention of fats (i.e. fatty acids) by aquaculture species is critical to the industry insofar as it offsets the cost of incorporating expensive lipids such as fish oil into aquafeeds

and it satisfies consumer demand for fat-rich fish, high in n-3 long chain polyunsaturated fatty acids [48]. Similarly, the microbial composition of the gut also impacts the uptake of proteins in feeds, which are a high cost component of fish diets [49]. Changes in the composition of the microbiome appear to influence the uptake of proteins and fats in fish, potentially impacting fish growth and meat quality [22, 38, 49].

There is now good evidence that the composition of the gut microbiome influences host digestion and nutrient assimilation [22, 27, 38]. However, the mechanisms by which this influence is achieved are still poorly understood and may vary considerably between fish species [34].

1.2.4 Gut Epithelial Health

Maintenance of a healthy intestinal mucus layer and epithelium is essential for disease resistance and efficient feed assimilation [7, 50]. The gastrointestinal microbiota significantly impacts development and preservation of gut morphology, inducing alterations in blood vessel and goblet cell density and mucus-layer properties [49, 51]. These changes, in turn, impact the effectiveness of the mucus layer as a barrier to prevent pathogenic colonisation and the level of secretory immune-related molecules present in the mucus. Commensal microbiota also impact the rate of epithelial cell proliferation, thus contributing to a healthy gut wall that is better able to absorb nutrients and maintain a sufficient population of immune cells [27, 52]. The microbiome, especially in the gut, modulates mucosal thickness and the underlying characteristics of the epithelium, contributing to disease resistance and assisting the gut in absorbing of nutrients [35, 49, 53].

1.3 Analysing the Microbiome

Historically much of the work performed on the microbiome in fish and other species has been through conventional cultivation techniques [27]. Estimates indicate that less than 1% of gut microbial species can be cultivated and therefore studies utilising culture-dependent techniques do not provide a complete representation of the fish microbiome [27]. The advent of next-generation DNA sequencing technologies has revolutionised the study of the microbiome and has facilitated the development of techniques for determination of the composition of the entire microbiome [54].

The vast majority of microbiome studies using next-generation sequencing (NGS) have utilised the small-subunit ribosomal RNA (16S rRNA) gene to taxonomically determine the species of bacteria and archaea present in the microbiome [14]. Both the Human Microbiome Project and the Earth Microbiome Project use the 16S rRNA gene for microbial population assessments [55, 56].

Sequencing of regions of this gene allows phylogenetic comparison of populations as well as providing details on diversity, richness, and relative abundance [14]. NGS microbiome reads are assigned likely phylogeny through bioinformatic tools that make use of reference databases containing 16S rRNA gene sequences that have taxonomic information assigned [57, 58].

The wide use of the 16S rRNA gene in microbial studies provides a platform for comparison between studies; however, there are a number of factors that hamper this. Firstly, 16S rRNA genes differ in their genomic copy number which can potentially result in inaccurate estimation of relative abundance dependent on copy number [59]. Secondly, the storage of samples and extraction technique, such as the brand of commercial extraction kit used, can impact the composition of microbial communities observed [60, 61]. For instance, multiple studies have shown that the method of cell lysis used during extraction impacts observed community structure, with mechanical lysis being the most effective at capturing the community accurately [14, 62]. Thirdly, different NGS platforms each have their relative limitations and are partially error-prone, meaning inferred composition may be affected by the sequencing platform [63]. Finally, the methods used to bioinformatically analyse the sequence data can impact the observed microbial composition [64]. Allali and colleagues (2017) found that the choice of bioinformatic analysis tool influenced the inferred diversity and abundance; however, each resulted in comparable biological conclusions [65]. Despite the aforementioned methodological issues associated with 16S rRNA studies, the 16S rRNA gene remains the target of choice for microbiome research due to its ubiquitous nature and the wealth of phylogenetic information available [14].

1.4 The Composition of the Fish Microbiome

Fish microbiomes are complex and dynamic, and their composition can vary greatly between species and even body sites of the same individual [35]. As fish pass through different developmental stages, there are corresponding shifts in the complexity and composition of their microbiome. Initially, the microbiome is strongly dictated by the rearing environment, as demonstrated in experimental studies in Nile tilapia (*Oreochromis niloticus*) larvae [24]. Following initial colonisation, the microbiome shifts in response to host manipulation and adult lifestyle, with the composition becoming increasingly distinct from the environment [25]. This adult microbiome shares few operational taxonomic units (OTUs) with the surrounding water and is generally species-specific [27, 34].

The composition and richness of the microbiome is often distinct between fish species [34]. A number of factors drive these differences, including host genetics and functional niche [20]. Whilst there are clear differences between species and between body sites of the same individuals, there remain several core bacterial taxa that are common [14]. The core microbiome is often more similar in closely related species, reinforcing the theory that the microbiome plays a key role for the host; however, the functionality of these 'core' taxa and how they contribute to host biology remain unclear [20, 34].

1.4.1 The Gut

The gastrointestinal tract is a tubular structure running from mouth to anus, with a primary function of digestion and absorption of nutrients from ingested food [66]. The gastrointestinal tract of carnivorous teleost fish is relatively simple and short in comparison to that of most vertebrates and is generally separated into seven main compartments: oesophagus, stomach, pyloric caeca, proximal intestine, mid intestine, distal intestine, and rectum [66]. Figure 1 details the main sections of the Yellowtail Kingfish gastrointestinal tract. The structure of the tract varies along its length dependent on the function of the specific region. The intestine of fish consists of the submucosa, being composed of connective tissue, blood vessels and nerves that has been coated with a mucosal lining secreted from goblet cells [67].

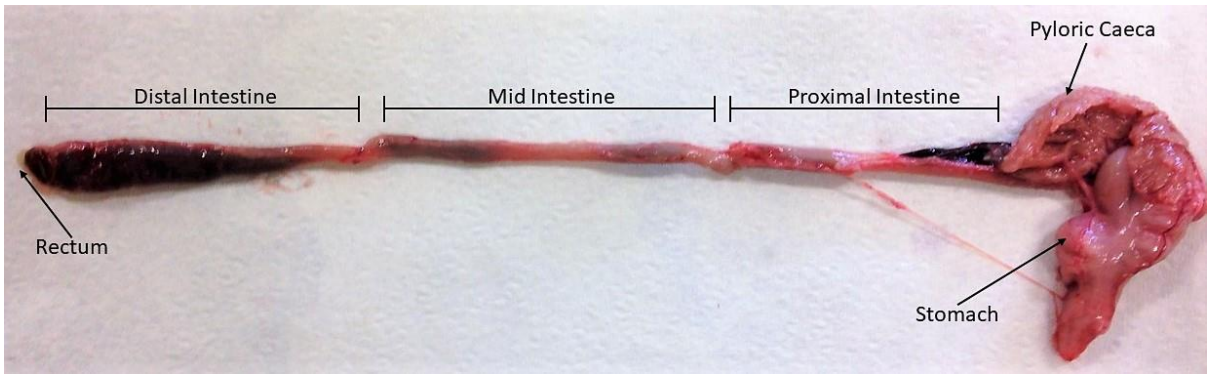

Figure 1 | Gastrointestinal tract of the Yellowtail Kingfish.

Current information indicates much of the fish gut is colonised by anaerobic and obligate anaerobic bacteria, with the dominant phylum being *Proteobacteria* [35, 68]. *Bacteroidetes, Actinobacteria, Firmicutes*, and *Fusobacterium* are also commonly present at high levels and, combined with *Proteobacteria*, can make up 90% of the microbial population of the gut [14, 27]. Whilst these phyla are predominant throughout, the fish gut is not a homogenous environment [26]. The microbial communities of the gut are separated into two main categories that differ in composition and richness: allochthonous (digesta), those which are transient and pass through the gut with food; and

autochthonous (gut mucosa), which are resident and intimately associated with the mucosa [34, 35]. In Atlantic salmon (*Salmo salar*), the digesta bacterial community was found to be richer, harbour more diversity, and have a significantly different composition than that of the gut mucosa [69]. Similarly, the gut mucosa and digesta microbial communities of rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchus labrax*) were distinct [70, 71]. Differences in composition between the gut mucosa and digesta communities are likely driven by the environment in which the bacteria reside. The autochothonous bacteria inhabit a complex layer of mucins secreted by goblet cells [26]. The physical properties of this mucus layer are determined by the patterns of glycosylation on the O-glycans of the mucins [72]. The mucus layer also contains antimicrobial defence molecules such as myeloperoxidase, cytokines, and antibodies [31, 73]. The composition of mucins and antimicrobials influences the habitability of the gut mucosa, therefore impacting the microbial community present [23, 26]. In contrast, the digesta is comprised mainly of food matter at different stages of digestion depending on the region of the gut [34, 35]. This environment is dictated mainly by the contents of the diet; however, the digesta also contains secreted immune defence molecules and digestive enzymes that impact microbial composition [23, 27, 41].

The composition of the gut microbiome also differs between different regions of the gut and generally increases in complexity and richness towards the distal intestine [27]. Differences in composition of phyla and species richness are evident between the gut regions in both gilthead sea bream (*Sparus aurata*) and Atlantic salmon [69, 74]. A recent literature review by Tarnecki and colleagues (2017) indicated that many studies are not accounting for the distinct differences between different sections of the gut [34]. A number of studies included in the review analysed the allochthonous and autochthonous communities together and many did not sufficiently describe the region of the gut sampled [34].

1.4.2 The Skin

The skin mucosa of fish acts as the primary barrier separating the skin epithelia from the surrounding environment, and therefore the maintenance of the mucosa is key for health [75]. The skin mucosa is relatively similar in composition to that of the gut mucosa in that it is comprised of mucins and a number of immune related defence molecules [75, 76]. A complex community of microbes inhabits external mucosal surfaces, and these play a significant role in protection from disease through immune homoeostasis and competitive exclusion [77]. The skin microbiome of a number of fish species from wild and aquaculture-reared specimens has been characterised, focusing on both healthy and diseased animals [76, 78, 79]. This work indicates that there is considerable variability

in skin microbiome community composition between species, and within species exposed to different environments, stressors, and diets [35, 80]. As with the gut, the composition of the skin microbiome is dependent on a variety of factors such as environment and phylogeny [78]. Investigations to date show that, similar to the gut, the dominant phylum associated with the skin mucosa is *Proteobacteria*, followed by phyla including *Firmicutes, Actinobacteria*, and *Bacteroidetes* [78, 79]. These dominant phyla were species dependent and independent of the surrounding water, as evidenced by surprisingly few shared OTUs with the water column [76, 78].

1.4.3 The Yellowtail Kingfish Microbiome

Ramirez and Romero (2017) recently published the first microbiome study involving Yellowtail Kingfish using next-generation sequencing rather than cultivation techniques [81, 82]. These authors investigated the differences in the faecal (digesta) microbiome between healthy wild and aquaculture reared Yellowtail Kingfish using high-throughput sequencing of the V4-region of the 16S rRNA gene. They report a significant difference between wild and aquaculture fish, with the dominant phylum in farmed fish being *Firmicutes* in comparison to *Proteobacteria* in wild caught fish [82]. The differences in microbial composition between wild and aquaculture Yellowtail Kingfish indicate a strong influence of diet and environment on the digesta microbiome, potentially impacting microbial functionality. Although research has been carried out on the digesta microbiome, no study investigating the gut mucosal microbiome of Yellowtail Kingfish exists.

To date there has only been one study investigating the composition of the skin and gill microbiome of Yellowtail Kingfish. Legrand and colleagues (2018) used high-throughput sequencing to determine the composition of the skin and gill microbiome of farmed and wild Yellowtail Kingfish with varying levels of enteritis [83]. As with other fish species, *Proteobacteria* was the dominant skin phylum; however, the relative proportions of other common phyla, such as *Bacteroidetes*, differed from the skin microbiome of other fish species [78, 83]. Microbial composition significantly differed between healthy fish and those exhibiting signs of enteritis, indicating that the microbiome may be a suitable indicator of health status [83]. As with the gut microbiome of Yellowtail Kingfish, the skin microbiome differed between wild and farmed fish, suggesting that environment and diet also influence the skin microbiome [82, 83].

1.5 The Impact of Aquaculture on the Microbiome and Fish Health

The fish mucosal microbiome can provide significant symbiotic benefits to the host; however, when this composition shifts unfavourably it can negatively impact health, nutrient absorption, and immunity [27]. Adverse shifts in microbial populations can expose fish to an increased risk of

pathogenic infection through reducing competitive exclusion and dampening the readiness of the immune response, which is usually stimulated by commensal microbial populations [38]. These shifts can also result in reduced nutrient digestion and damage the associated epithelial layer [84]. Negative shifts in microbial composition can be caused by a variety of factors such as changes in environmental factors and diet and exposure to stressful situations [20, 85].

1.5.1 Alternative Feeds and Fish Health

With the rise in aquaculture production globally, there has been a corresponding increase in the demand for fish meal and fish oil that comprise the majority of aquaculture diets [10, 86]. The growth in farming of high metabolic demand species such as Yellowtail Kingfish that require high levels of feed has exacerbated the growth in demand [87]. Increased demand and dwindling fish stocks mean that resources of wild fishmeal and fish oil are not sufficient to sustain the aquaculture feed industry, resulting in inflated prices [87]. The high cost of fishmeal and fish oil has created an economic incentive to diversify aquaculture feeds, with the aim of finding suitable and cost-effective alternatives to fishmeal and fish oil based aquafeeds [10].

Many plant-based products, including soybean products and those produced from legumes, have emerged as potential substitutes for fish meal [88, 89]. These plant-based protein sources are both cheaper and often more sustainable than fish meal protein sources, resulting in lower feed costs and higher consumer acceptance of aquaculture products [10]. Whilst dietary inclusion of plant proteins in fish diets has developed considerably and is more suited for species naturally exploiting herbivorous or omnivorous niches they are not wholly suitable for many carnivorous fish [89, 90]. Carnivorous fish have not evolved a gut that is suited for the consumption of plant-based raw feed materials, and elevated levels can cause gut epithelial damage, reduce nutrient digestibility, and affect fish immunity [90]. Many of these issues are due to the anti-nutritional factors contained in unrefined plant proteins [91]. Anti-nutritional factors are a broad range of compounds found in some agricultural plants that negatively impact protein, vitamin, and mineral utilisation, thus influencing digestion and growth [91, 92] Methods of raw feed material refinement or improvement, such as alcohol extraction and cooking, can remove or reduce some of these anti-nutritional factors. Refined plant products are often more suitable for carnivorous fish [7, 93]. For example, the use of unrefined soy products in Yellowtail Kingfish diets had a negative influence on gut histology when compared to refined soy products and have been found to contribute to the development of enteritis [7, 94]. Given this, the use of less refined products like soybean meal is not considered suitable for Yellowtail Kingfish [94]. However, while more refined, higher quality plant

proteins are considered more suitable, there often remain negative side effects at higher feed inclusion levels [91]. Plant-based protein sources also lack many of the additional components of fish meal that, whilst poorly understood, are believed to promote epithelial health, efficient feed digestion and nutrient assimilation [93, 95].

In Yellowtail Kingfish, replacement of fish meal with soybean meal and soy protein concentrate has been shown to negatively impact fish growth and health [94, 96]. Stone and colleagues (2018) observed a significant reduction in growth and myeloperoxidase levels (an innate immune defence molecule) in Yellowtail Kingfish fed a diet including 30% soybean meal [94]. Similarly, Yellowtail Kingfish fed soy protein concentrate experienced reduced growth; however, this reduction in growth was only noted in fish fed diets in which soy protein concentrate was greater than 20% [96]. Interestingly, dietary inclusion of 46% soy protein concentrate had no significant impact on closely related longfin Yellowtail (*Seriola rivoliana*). Studies in other species have also shown that fishmeal replacement with a variety of plant proteins is appropriate up to a limit and that inclusion at higher levels has a negative impact on fish growth and health [93, 95, 96]. For example, the replacement of fish meal with soy protein concentrate in the starry flounder (*Platichthys stellatus*) was appropriate up to 40%, after which negative impacts on growth, mortality, and nutrient assimilation were noted [97].

1.5.2 Alternative Feeds and the Microbiome

Recent studies in a variety of fish species show that dietary supplementation with plant-based products has a marked impact on the composition and diversity of the microbiome [71, 74, 98, 99]. Given the importance of the microbiome in a variety of host functions, these feed-associated shifts in microbial populations are potentially driving negative health and growth outcomes. Due to the range of species and alternative feeds investigated to date, it can be difficult to develop a clear picture of how these microbial shifts impact health and growth [66]. For example, rainbow trout fed a variety of functional diets experienced significant shifts in the composition of their microbiomes, with plant-based diets associated with a higher *Firmicutes:Proteobacteria* ratio [85]. Conversely, the inclusion of plant proteins in the diet of sea bream resulted in a lower relative abundance of *Firmicutes* [74]. Despite being variable between species, dietary shifts in the microbiome appear to be dose-dependent, with higher inclusion levels of alternative raw feed materials having a greater impact on microbial composition [91]. In the northern snakehead (*Channa argus*), dietary soybean meal led to reduced relative abundance of *Firmicutes* and elevated levels of the potentially pathogenic bacteria *Shewanella* in a dose-dependent manner [100]. Not

only does the inclusion level of alternative proteins impact the microbiome composition, but the quality of the protein source also has a significant effect [91].

The level of refinement and processing that an alternative protein source has undergone can determine the severity of the diet-induced shift in microbial composition in fish, with high-quality proteins having a less detrimental effect [91]. In rainbow trout, the least processed meal (soybean meal) resulted in the greatest reduction in microbial diversity and richness [101]. Similarly, in Atlantic salmon, soybean meal diets significantly impacted overall microbial community composition whereas soy protein concentrate did not significantly impact the community profile [102]. These results indicate that higher-quality refined meals have less of an impact on the overall microbial community than those that have undergone less refinement. Understandably, the majority of the work to date investigating the impact of alternative proteins on the microbiome of fish has involved the most economically important species, such as salmon and trout [27]. Despite their growing importance commercially, there have been no studies to date investigating the impact of alternative feeds on the microbiome of Yellowtail Kingfish, or any other *Seriola* species.

1.5.3 Alternative Feeds and Temperature

Fish raised in captivity are limited in their ability to move in response to environmental shifts and are therefore often subjected to environmental water temperatures outside their natural range [8, 103]. These non-optimal temperatures can negatively affect farmed fish by altering their metabolism and health, leading to reductions in feed intake, growth rate and resistance to disease [103, 104]. The optimal growing temperature for farmed Yellowtail Kingfish has not been fully determined; however, evidence suggests that the optimal temperature is dependent on fish size [105]. Abbink and colleagues (2012) found that the optimal temperature for juvenile Yellowtail Kingfish weighing around 4g was 26.5°C, whereas Pirozzi and Booth (2009) determined the optimum temperature for more mature fish weighing around 200g to be 22.8°C [105, 106]. Sub and supra-optimal water temperature have also been found to exacerbate the detrimental effects of using alternative protein sources in Yellowtail Kingfish and Atlantic salmon [7, 96, 107]. Research has shown that Atlantic salmon experience elevated levels of soybean induced enteritis at higher water temperatures, and similar detrimental impacts in Yellowtail Kingfish have been noted at sub-optimal water temperatures (18°C) [96, 107]. Despite the evidence that water temperature and the choice of raw feed material can negatively impact the animal, there have been limited investigations into the combined effects of water temperature and protein source on the microbiome of fish. Some have suggested the interplay of these factors is important. For example Green and colleagues (2013)

found that the gut microbiome of Atlantic salmon shifted with seasonality, and they hypothesised that changes in water temperature caused shifts in microbial composition which in turn led to development of gut disorders related to the consumption of soybean meal [99]. Whilst this work and other non-microbiome based research indicate that water temperature is an important factor to consider when assessing the suitability of alternative raw feed materials for farmed fish, conclusive evidence as to the dependence of one factor on the other is lacking. This dilemma can only be resolved by conducting carefully controlled and replicable studies.

1.6 Scope of the Project

The growth of the aquaculture industry has created economic and environmental pressure to find suitable alternatives to fishmeal [2, 10, 91]. Many of these potential alternatives, such as soy-based products, have negative health and growth outcomes for fish when included at high levels, especially in carnivorous species [90]. The exact causes of these detrimental health and growth impacts are not entirely clear; however, dietary-induced shifts in the fish microbiome are likely invovled [66]. While early evidence suggests that some alternative proteins have a negative impact on microbial composition, our understanding of how shifts in the microbiome influence the host remains limited for many commercial species [91].

The primary aim of this project is to investigate how the selection of raw feed materials impacts the microbiome of Yellowtail Kingfish and understand how this shift is influenced by water temperature. This project also aims to understand how raw feed material selection and microbial composition impact biometric and health parameters that are relevant to the aquaculture industry, including growth, feed conversion ratio, and immune status. The research presented here will contribute to a deeper understanding of how alternative raw feed materials impact the microbiome of Yellowtail Kingfish and how changes in the microbiome relate to host health.

1.7 Specific Objectives

The central objective of this project was to understand how the dietary inclusion of a high-quality soy protein concentrate (SPC) impacts the microbiome of Yellowtail Kingfish. The second objective was to investigate whether the microbiome of Yellowtail Kingfish is affected by the interaction between raw feed material selection (i.e. fish meal vs SPC) and water temperature (i.e. optimal vs supra-optimal). The third objective was to determine if observed shifts in microbial composition were correlated with changes in selected growth and immune-related health parameters. The final objective, given the limited information available on the Yellowtail Kingfish microbiome, was to provide a detailed overview of the microbial composition of farmed Yellowtail Kingfish across

multiple body sites. These objectives were addressed by conducting a controlled experiment with Yellowtail Kingfish in which replicated groups of fish were fed a control diet based on fish meal (FM) or the control diet substituted with 30% soy protein concentrate (SPC). These diets were fed to fish at two water temperatures; an optimal temperature of 22°C and a supra-optimal temperature of 26°C.

2. Materials & Methods

2.1 Composition of experimental diets

The FM control diet was composed mainly of fishmeal (68%) and wheat flour (27%) with a small amount of fish oil (3.5%). The SPC diet was made by blending the FM mash and SPC in a 70:30 ratio, however supplements such choline chloride, vitamin-C and mineral vitamin premix were kept constant between diets. The crude protein and gross energy content of the FM and SPC diets were approximately 58% and 20.8 MJ kg^{-1}, respectively. The formulation, proximate composition and amino acid composition of the diets are detailed in Supplementary Table 1.

2.2 System Design

Fish were housed in a custom-built and designed seawater flow-through system, separated for the two temperatures (22°C and 26°C) as shown in Figure 2. A flow-through system was used to ensure that there was no recirculation of water between replicate tanks, thus maintaining separation and independence. Both systems were supplied with filtered seawater from the same source. Filtration was carried out by passing raw estuarine water through a system consisting of two sand filters, a cartridge particle filter, and two bag filters with a final filtration diameter of 5 µm. Filtered water was used to constantly fill two 10,000 L fibreglass supply tanks for the 26°C system and one 10,000 L fibreglass supply tank with a 4,000 L fibreglass sump for the 22°C system. The water in the supply tanks and sump for the two systems was constantly re-circulated through a temperature control unit capable of heating or chilling to maintain a consistent temperature. Oxygen was supplied to the supply tanks using an oxygen diffuser to maintain suitable and stable levels of dissolved oxygen in the experimental tanks housing the fish. The temperature controlled filtered seawater from each system was supplied at a constant rate of 6-7 L min^{-1} to six 200 L cylindrical, flat bottom, experimental polythene tanks. Each of the twelve experimental tanks was fitted with a black 10 mm mesh lid to prevent fish escape. All overflow water was directed to waste, ensuring the isolation of each of the experimental tanks. Water quality parameters were recorded using a hand-held probe twice daily and are detailed in Table 1. Fluidised-sand particle filters were cleaned by backwashing

twice daily and the cartridge and bag filters were changed twice daily. Fish tanks were cleaned every other day using a syphon cleaner.

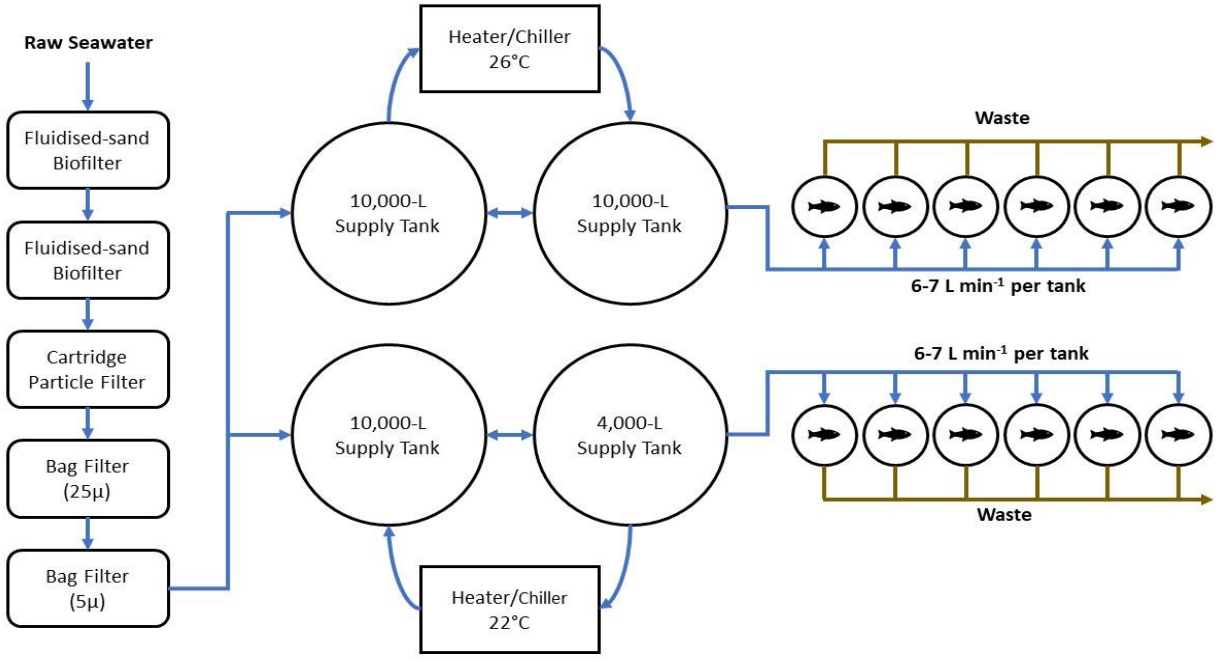

Figure 2 | Schematic representation of the flow-through aquaculture system.

Table 1 | Mean water parameters for each treatment.

Water Parameter	22 FM	22 SPC	26 FM	26 SPC
Temperature (°C)	22.1 ± 0.3	22.1 ± 0.3	26.1 ± 0.2	26 ± 0.2
pH	8.6 ± 0.1	8.6 ± 0.1	8.6 ± 0.2	8.6 ± 0.1
Dissolved Oxygen (%)	101.4 ± 4.4	102 ± 4.7	99.4 ± 5.5	98.6 ± 5.8
Salinity (%)	3.7 ± 0.04	3.7 ± 0.05	3.7 ± 0.05	3.7 ± 0.04

2.3 Stocking

Prior to the trial, fish were reared at low densities in 10,000 L fibreglass tanks. Prior to all stocking procedures, fish were maintained on a 6 mm commercial marine finfish feed (Skretting, Australia) and housed at ambient temperature.

Before stocking, fish were starved for 24 h and lightly anesthetised (10 mg L^{-1}, AQUI-S®). Fish used in this experiment were graded from a larger population and allocated to the experimental tanks ensuring that the overall biomass of all tanks was consistent (individual fish weight = 287± 32g and tank biomass = 1,719g ± 32g). During allocation, fish were individually tagged by fin-clipping of the pectoral or dorsal fin for identification upon subsequent sampling.

Fish within each system were slowly acclimatised to the temperature regimes of 22°C and 26°C over a period of nine days. During this period fish were handfed once daily (11:00 hours) at 2% of their body mass on a 6mm commercial marine finfish feed (Skretting, Australia). On day nine, all fish were lightly anesthetised (10 mg L-1, AQUI-S®), identified by fin-clip, and individually measured for length and weight. Two fish from each tank were selected for sampling to collect baseline health and microbiome data (Figure 3).

Following acclimatisation, each of the two experimental diets (FM and SPC) were randomly assigned to triplicate experimental tanks in each of the temperature-controlled systems (Figure 3). Fish were handfed experimental diets once daily (11:00 hours) to slight excess at 3% of measured body weight for the first for 9 days. At day 10, expected weight gain was calculated using a feed conversion ratio of 1.0, based on previous Yellowtail Kingfish research using soy-based diets [96]. Expected weight on day 10 was then used to determine the 3% bodyweight ratio for the remaining 11 days of the trial. The trial was concluded after 21 days, and all remaining fish were sampled for health and microbiome measurements (Figure 3).

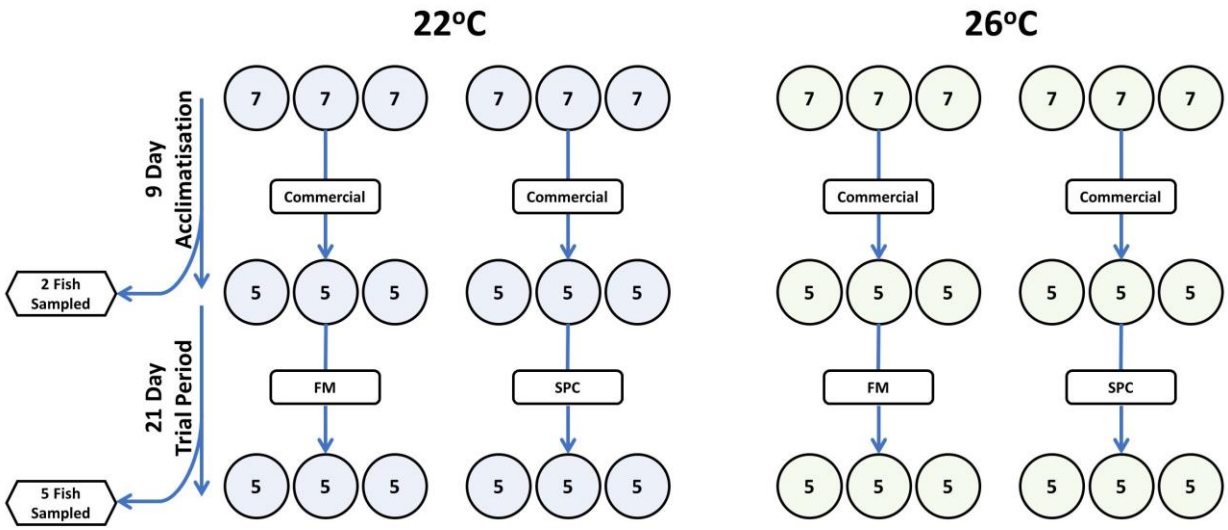

Figure 3 | Schematic representation of the experimental design.
Circles represent experimental tanks with fish numbers detailed. Diet type fed during stages of the experiment are detailed in the rectangular boxes.

2.4 Sampling Procedures

Sampling procedures were standardised throughout the experiment. Samples of three microbial communities from each fish were taken from different parts of the fish (the body sites); namely skin mucosa (skin), distal digesta (digesta), and adhered distal gut mucosa (gut mucosa). On the day prior to sampling, fish in the 22°C and 26°C treatments were fed 20 and 15 hours respectively prior to sampling time to ensure that sufficient digesta was present in the gut upon sampling. The 200-L

experimental tanks were lowered to c.80 L and fish were lightly anesthetised (5 mg L^{-1}, AQUI-S®) to allow safe removal from tanks for individual sampling. Following sterilisation of hands with 80% ethanol, fish were transferred to a sterilised stainless-steel benchtop and euthanised. The skin mucus from the right side of the fish between the pectoral and caudal fin was gently scraped with a sterile scalpel blade and transferred to a labelled sterile 1.5 mL tube before being immediately stored on ice. Blood was drawn from the caudal vein at the base of the tail using a 23G sterile hypodermic needle and transferred to a 1 mL K3E KEDTA Minicollect tube (Grenier Bio-one) and immediately stored on ice. Fish were measured for fork length and weight. The exterior of the fish was then spray sterilised (100% ethanol), and the right gill operculum was removed with sterilised surgical scissors to gain access to the gills. A section of the second-gill arch was removed using sterile surgical scissors and tweezers and transferred to a sterile 1.5 mL tube before immediate storage on ice. Fish were dissected through the ventral surface using sterile surgical scissors, and the tissue surrounding the visceral fat was removed and the intestine transferred to a separate sterilised section of the stainless-steel surface. The fish, less viscera, and the liver were individually weighed. The distal gut digesta was aseptically excised into a sterile 1.5 mL tube and immediately stored on ice. The intestine was transferred to a separate sterilised section of the stainless-steel surface, opened lengthways using sterile surgical scissors, and gently washed in sterile phosphate buffered saline (0.01 M) to remove non-adhered luminal gut contents. The gut mucosa was then gently scraped using a sterile scalpel blade and the mucosa transferred to a sterile 1.5 mL tube before immediate storage on ice. The four microbiota samples were then immediately stored at -80°C until further analysis. Blood samples were centrifuged at 11,300 rpm for 14 min and the plasma carefully transferred to a sterile 1.5 mL tube and stored at -20°C until further analysis.

Forty-five mL of filtered input estuarine water, in duplicate, was sampled on the final day of the trial and stored at -80°C until further analysis.

2.5 Plasma Lysozyme Activity

Plasma lysozyme activity was determined by a turbidometric assay utilising lyophilised *Micrococcus lysodeikticus* cells (Sigma, Australia) using a method modified from [108]. Plasma was diluted 1:40 times in 0.02 M sodium citrate buffer. Lysozyme from chicken egg white (Sigma, Australia) was used as a standard at seven serially diluted concentrations of 2,000 – 31.25 units/mL. In a 96 well plate, 15 µL of the diluted plasma, the seven lysozyme standards, and a blank of sodium citrate buffer were added to 150 µL of *M. lysodeikticus* suspended in 0.02 M sodium citrate buffer at a concentration of 0.2 mg/mL. The absorbance was immediately measured at 450 nm, and

subsequent measurements were taken every 5 min for 60 min using a PHERAstar FS (BMG Labtech) microplate reader. A unit of lysozyme activity was defined as the quantity of enzyme that caused a reduction in the absorbance of 0.001 min^{-1} [108].

2.6 Plasma & Digesta Myeloperoxidase Activity

The myeloperoxidase (MPO) activity of plasma and digesta samples was determined using a colourimetric assay utilising 3,3',5,5'- tetramethylbenzidine hydrochloride (TMB) (Sigma, Australia), following the method of Quade and Roth (1995) with minor modifications [109].

To prepare equal solutions of the digesta samples for measuring MPO activity, c. 250 mg of sample was added to a previously weighed 1.5 mL tube and an exact weight taken for each. Three microliters of 0.02M sodium citrate buffer was added for each mg of sample to ensure equal dilution across samples. Samples were heated to 55°C for two min and briefly vortexed, this step was repeated twice. Samples were then centrifuged at 3000 x g for 5 min and the supernatant collected for further analysis.

For the assay, 5 µL of plasma or digesta supernatant were added to 95 µL of Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} (Thermo Fisher Scientific, Australia) in a 96 well plate. Next, 35 µL of freshly prepared 20 mM TMB and 5 mM H_2O_2 was added to each well using a multichannel pipette. The colour change reaction was stopped after 2 min by adding 35 µL of 4 M sulphuric acid and the optical density was read at 450nm using a PHERAstar FS (BMG Labtech) microplate reader. The reduction in absorbance was determined as the relative level of MPO present in the sample.

2.7 16S rRNA Gene Amplicon Sequencing

DNA extractions were performed on all microbiome samples using the FastDNA spin kit (MP Biomedicals, Australia). A number of modifications to the manufacturer's recommended protocol were implemented after initial testing of a sub-set of samples representing all fish body sites indicated that extracted DNA was not PCR competent. In order to identify whether there was an issue with the extraction kit or the samples, the FastDNA spin kit was used to isolate mouse faecal DNA from archived samples kindly provided by Dr. Hasinika Gamage (Macquarie University, Australia). Parallel extractions with mouse and fish material resulted in PCR competent DNA for mouse faecal material only, indicating that there was likely some form of PCR inhibiting substance in the Yellowtail Kingfish samples following extraction with the commercial kit. Initially, approaches to improve PCR efficiency were trialled. Briefly, these included the addition of bovine serine albumin to the PCR reaction, use of GeneReleaser (Bioventures, USA), dilution of the extracted DNA, and alteration of the PCR cycling parameters; however, none of these resulted in a positive PCR product

for fish microbiome samples. Subsequently additional DNA cleaning steps were trialled (ethanol precipitation of extracted DNA and application of DNA Clean & Concentrator (Zymo Research, USA)); however, these also failed to produce PCR competent DNA from fish microbiome samples. Alterations of the FastDNA spin kit manufacturer's protocol were subsequently trialled based on a survey of literature on overcoming PCR inhibition issues associated with fish microbiome samples, particularly the report of Hart and colleagues (2015) that found isopropanol extraction of DNA after cell lysis assisted with isolating PCR competent DNA from Zebrafish (*Danio rerio*) [61]. The inclusion of a modified isopropanol extraction together with the application of Protein Precipitation Solution (MP Biomedicals, Australia), as described below, were found to substantially reduce issues with PCR inhibition. This method was therefore employed for the isolation of the total community DNA from the skin, gut mucosal, digesta, and water samples analysed in this study. PCR inhibition could not be corrected for gill samples therefore that data has been excluded from this report.

All samples reported were extracted using the following modified FastDNA spin kit (MP Biomedicals, Australia) DNA extraction procedure. Samples were removed from – 80°C, thawed for 15 min, then approximately 25 mg was added to a lysing matrix A tube (MP Biomedicals, Australia) containing 1 mL CLS-TC lysis buffer (MP Biomedicals, Australia). For one tube, no sample was added to act as an extraction blank. Samples were homogenised in a FastPrep® Instrument (MP Biomedicals, Australia) for 40 sec at a speed setting of 6.0 and immediately chilled on ice for 5 min before being subjected to a second round of homogenisation. Following homogenisation, samples were incubated at 70°C for 20 min with periodic vortexing, to aid lysis of *Fusobacteria* as reported previously [34]. Samples were centrifuged at 14,000 x g for 8 min to pellet debris. Seven hundred microliters of the supernatant were transferred to a 2 mL tube containing 200 µL of 10 mM ammonium acetate and 800 µL of chilled isopropanol and mixed by inversion. Samples were incubated on ice for 30 min and then centrifuged at 16,000 × g at 4°C for 15 min to pellet the DNA. The pellets were washed with chilled 70% ethanol and resuspended in 650 µL DNAse free water. One hundred and fifty microliters of Protein Precipitation Solution (MP Biomedicals, Australia) was added, mixed by inversion, and incubated at room temperature for 5 min to precipitate any residual proteins. Samples were centrifuged at 14,000 x g for 5 min to pellet the precipitate and supernatant transferred to a clean 1.5 mL tube containing an equal volume of binding matrix (MP Biomedicals, Australia). The DNA was the further purified following the manufacturer's instructions.

The water samples were filtered through a Sterivex™ GP 0.22 µm filter unit (Millipore, Australia) to isolate the bacteria from the water. The filter was removed and processed in the same manner as the other samples, as described above.

Following extraction, the V4 region of the 16S rRNA gene was amplified using 515 forward (5'-GTGCCAGCMGCCGCGGTAA-3') and 806 reverse (5'-GGACTACHVGGGTWTCTAAT-3') primers with custom barcodes, based upon the earth microbiome primer protocols [56, 110]. PCR amplification was performed on 1:50 dilutions of extracted DNA using MyFi Mix (Bioline, Australia) with a primer concentration of 400 nM in a final volume of 30 µL. Samples were PCR amplified with 35 cycles at 95°C for 15 seconds, 50°C for 15 seconds and 72°C for 60 seconds. Previously isolated bacterial genomic DNA was used as a positive control and DNAse free water as a negative control. Five microliters of resulting amplicons were visualised on 2% agarose gels (Bioline, Australia) using 5X loading dye (Bioline, Australia) and a 100 bp Hyperladder (Bioline, Australia). Samples that were positive for amplification, along with 25 µL of the extraction blank, were then quantified using Quant-iT™ PicoGreen® (Invitrogen, Australia). Barcoded amplicons were pooled at equimolar concentrations and gel purified using a Wizard® SV gel and PCR clean up system (Promega, Australia). The purified pooled barcoded amplicons were diluted to a final concentration of 5 nM and sequenced using the Illumina MiSeq platform (MiSeq V2 2 x 250 bp paired-end sequencing run) at the Ramaciotti Centre for Genomics, Sydney, Australia.

2.8 PCR assay to screen for plpV & sequencing of PCR Products

Amplification of a region of the plpV gene from *Photobacterium* was carried out using forward (5'-TCTCATAATAGCAGTAATCT-3') and reverse (5'-TTACTAAGCAGAATCCAGCC-3') primers, as described by Vences and colleagues (2017) [111]. PCR amplification was performed on 1:50 dilutions of extracted DNA using MyFi Mix (Bioline, Australia) with a primer concentration of 400 nM in a final volume of 30 µL. Samples were PCR amplified with 35 cycles at 95°C for 30 seconds, 54.5°C for 30 seconds and 72°C for 60 seconds. Five microliters of resulting amplicons were visualised on 2% agarose gels (Bioline, Australia) using 5X loading dye (Bioline, Australia) and a 1 kbp Hyperladder (Bioline, Australia).

Three samples were selected to confirm the identity of the amplified product, with Sanger sequencing of bands gel purified using a Wizard® SV gel and PCR clean up system (Promega, Australia), carried out by Macrogen (South Korea). Sequence identity was checked by blastn and blastx searches of the NCBI nucleotide and protein databases using Geneious 11.1.5 (https://www.geneious.com) [112, 113].

2.9 Bioinformatic & Statistical Analysis of Microbiome Data

Raw sequences were demultiplexed by the Ramaciotti Centre for Genomics, Sydney, Australia. Demultiplexed sequences were processed using Quantitative Insights Into Microbial Ecology 2 (QIIME2) software (version 2018.4) [57]. Quality control was performed within QIIME2 using DADA2 to remove low-quality sequence regions and chimeric sequences through the 'consensus' method [114]. No truncation of the forward or reverse reads was required based upon the quality scores. DADA2 also performed dereplication by combining identical sequences to construct a high-resolution amplicon variant table. Taxonomy was assigned using the QIIME2 q2-feature-classifier plugin and a Naïve Bayes classifier that was trained on the SILVA 99% OTU database trimmed to the V4 region of the 16S rRNA gene [57, 115]. Samples with a total number of reads less than 10,000 were discarded from further analysis.

Statistical calculations and graphical construction analysis of amplicon sequence data were performed in the RStudio statistical package (version 1.1.453). Alpha diversity analyses were performed in the phyloseq R package (V.1.24.0) using multifactor analysis of variance followed by a post hoc Tukey honest significant difference test [116]. Non-metric multidimensional scaling (NMDS) plots were constructed using the phyloseq R package (V.1.24.0) [116]. The phyloseq R package (V.2.5-2) was used to perform permutational multivariate analysis of variance (PERMANOVA) with 999 permutations on Bray Curtis distance matrices (V.1.24.0) [117]. The R package phyloseq (V.1.24.0) was used for the construction of heatmaps [116].

Differential abundance of OTUs between treatments were identified using the linear discriminant analysis (LDA) effect size (LEfSe) method, available at http://huttenhower.sph.harvard.edu/galaxy/ [118]. OTU relative abundance with treatments as the classes of subjects was used as the input. Alpha values of 0.05 were used for the the factorial Kruskal-Wallis sum test and the pairwise Wilcoxen test between classes. A threshold of 2.0 was chosen for logarithmic LDA scores.

Spearman correlations and node weightings for network analysis were calcualted using R package Hmisc [119]. Correlations were considered significant when the correlation p-value was < 0.05. Significant correlations were visualised in Cytoscape v.3.6.1 [120].

2.10 Statistical Analysis of Physiological Parameters

Significant differences between treatments for the physiological parameters, such as growth and plasma lysozyme, were determined by two-way analysis of variance (ANOVA). The fixed factors were diet type (FM vs SPC) and water temperature (22°C vs 26°C). Each treatment was applied in triplicate. If ANOVA proved significant ($p<0.05$), a Tukeys honestly significant difference test was

used to separate the treatment means. Statistical analysis was performed using GraphPad Prism (version 7) software (GraphPad Software, USA).

2.11 Data deposition

The 16S rRNA gene sequence data generated and analysed in this study can be found in the GenBank Sequence Read Archive (SRA) database under accession number SUB4557405.

3. Results

Yellowtail Kingfish were fed a fishmeal diet (FM) or a FM diet partially replaced with soy-protein concentrate (SPC) and housed at optimal and non-optimal temperatures (22 and 26°C) to investigate impacts on fish health and microbiome composition within skin mucosa, digesta, and gut mucosa. The combination of diet and temperature resulted in four treatments; one control treatment (22 FM) and three experimental treatments (22 SPC, 26 FM, and 26 SPC).

3.1 Analysis of Physical and Immune Parameters

After 21 days, differences in a number of standard physical, measurable factors were noted between experimental treatments (Table 2).

Table 2 | Growth performance, feed conversion ratio, Fulton's body condition, and hepatosomatic index of Yellowtail Kingfish for each treatment.

Factors	22 FM	22 SPC	26 FM	26 SPC
Weight Gain (%)	70.37% ± 2.8%	62.68% ± 3.21%	**59.26% ± 5.14%**	**54.04% ± 4.87%**
Fork Length Growth (%)	17.38% ± 0.76%	16.72% ± 1.57%	13.35% ± 0.97%	13.07% ± 1.54%
Feed conversion ratio	0.93 ± 0.02	0.99 ± 0.02	1.09 ± 0.06	**1.19 ± 0.04**
Fulton's body condition	1.43 ± 0.03	1.42 ± 0.01	1.45 ± 0.01	1.49 ± 0.02
Hepatosomatic index	1.07 ± 0.04	1.03 ± 0.03	1.18 ± 0.04	1.16 ± 0.03

Means of triplicate tanks (5 fish per tank) ± standard deviation. Feed conversion ratio = feed given (g)/ weight gain (g). Fulton's body condition = 100 × body weight (g) /length (cm) ^3. Hepatosomatic index = 100 × liver weight (g) / body weight (g). Factors that were statistically significantly different to the control ($p < 0.05$) by ANOVA are highlighted bold.

Two-way ANOVA indicated that percent weight gain was statistically significantly affected by water temperature and by diet ($P < 0.05$); however, their interaction was not significant ($P > 0.05$). Overall, the fish fed the control treatment (22 FM), experienced the greatest increase in weight and fork length and had the lowest feed conversion ratio (Table 2). Elevated temperature (26°C) resulted in a statistically significant reduction in weight gain, regardless of the diet fed (Table 2). The

combination of SPC and 26°C resulted in a significant reduction in weight gain and a significant increase in feed conversion ratio (Table 2). There were no significant differences in Fulton's body condition index or the hepatosomatic index between treatments (Table 2).

Assays of innate immune parameters were conducted on digesta and blood plasma as an indicator of fish health. Digesta myeloperoxidase (MPO), an innate immune defence against pathogenic bacterial infection which is produced by neutrophils, was statistically significantly lower in fish fed SPC regardless of temperature (Figure 4) [73]. Fish housed at 26°C experienced a slight reduction in digesta MPO levels in both diets, although these differences were not statistically significant (Figure 4). Fish fed SPC and maintained at 26°C exhibited the lowest levels of digesta MPO (Figure 4). No statistically significant differences were noted between treatments for measured plasma MPO (Supplementary Figure 1). Neither SPC nor maintenance at 26°C alone had a significant impact on the levels of plasma lysozyme, an innate immune defence molecule that causes lysis of bacteria through breaking linkages in the cell wall (Figure 4) [121]. However, fish fed SPC and housed at 26°C showed a statistically significant increase in plasma lysozyme levels when compared to the control treatment (Figure 4).

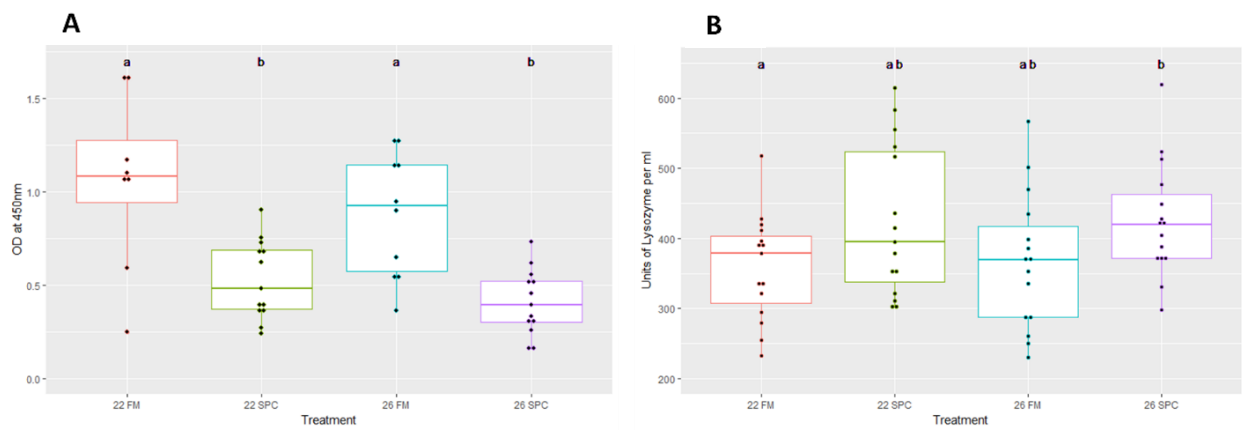

Figure 4 | Levels of (A) digesta MPO and (B) plasma lysozyme in Yellowtail Kingfish for each treatment.
Different letters denote statistically significant ($p < 0.05$) differences between treatments by ANOVA.

3.2 Characteristics of the High-Throughput Sequence Data

The bacterial communities of the skin, digesta, and gut mucosa were surveyed from a total of 84 Yellowtail Kingfish, along with two input water samples. Samples were collected from each body site for each fish, and DNA extractions were carried out on all samples; however, successful PCR amplification was only achieved for a subset of samples (Table 3). The extraction blank generated no visible PCR product based on gel electrophoresis examination; however, this sample was

included in the full set of pooled products for which sequencing was performed. A relatively small number of amplicon sequence reads (1,901) were attributed to this sample following demultiplexing. For each of the fish body sites, each sample had on average ≥ 47,556 reads while water samples had an average of 33,371 reads following sequence quality control and filtering (Table 3). Overall, a total of 7,787 unique sequence variants were detected, which were assigned to 1,713 operational taxonomic units (OTUs) at a 99% similarity level.

Table 3 | Sequencing reads post quality filtering for each body site and water.

Sampling Site	Total Reads	Baseline Samples	Final Samples	Average reads per sample
Gut Mucosa	2,983,500	15	33	62,156 ± 29,702
Digesta	2,805,841	16	43	47,556 ± 11,019
Skin	2,731,892	15	41	48,784 ± 18,976
Water	66,741	-	2	33,371 ± 4,060
Total	**8,587,974**	**46**	**119**	**52,048 ± 21,486**

Total reads, number of samples, and mean reads (± standard deviation) per sample for the gut mucosa, digesta, skin, and water samples.

3.3 Baseline Microbial Community Composition

Following the nine-day acclimation period and prior to commencing the treatments, two fish were sampled from each of the twelve experimental tanks. These samples were taken to assess whether there were any differences in the baseline microbial communities between the tanks assigned to each experimental treatment. Pairwise permutation multivariate analysis of variance tests (PERMANOVA) indicated that there were no statistically significant differences in the baseline microbial community profiles at the examined body sites for the different treatments (Supplementary Table 2).

3.4 Comparison of Microbial Composition across Body Sites and the Water

The alpha diversity (Shannon), richness (Chao1), and evenness (Simpson) of samples were analysed to investigate how the microbiome of Yellowtail Kingfish differed between body sites and the water (Figure 5). The richness, evenness, and diversity observed in the skin samples were statistically significantly higher to that of the gut mucosa and digesta. There was no statistically significant difference in the alpha diversity measures between the digesta and gut mucosa (Figure 5). The diversity (Shannon) and evenness (Simpsons) of the microbial communities in the water samples were similar to that of the skin, whereas the richness (Chao1) of the water samples was lower than the skin (Figure 5).

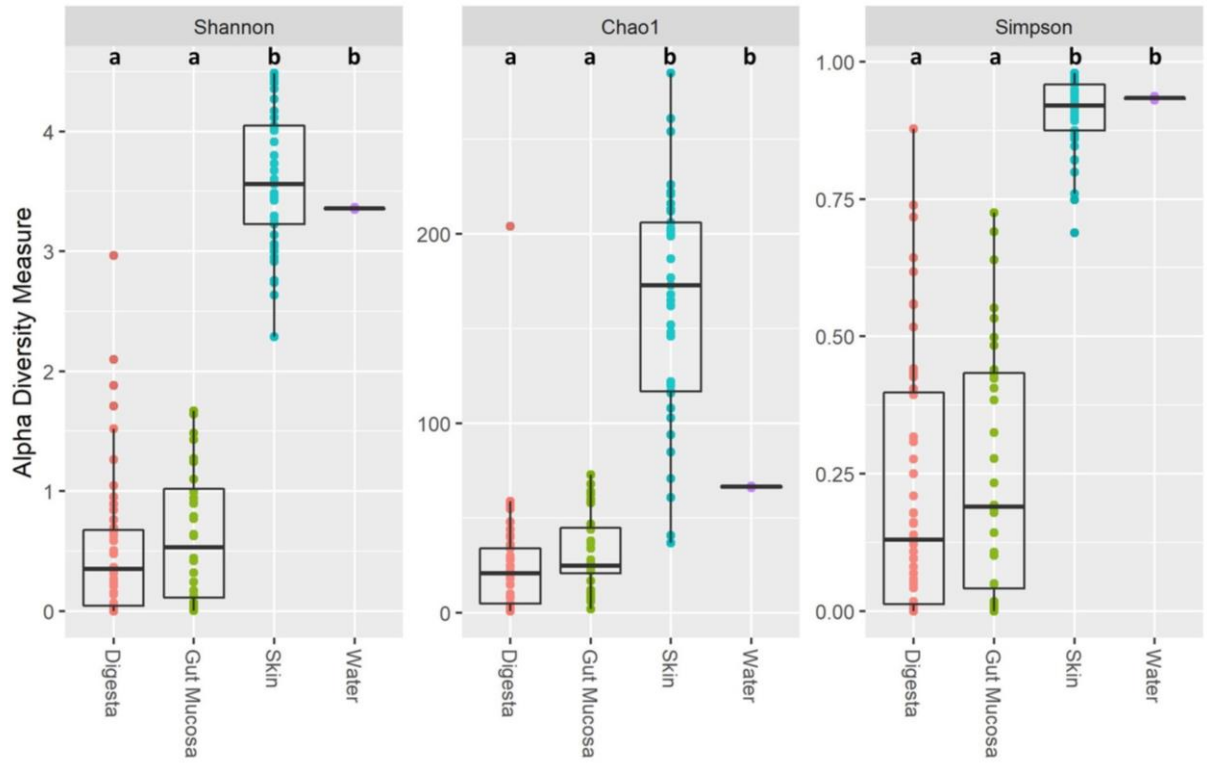

Figure 5 | Alpha diversity (Shannon), richness (Chao1), and evenness (Simpson) of digesta, gut mucosa, skin, and water microbial communities.
Shannon, Chao1, and Simpson alpha diversity metrics for each sample, grouped by sampling site. Different letters denote significant ($p < 0.05$) differences between treatments by ANOVA.

Of the 1,713 OTUs identified, only 58 were shared between the water and the three body sites of Yellowtail Kingfish (Figure 6). Of this 58 OTUs, 63% were assigned to *Proteobacteria*, 17% to *Actinobacteria*, 12% to *Firmicutes*, 5% to *Bacteroidetes*, and the remaining 3% to *Cyanobacteria*. The water shared more OTUs with the skin (92) than either the gut mucosa (79) or the digesta (62) (Figure 6). The skin also contained by far the highest number of unique OTUs (985). The gut mucosa and digesta both shared a higher number of OTUs with the skin (412 and 270 respectively) than they did with one another (239). The three body sites shared a core microbiome of 201 OTUs, of which 53% were assigned as *Proteobacteria*, 15% as *Firmicutes*, 14% as *Actinobacteria*, 8% as *Bacteroidetes*, 2% as *Cyanobacteria*, with the remaining 8% split between 16 additional phyla.

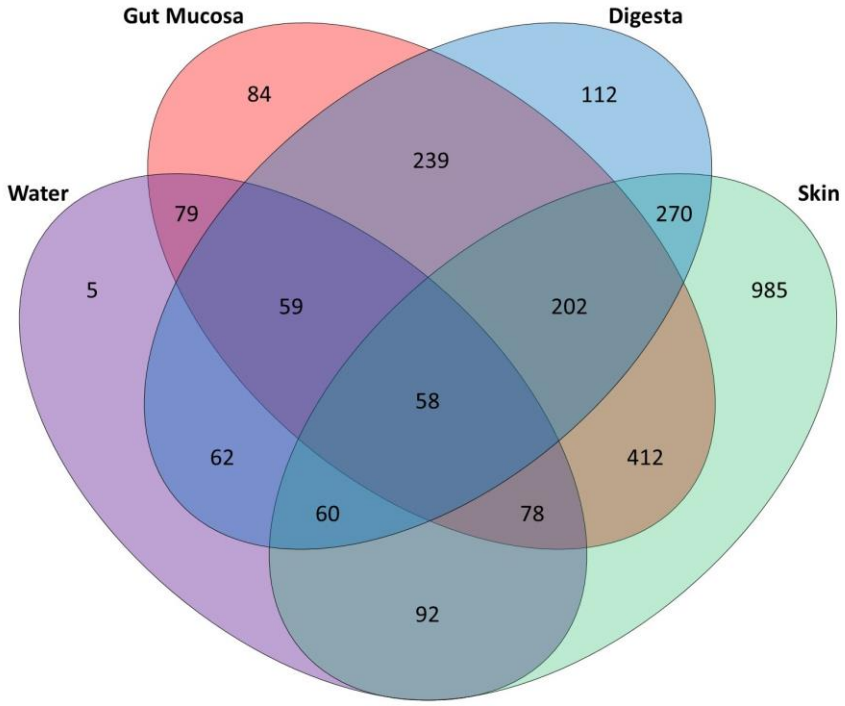

Figure 6 | Distribution of OTUs identified in the gut mucosa, digesta, skin, and water samples. Venn diagram detailing the number of OTUs that are unique to and shared between each of body sites and water.

Analyses were conducted to compare the overall community composition of the microbiomes sampled from each of the body sites and the surrounding water. PERMANOVA showed that microbial community composition was strongly influenced by body site (Table 4). The community composition of the skin was statistically significantly different from that of the gut mucosa and digesta across all four treatments when analysed by PERMANOVA (Table 4). This statistical difference was reflected in the non-metric multidimensional scaling (nMDS) plots which show some separation of the skin communities from the other two body sites; however, the separation is more distinct at 22°C than at 26°C (Figure 7). The gut mucosa and digesta bacterial communities were not statistically significantly different in the fish fed FM diets (Table 4). However, in fish fed SPC diets, the gut mucosa and digesta microbial communities were statistically significantly different (Table 4). The microbial communities in the two water samples appeared to align closely with the skin samples, apart from under control conditions where the water was oriented distinctly from each of the three body sites (Figure 7).

Table 4| Pairwise PERMANOVA comparing the microbial communities of gut mucosa, digesta, and skin samples by treatment.

Body site	Body Site	22 FM		22 SPC		26 FM		26 SPC	
		R^2	p	R^2	p	R^2	p	R^2	p
Gut Mucosa	Digesta	0.083	0.125	0.124	**0.025**	0.053	0.428	0.217	**0.002**
Gut Mucosa	Skin	0.285	**0.001**	0.231	**0.001**	0.181	**0.002**	0.138	**0.002**
Digesta	Skin	0.255	**0.001**	0.408	**0.001**	0.252	**0.002**	0.435	**0.001**

Pairwise PERMANOVA with 999 permutations was performed on a Bray-Curtis dissimilarity matrix. Comparisons for which p < 0.05 are presented in bold.

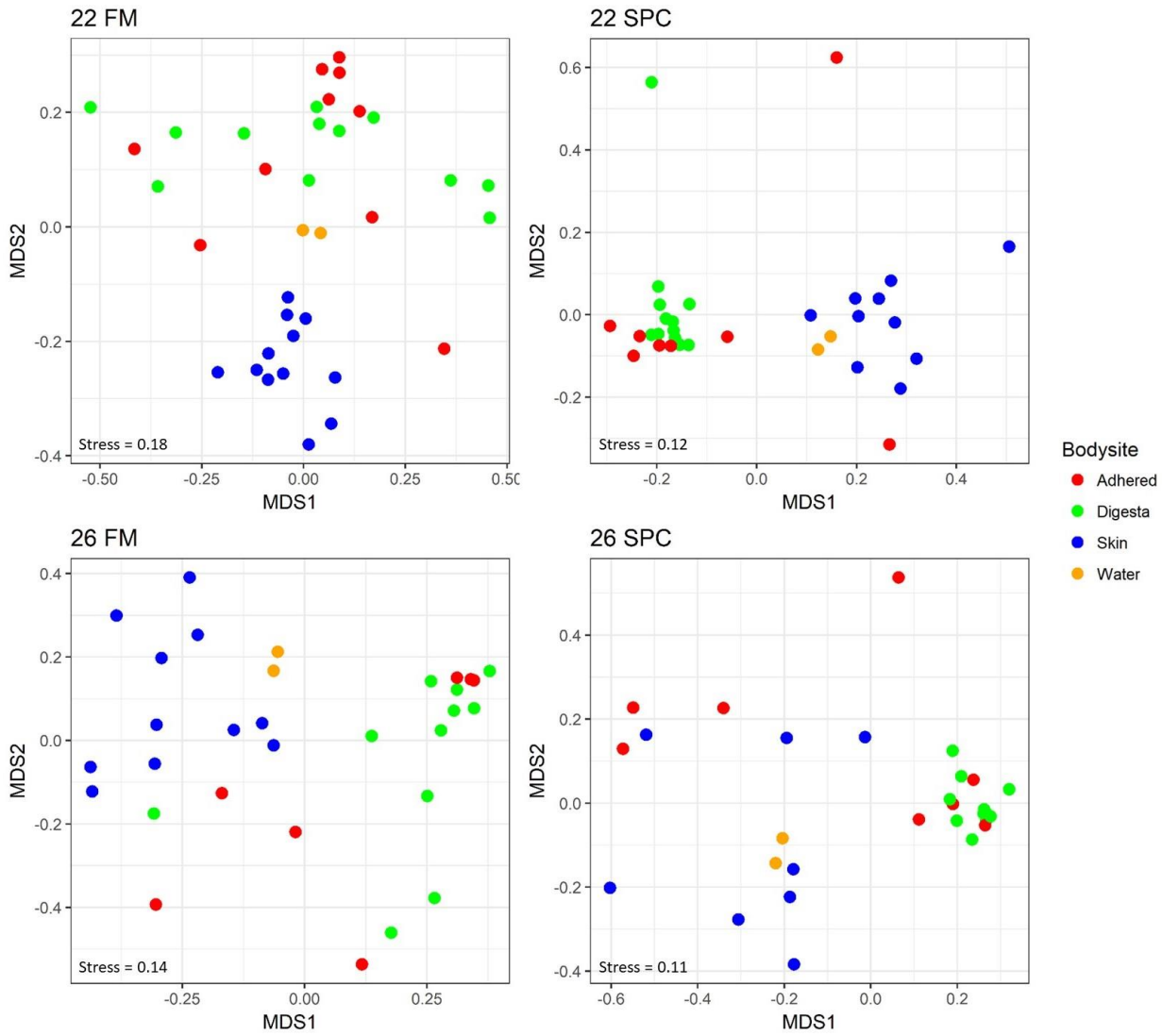

Figure 7 | Microbial community composition within gut mucosa, digesta, skin, and water samples for each treatment.

Non-metric multidimensional scaling plot of sample clustering based upon a Bray-Curtis dissimilarity matrix.

3.5 Effects of Diet and Temperature on Skin Microbial Community Structure and Composition

The alpha diversity (Shannon), richness (Chao1), and evenness (Simpson) of skin samples were analysed to determine the impact of diet and temperature on the microbial communities (Figure 8). Figure 6 shows that diet impacted alpha diversity independently of temperature, with fish fed SPC exhibiting increased microbial diversity, richness, and evenness, although differences were not statistically significant (Figure 8). Elevated temperature resulted in a reduction in the richness (Chao1) and diversity (Shannon) of microbial communities and increased community evenness (Simpson) when compared to the control (Figure 8). The combination of elevated temperature and SPC diet resulted in a significantly greater community evenness when compared to the control treatment; however, there was no significant impact on diversity and richness (Figure 8).

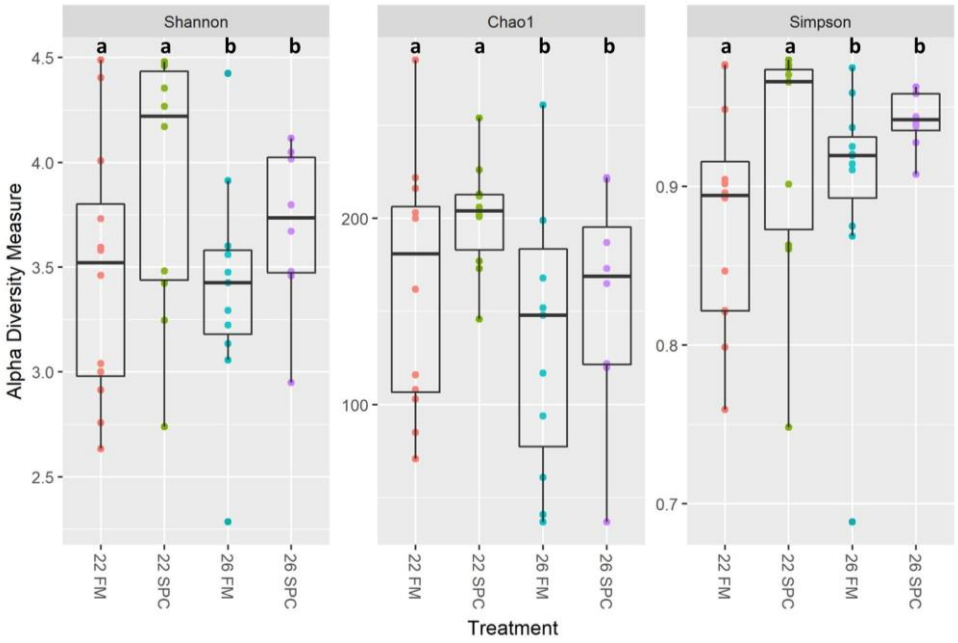

Figure 8 | Alpha diversity (Shannon), richness (Chao1), and evenness (Simpson) of skin microbial communities for each treatment.
Shannon, Chao1, and Simpson alpha diversity metrics for each skin sample, grouped by treatment. Different letters denote significant ($p < 0.05$) differences between treatments by ANOVA.

Analyses were performed to assess the impact of diet and temperature on the overall community microbial composition of the skin samples. Pairwise PERMANOVA tests indicated that diet is a key factor in shaping the community composition of the skin microbiome, with statistically significant differences noted between fish fed FM and SPC diets (Table 5). Maintenance at 26°C alone also impacted the microbial communities, with a statistically significant difference between the control

treatment and 26 FM (Table 5). PERMANOVA analysis showed that the combination of elevated temperature and SPC had the greatest impact on the skin microbiome as this treatment was associated with the strongest shift in composition from the community of the control treatment (Table 5).

Table 5 | Pairwise PERMANOVA comparing the microbial communities of experimental treatments to the control in the skin.

Control	Treatment	Differ by	R^2	p
22 FM	22 SPC	Diet	0.107	**0.005**
22 FM	26 FM	Temp	0.083	**0.008**
22 FM	26 SPC	Diet + Temp	0.117	**0.003**

Pairwise PERMANOVA with 999 permutations was performed on a Bray-Curtis dissimilarity matrix. Comparisons for which $p < 0.05$ are presented in bold.

These four phyla comprised the vast majority of the resident skin bacteria present in all treatments, with only minor differences in the relative abundance across treatments noted (Figure 9). At a genus level, across all four treatments, only four genera comprised over 5% of the population of the skin microbiome: *Ralstonia, Photobacterium*, unassigned bacterium of the family *Rhodobacteraceae*, and *Thalassotalea* (Figure 10). Approximately 35% of the inferred microbial population in each treatment was assigned to these four genera. The remaining population was comprised of 949 lower abundance genera, of which 916 had a relative abundance of less than 1%.

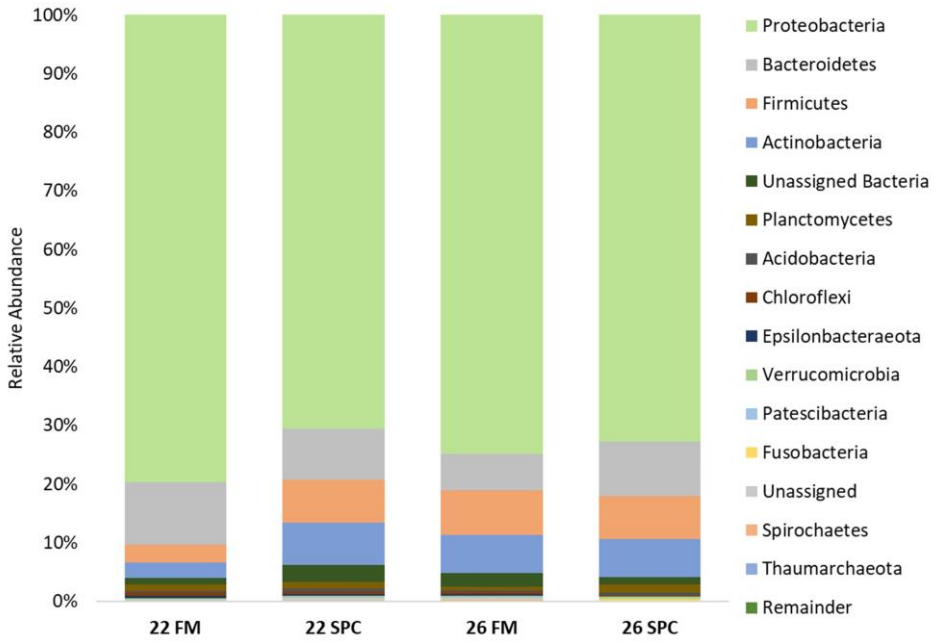

Figure 9 | Skin microbial composition (mean relative abundance of OTUs) for each treatment grouped by phylum.
Phyla comprising less than 0.1% of the population are grouped under remainder.

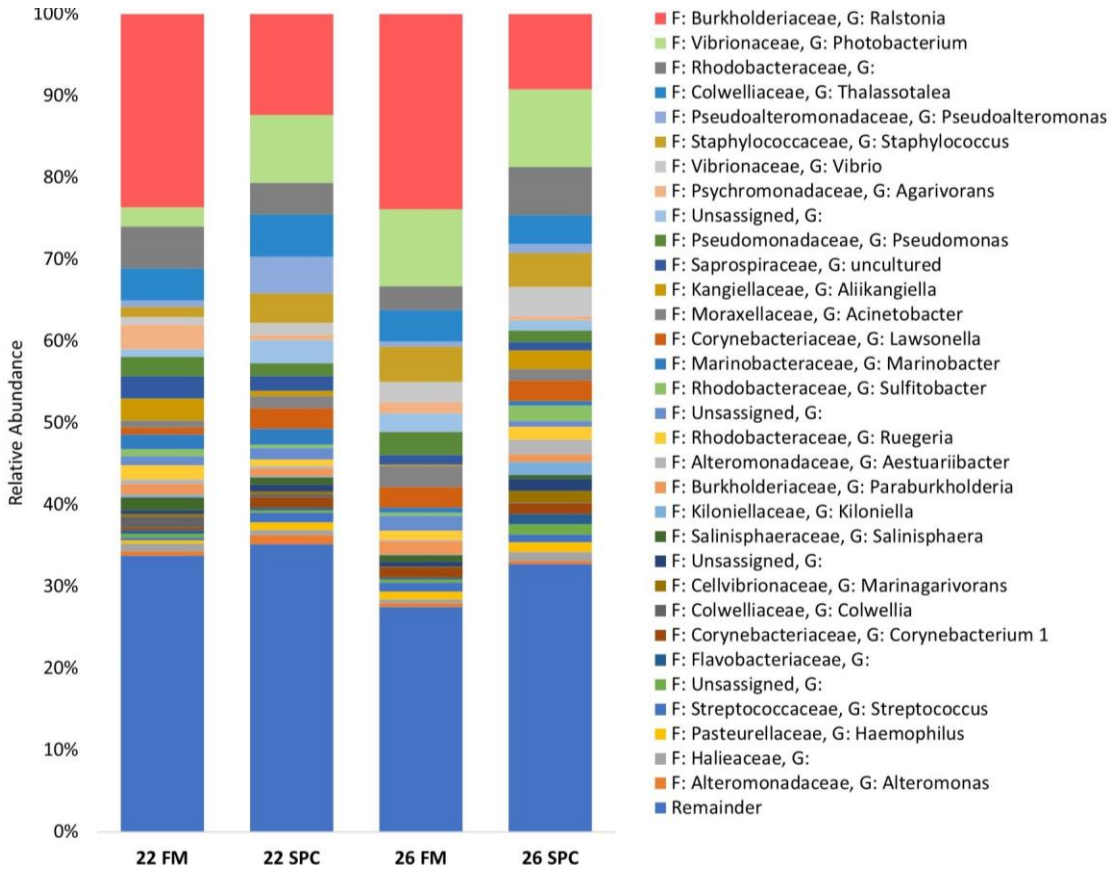

Figure 10 | Skin microbial composition (mean relative abundance of OTUs) for each treatment grouped by genus with assigned family detailed.

Where no genus has been taxonomically assigned, the genus is left blank. Families that comprise less than 1% of the population are grouped together under remainder.

Differential abundance testing via linear discriminant analysis (LDA) effect size (LEfse) was performed to investigate which OTUs significantly contributed to the differences in skin microbiome composition associated with diet and temperature treatments. LEfse analysis revealed 25, 23, and 12 statistically significantly differentially abundant OTUs in the 22 SPC, 26 FM, and 26 SPC treatments respectively when compared to the control treatment (Figure 11). For all three of the experimental treatments, the relative abundance of OTUs assigned to *Photobacterium* and *Lawsonella* were increased when compared to the control treatment, while OTUs assigned to *Chitinophagales* and *Salegentibacter* were signficantly decreased. The relative abundance of OTUs assigned to *Staphylococcus*, *Corynebacterium*, and *Acinetobacter* was statistically significantly higher in fish fed SPC or housed at 26°C compared to the control but not in the 26 SPC treatment. Similarly, the relative abundance of an OTU assigned as *Lewinella* was only significantly decreased in the 22 SPC and 26 FM treatments, not in the 26 SPC treatment. The 22 SPC treatment was associated with increased abundance of several OTUs that were not significantly increased in the other treatments, including *Acinetobacter, Streptococcus, Haemophilus*, and *Micrococcus*

representatives (Figure 11). Elevated temperature, regardless of diet, was associated with lower levels of OTUs assigned to *Alteromonadaceae*. OTUs assigned to *Rhodobacteraceae,* and *Ralstonia* decreased under 26 FM and 26 SPC treatments respectively when compared to the control. Among the significantly impacted OTUs there is considerable variability in the response of individuals within the treatments (Figure 11). However, the variability of individuals within each treatment did not appear to be impacted by which tank fish were housed (Figure 11).

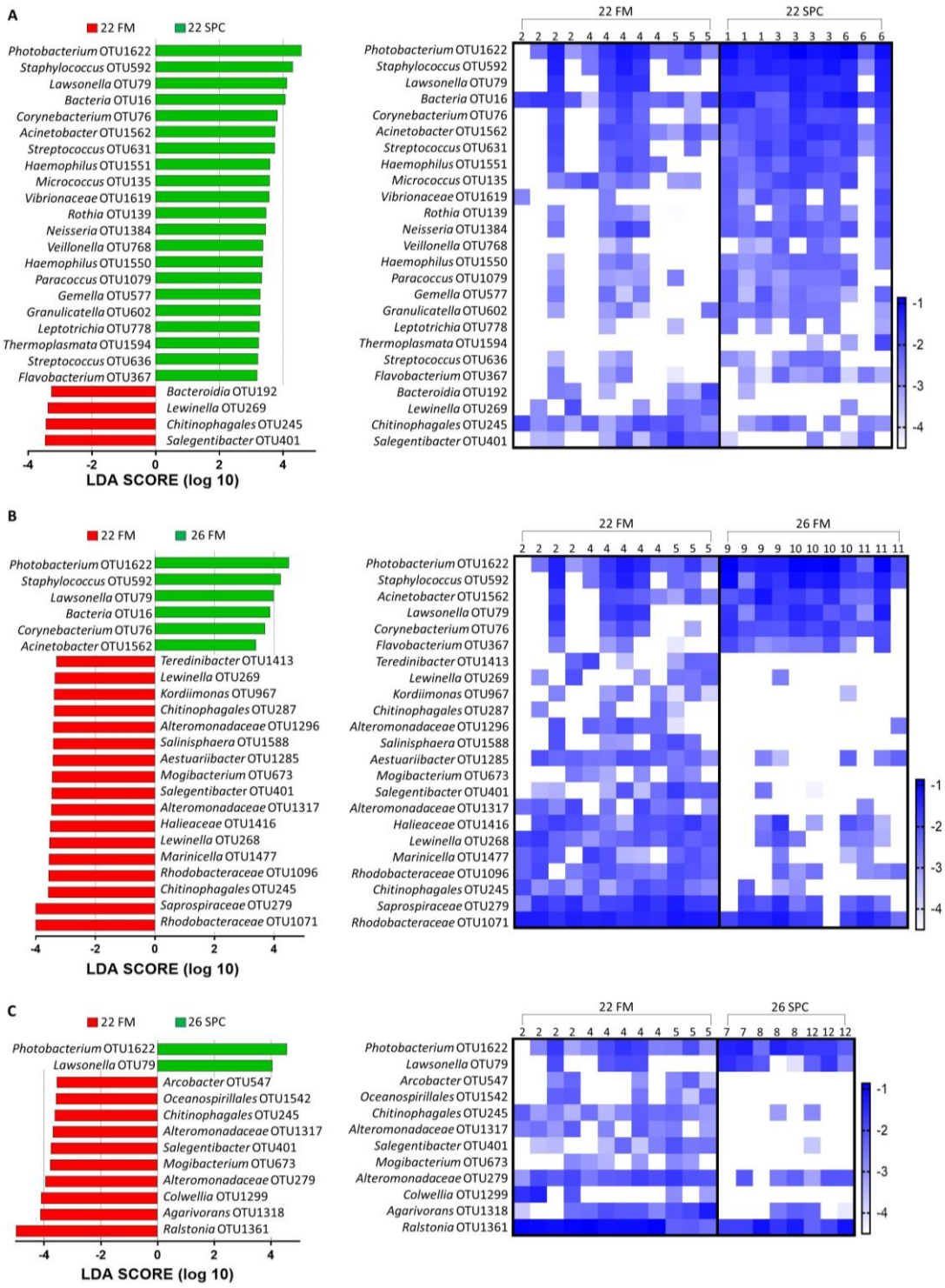

Figure 11 | Impact of diet and temperature on the abundance of OTUs in the skin microbiome. Differentially abundant OTUs were determined by LEfse analysis between control treatment and (A) 22 SPC, (B) 26 FM, and (C) 26 SPC. The left histograms show the significant ($p<0.05$) LDA scores calculated for each OTU and the right heat maps show the relative abundance (Log_{10} transformed) for each biological sample. Rows of the heat map correspond to OTUs and columns to biological samples. Biological samples in the heat map are grouped by treatment and are labelled with their corresponding experimental tank number. Blue and white denote the highest and lowest relative abundance respectively. OTUs were assigned at a genus level where possible, otherwise, the lowest inferred taxonomic level available was given.

3.6 Effects of Diet and Temperature on the Digesta Microbial Community Structure and Composition

To determine how diet and temperature impact the digesta microbiome, the overall microbial composition was analysed via PERMANOVA. These tests indicated that the microbial communities of fish fed SPC at 22°C were statistically significantly different from the control (Table 6). Similar differences were noted in fish housed at 26°C, which had significantly altered community profiles compared to the control treatment (Table 6). As with the skin, the most pronounced shift in digesta community composition from the control treatment was noted in fish fed SPC and maintained at 26°C (Table 6).

Table 6 | Pairwise PERMANOVA comparing the microbial communities of experimental treatments to the control in the digesta.

Control	Treatment	Differ by	R^2	p
22 FM	22 SPC	Diet	0.173	**0.005**
22 FM	26 FM	Temp	0.118	**0.029**
22 FM	26 SPC	Diet + Temp	0.267	**0.001**

Pairwise PERMANOVA with 999 permutations was performed on a Bray-Curtis dissimilarity matrix. Comparisons for which $p < 0.05$ are presented in bold.

Visual analysis of the communities by nMDS plotting also highlights the impact of diet on the overall digesta microbial composition (Figure 12). The large confidence ellipses of the two FM treatments indicate a high level of variability between samples within the treatments (Figure 12). In contrast, the tight clustering of the samples in the two SPC treatments, as indicated by the small ellipses, suggests that the SPC diet is associated with a more uniform community profile across the majority of the samples (Figure 12).

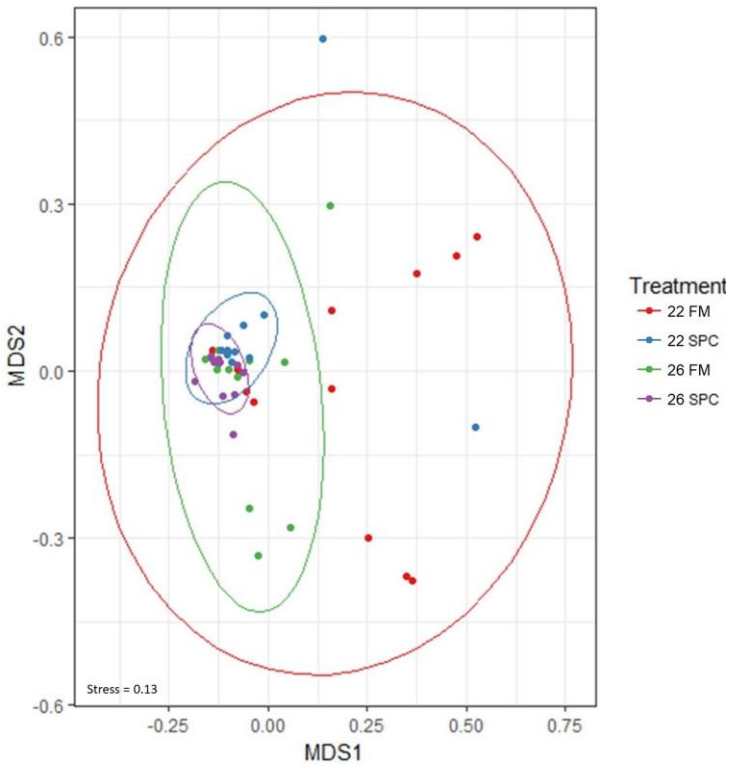

Figure 12 | Microbial community composition of the digesta by treatment.
Non-metric multidimensional scaling plot of sample clustering based upon a Bray-Curtis dissimilarity matrix. Ellipses represent a 90% confidence bubble around the mean for each treatment.

There was no significant difference between treatments in the alpha diversity (Shannon), richness (Chao1), and evenness (Simpson) of digesta samples when analysed by ANOVA (Supplementary Figure 2). The lowest diversity, richness, and evenness scores were observed in the 26 SPC treatment (Supplementary Figure 2).

To establish how the digesta microbiome changes in respect to altered diet and elevated temperature, the relative abundance of taxa in different experimental treatments were evaluated. Digesta microbial communities across all four treatments were dominated by the family *Vibrionaceae,* with a relative abundance of over 80% across all four treatments (Figure 13). No other family comprised more than 5% of the population in any treatment. Whilst the relative abundance of the family *Vibrionaceae* was relatively consistent across treatments, specific genera within this family were strongly impacted by both diet and temperature (Figure 13). Differential abundance testing via LEfse was performed to statistically analyse how diet and temperature impacted the composition of the digesta samples. A small number of OTUs were statistically significantly impacted by diet and temperature. LEfse analysis revealed 5, 3, and 10 differentially abundant OTUs in the 22 SPC, 26 FM, and 26 SPC treatments respectively when compared to the control treatment (Figure

14). Across each of the three experimental treatments, the relative abundance of OTU 1622, assigned to genus *Photobacterium,* was statistically significantly increased with respect to the control treatment (Figure 14). The 22 SPC treatment, when compared to the control, was also associated with higher levels of OTUs assigned as *Clostridiales, Streptococcus*, and *Virgibacillus* and decreased levels of OTU 1626 assigned to the genera *Vibrio* (Figure 14). Three OTUs were statistically significantly impacted by the elevated temperature when compared to the control (Figure 14). These were assigned as *Photobacterium* and *Planctomycetes*, which were increased, and *Vibrionaceae* which decreased in relative abundance (Figure 14). The combination of 26°C and SPC diet was associated with nine OTUs that were lower in abundance relative to the control treatment, whereas the 22 SPC and 26 FM treatments were only associated with a decrease in one OTU respectively (Figure 14). The levels of OTUs assigned as *Vibrionaceae, Acinetobacter*, *Oligoflexales,* and *Pseudomonas* were particularly low in the 26 SPC treatment, as indicated by the heat map in Figure 14. As with the skin samples, variability between biological samples within each treatment was noted (Figure 14). Within each treatment there was no common trend in the relative abundance of OTUs for each tank, indicating that the tank did not have an impact on the microbial composition (Figure 14).

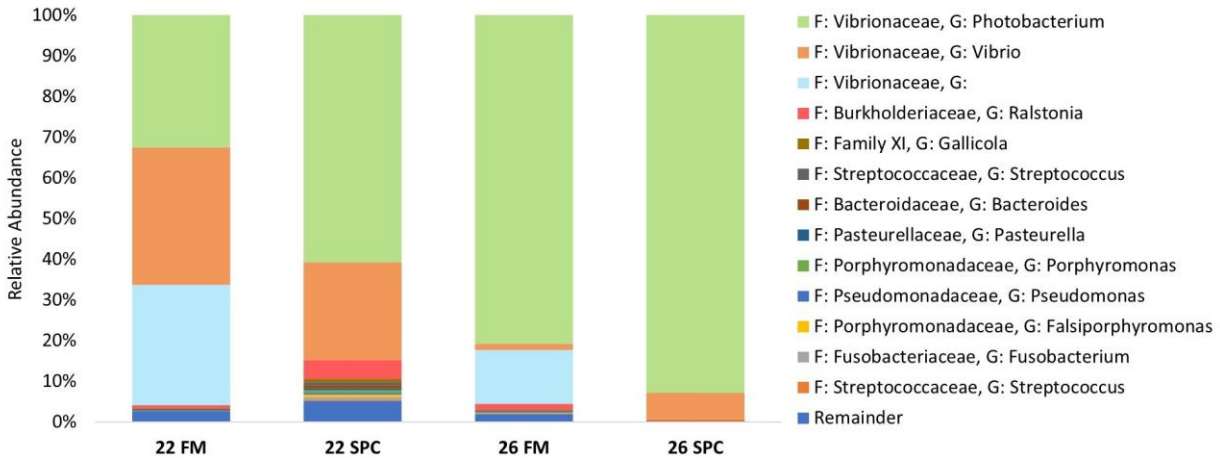

Figure 13 | Digesta microbial composition (mean relative abundance of OTUs) for each treatment grouped by genus with assigned family detailed.

Where no genus has been taxonomically assigned, the genus is left blank. Genera that comprise less than 0.5% of the population in all treatments are grouped together under remainder.

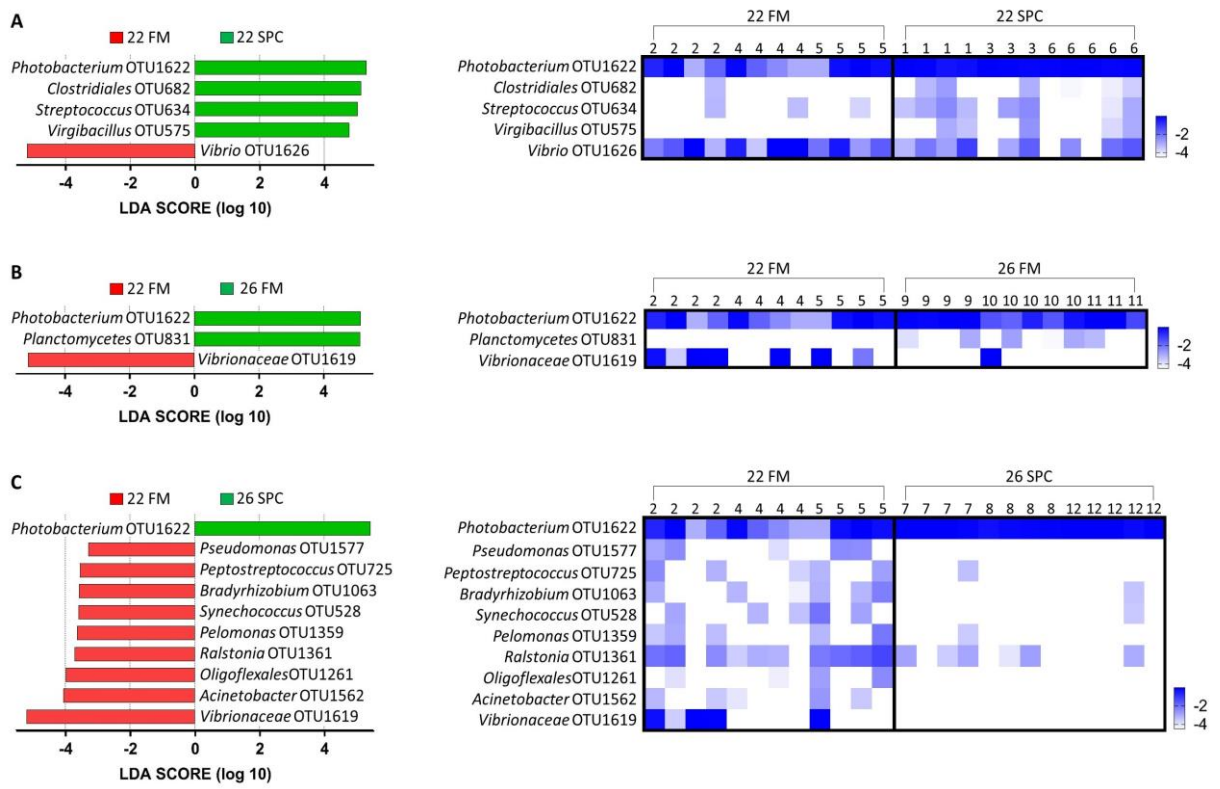

Figure 14 | Impact of diet and temperature on the abundance of OTUs in the digesta microbiome. Differentially abundant OTUs were determined by LEfse analysis between control treatment and (A) 22 SPC, (B) 26 FM, and (C) 26 SPC. The left histogram shows the significant ($p<0.05$) LDA scores calculated for each OTU and the right heat map shows the relative abundance (Log_{10} transformed) for each biological sample. Rows of the heat map correspond to OTUs and columns to biological samples. Biological samples in the heat map are grouped by treatment and are labelled with their corresponding experimental tank number. Blue and white denote the highest and lowest relative abundance respectively**.** OTUs were assigned at a genus level where possible; otherwise, the lowest taxonomic level available was assigned.

OTU 1622, assigned to genus *Photobacterium,* was dominated (94%) by a single sequence variant. A blastN search of the NCBI nucleotide database (July 2018) showed this sequence variant shares 100% identity with characterised strains of species *Photobacterium damselae*, including *Photobacterium damselae* subsp. *damselae* (GenBank ID MG077071.1) and *Photobacterium damselae* subsp. *piscicida* (GenBank ID MH472944.1), indicating this OTU can be tentatively assigned at a species level, but not to a specific strain. For OTU 1626, assigned to genus *Vibrio*, two sequence variants comprised 96% of assigned sequences, however in this case blastN searches indicated that the sequenced region for these variants was not sufficiently informative to identify taxonomy below the level of genus.

Certain strains of the *Photobacterium damselae* are pathogenic and can cause lethal infections in fish [122, 123]. Given that taxonomic characterisation of OTU 1622 at a strain level was not possible, methods to identify the presence of pathogenic markers common to virulent strains of *Photobacterium* were employed. Virulent strains of *Photobacterium* have been found to contain a chromosome-encoded phospholipase (plpV) that has haemolytic activity, and this gene was therefore determined as a marker of virulence [1]. PCR assays to amplify a region of this virulence gene using primers designed by Vences and colleagues (2017) were carried out using the extracted community DNA from the 43 final digesta samples [111]. A PCR product of the expected size (1,618 bp) was noted in 38 digesta samples, all of which had a relative abundance of OTU 1622 greater than 20% (Table 7). This product was not observed in 5 samples that had a relative abundance of OTU 1622 of less than 2%. The positive bands from three randomly selected samples were sequenced and all found to have high similarity (>95%) to the coding region of haemolysin genes found in both *Photobacterium damselae* subsp. *piscicida* and *Photobacterium damselae* subsp. *piscicida* (Supplementary Table 3). The translated nucleotide sequence of the three sequenced plpV PCR products were all found to share 100% amino acid identity with sequenced thermolabile haemolysin proteins from *Photobacterium Damselae* (Supplementary table 4).

Table 7 | Presence of *Photobacterium* plpV gene in digesta samples

	No. of samples	Presence of plpV	%
Relative Abundance > 20%	38	38	100%
Relative Abundance < 2%	5	0	0%

Network analysis was performed to analyse and visualise how specific OTUs correlate with diet, temperature, and measured health and growth parameters. Network analysis visually represents statistically significant correlations between continuous or categorical variables. This analysis revealed that OTU 1622, assigned to genus *Photobacterium*, was significantly correlated with decreased levels of digesta myeloperoxidase (Figure 15). OTU 1622 was also significantly positively correlated with SPC diet and negatively with FM diet (Figure 15). OTU 1622 was the only OTU that was significantly correlated with either FM or SPC diets. Higher levels of digesta peroxidase were positively associated with the FM diet and negatively associated with the SPC diet. Fourteen OTUs were positively correlated with levels of digesta peroxidase (Figure 15). The most abundant of these was OTU 1619, only classified to family *Vibrionaceae,* which had a maximum relative abundance of 66% across all digesta samples. The other 13 OTUs all had a relative abundance of less than 4% in any given sample and were diverse in their taxonomic classification, spanning seven different taxonomic orders (Supplementary Table 5).

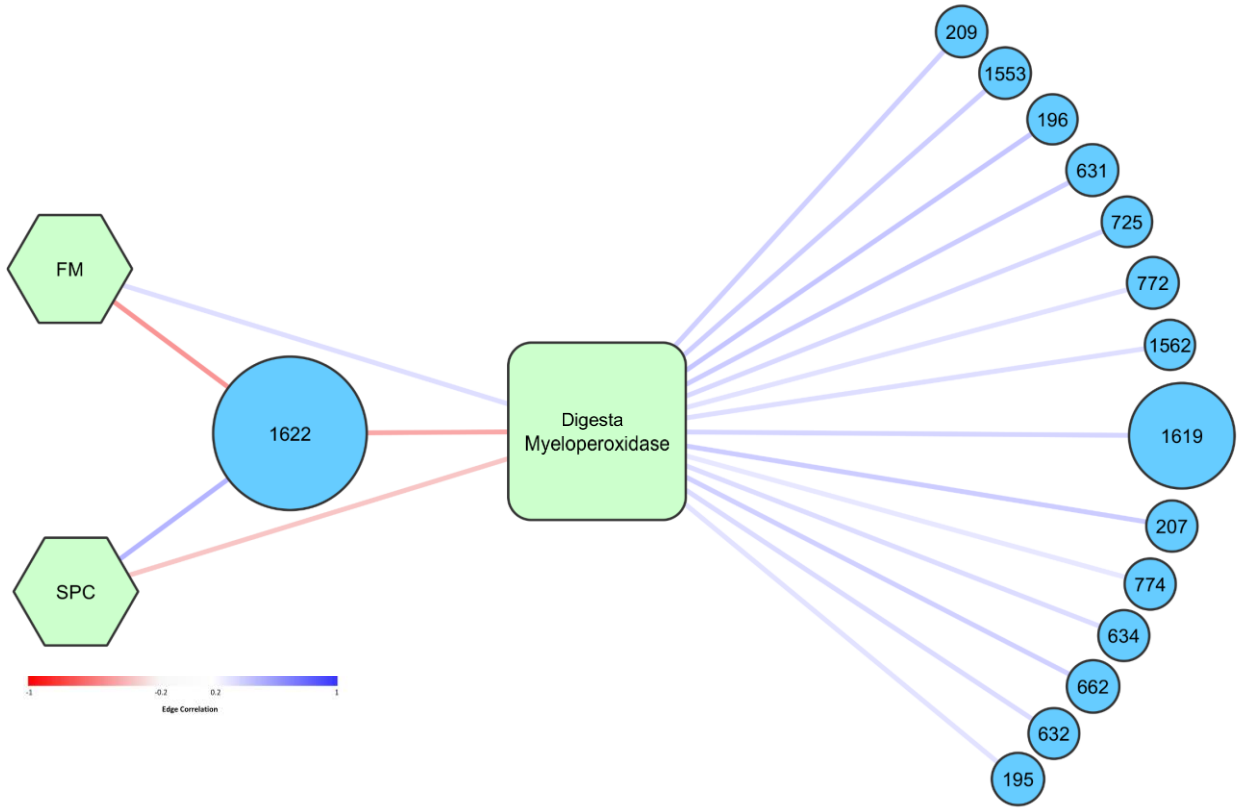

Figure 15| Graphical network representing the interactions in digesta samples between OTUs, diet, and digesta myeloperoxidase.

Only significant correlations ($p < 0.05$) are shown. Circular nodes correspond to OTUs and the size of these nodes represents the maximum relative abundance of that OTU across all digesta samples. Square nodes correspond to physiological measurements taken, and hexagonal nodes to diets. Red edges represent negative correlations, whilst blue edges represent positive correlations. The strength of the red or blue colour represents the strength of the correlation. Taxonomy assigned to OTUs is presented in Supplementary Table 5.

3.7 Effects of Diet and Temperature on Gut Mucosa Microbial Community Structure and Composition

In a similar manner to the skin and digesta communities, the impact of diet and temperature on the microbial community of the gut mucosa samples was analysed. Comparison of the overall gut mucosa microbial community by pairwise PERMANOVA tests showed no statistically significant differences between the experimental treatments, indicating that diet and temperature had no statistically significant impact on overall community composition (Supplementary Table 6). As with the digesta and skin samples, the taxonomic investigation revealed that the gut mucosa microbiome, across all four treatments, was dominated by three genera of the family *Vibrionaceae* (Figure 16.A). These three genera had a combined relative abundance of 91% in 22 FM, 89% in 22 SPC, 87% in 26 FM, and 76% in 26 SPC treatments (Figure 16.A). Within the family *Vibrionaceae* the mean relative abundance of OTUs assigned as *Photobacterium* differed between treatments (Figure

16.A). The only other genus that comprised over 2% of the population in any of the treatments was *Ralstonia* (Figure 16.A). The remainder of the bacterial population detected in the gut mucosa was assigned to 264 genera with an abundance of less than 2%. Figure 16.A shows marked differences in the mean relative abundance of different genera by treatment; however, Figure 16.B reveals that there is a high degree of variability in individuals that make up the mean relative abundance within each treatment. LEfse analysis revealed only one OTU that had a statistically significantly different relative abundance between treatments. OTU 1619, which is only classified to the family level of *Vibrionaceae* was statistically significantly lower in the 22 SPC (LDA -5.5), 26 FM (LDA -5.4), and 26 SPC (LDA -5.2) treatments.

There was no significant difference between treatments in the alpha diversity (Shannon), richness (Chao1), and evenness (Simpson) of gut mucosa samples when analysed by ANOVA (Supplementary Figure 3).

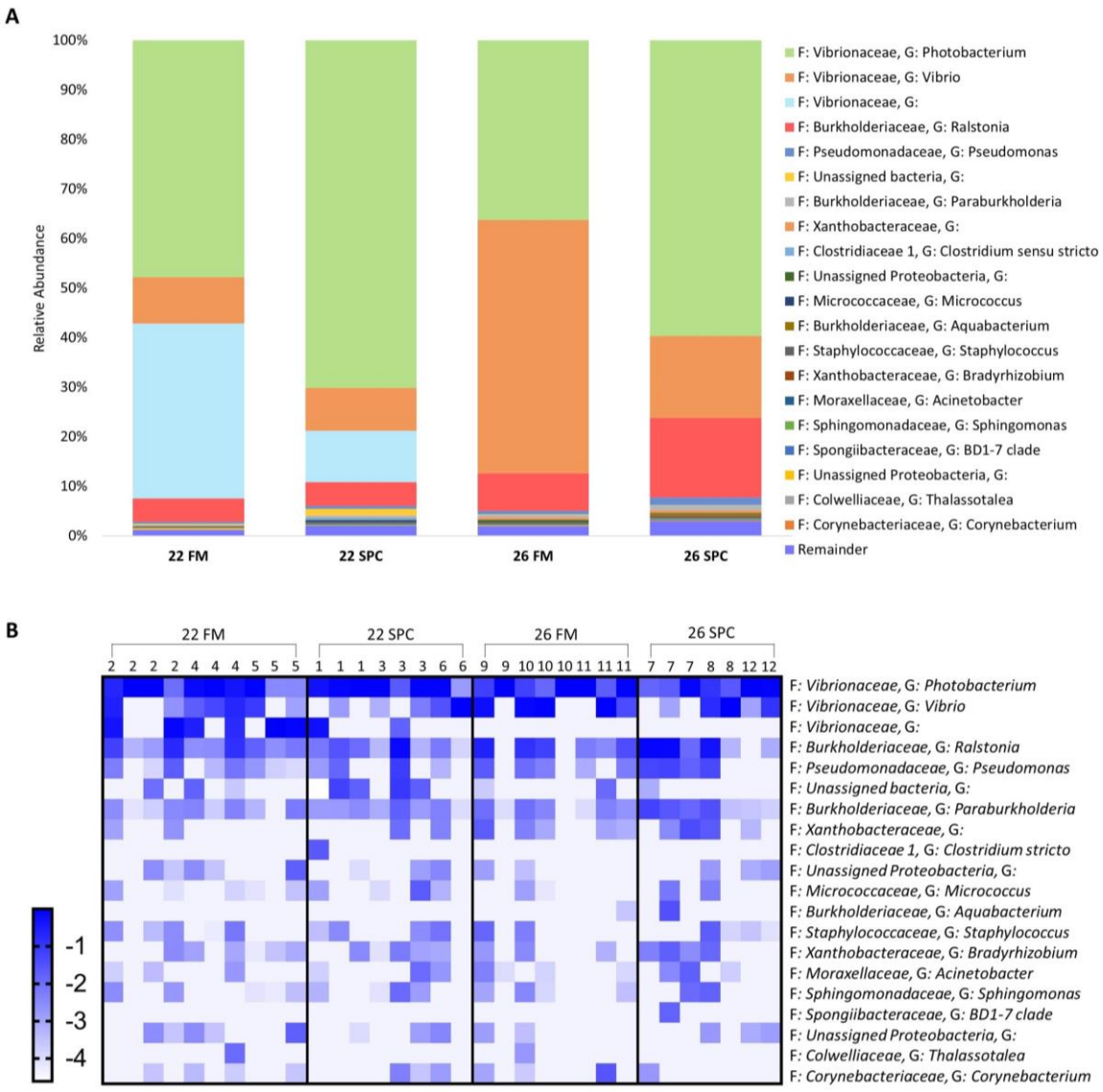

Figure 16 | Mean relative abundance of OTUs present in gut mucosal samples grouped by genus. (A) Mean relative abundance of OTUs in each treatment grouped by genera with family detailed. Genera comprising less than 0.2% grouped under remainder. (B) Mean relative abundance (Log_{10} transformed) of OTUs grouped by genera that comprise >0.2% of each treatment. Rows of the heat map correspond to OTUs and columns to biological samples. Biological samples in the heat map are grouped by treatment and are labelled with their corresponding tank number. Blue and white denote the highest and lowest relative abundance respectively. Where no genus has been taxonomically assigned, the genus is left blank.

4. Discussion

This study aimed to investigate how replacement of fishmeal with SPC impacted the health and microbiome of Yellowtail Kingfish at optimal (22°C) and supra-optimal (26°C) water temperature. This study found that a 30% inclusion of SPC altered the microbiome of the skin mucosa, digesta, and gut mucosa of Yellowtail Kingfish, with changes in the microbiome and host health being more pronounced in fish reared at 26°C.

4.1 Diet and Temperature Impact Growth and Innate Immunity

Both diet and water temperature had a significant impact on the growth of the Yellowtail Kingfish in this study. Given that the diets were very similar in nutrient and energy content, it is noteworthy that such a pronounced reduction in weight gain occurred within 21 days. Similar reductions in weight gain have been noted in juvenile Yellowtail Kingfish fed SPC diets; however, this was over a slightly longer duration of 34 days and in younger fish [96]. According to Pirozzi and Booth (2009), water temperatures above 22-24°C are not metabolically optimal for Yellowtail Kingfish of the size used in the present study and therefore the higher rearing temperature was predicted to induce slower growth [103, 106]. Given that all fish were fed the same slightly restricted ration, the reduced weight gain in fish reared at 26°C may also be due to the fact these fish were more metabolically active and thus expending more energy relative to those housed at 22°C [106].

The SPC diet was associated with increased levels of measured plasma lysozyme, an important immune defence molecule that causes lysis of bacteria through breaking linkages in the cell wall [121]. Higher levels of lysozyme activity have previously been observed in Atlantic salmon fed diets containing SPC [124]. It is hypothesised that dietary SPC causes inflammatory responses in fish that impact the levels of innate immune parameters such as lysozyme. Future work analysing the expression of inflammatory marker genes in response to SPC diets would allow examination of this theory [125].

The measured levels of MPO were significantly lower in fish fed SPC diets relative to the control treatment. This is further discussed in section 4.6.

4.2 Body Site Dictates Microbial Composition and Diversity

As expected, the microbial community of the Yellowtail Kingfish skin mucosa was distinct from that of the gut mucosa and the digesta. Prior work in rainbow trout and Atlantic salmon has also shown the skin microbiome to be distinct from that of the gut [37, 69, 76]. The skin microbiome in the

present study also exhibited significantly higher diversity than the gut mucosa and digesta samples, which is consistent with previous work in rainbow trout [37].

The high diversity of the skin mucosa is also consistent with previous findings for Yellowtail Kingfish. Legrand and colleagues (2018) in their study observed an average Shannon diversity of 2-4 for skin microbiomes in both farmed and wild Yellowtail Kingfish [83]. In contrast, the extremely low diversity in the digesta and gut mucosa noted in this study was not observed in the only previous gut microbiome study focusing on this species [83]. Ramírez and Romero (2017) found the Shannon diversity of wild and farmed Yellowtail Kingfish gut digesta samples to be 4 and 6, respectively, which is in keeping with reports for Atlantic salmon and gilthead seabream, where Shannon diversity measurements ranging from 4 to 7 are reported [69, 74, 82]. In this study the Shannon diversity of gut digesta was found to be less than one for control animals, and lower still in the experimental treatments [82]. While the diversity of the digesta and gut mucosa in this study was lower than generally reported; there are past fish microbiome studies reporting low diversity in these sites. Uren Webster and colleagues (2018) observed Shannon diversity index scores of less than 1 in Atlantic salmon [126]. A recent study investigating the gut microbiome of Atlantic cod *(Gadus morhua*) found similarly low levels of diversity, with an average Shannon diversity index of 1.4 and a minimum of 0.49 [127]. Interestingly, in accordance with the present study, the Atlantic cod microbiomes were dominated by an OTU assigned to genus *Photobacterium* [127]. It is possible, therefore, that the genus *Photobacterium* is able to rapidly colonise and become dominant in the gut of certain fish species.

The digesta and gut mucosa communities were not significantly different in their overall composition in fish fed FM diets; however, these communities were distinct in fish fed SPC diets. The similarity in the microbial communities of fish fed FM diets is in contrast to previous work in Atlantic salmon and rainbow trout which found the gut mucosal and digesta microbiomes to be significantly different [69, 71, 102]. As this is the first study to compare the gut mucosa and digesta microbiome in Yellowtail Kingfish, further studies are required to determine whether the communities of the digesta and gut mucosa of Yellowtail Kingfish are usually similar.

4.3 Diet and Temperature Influence Overall Microbial Composition, Growth and Health

Shifts in microbial composition were observed in the skin mucosa, digesta and gut mucosa of Yellowtail Kingfish fed the SPC diet and housed at 26°C. The results presented here on the impact of SPC on Yellowtail Kingfish are similar to past work in a number of other fish species where it has

been demonstrated that that partial fishmeal replacement with alternative proteins significantly influences the microbiome [74, 85, 91, 102, 128]. Changes in microbial community profiles in the present study were also associated with a reduction in fish growth and a significant alteration in measurable immune parameters. Given that the microbiome has previously been shown to influence digestion, nutrient assimilation, and stimulation of the immune system, it is reasonable to hypothesise that SPC and increased water temperature induced shifts in the microbial communities of Yellowtail Kingfish, subsequently influencing the growth and health of the animal [23, 43]. SPC-induced shifts in the microbiome may act in parallel with any anti-nutritional factors remaining in the processed SPC, with both factors impacting the digestive abilities of the fish [7, 66]. Determining how each of these factors individually impact digestion is complex due to the direct impact the anti-nutritional factors have on the microbial communities. Further studies incorporating a wider range of diets and that measure a greater number of health parameters would provide a more detailed understanding of how diet and the microbiome influence Kingfish health and growth.

One of the objectives of this study was to understand how the response of the microbiome to SPC is influenced by water temperature. Raising water temperature from 22°C to 26°C influenced the composition of the microbiome across all body sites; however, the combination of elevated water temperature and dietary inclusion of SPC resulted in the greatest shifts in microbial community composition. These results indicate that elevated temperature impacts the microbial composition of the skin and digesta and that water temperature also influences the response of the microbiome to altered diet. Previous studies have shown that water temperature can impact the development of intestinal disorders in Yellowtail Kingfish fed SPC and that elevated temperatures can enhance the severity of soybean meal induced enteritis in Atlantic salmon [7, 96, 107]. Despite this, we currently have only a sparse understanding of how perturbations in water temperature impact microbial composition and how water temperature influences the impact of FM replacement [129]. The results of the present study suggest that the impact of diet on the microbiome may be exacerbated by additional environmental stressors, such as non-optimal water temperature. Future studies of the impact of alternative diets would benefit from considering both optimal and non-optimal environments encountered in commercial aquaculture settings. This might include examination of changing abiotic factors such as salinity, pH and dissolved oxygen.

4.4 Yellowtail Kingfish have a Core Skin Microbiome that is Impacted by Diet and Temperature

There was a core bacterial community in the skin mucosa observed across all treatments. This core community was dominated by the phyla *Proteobacteria* followed by varying proportions of *Bacteroidetes, Firmicutes,* and *Actinobacteria*. These results are broadly consistent with the work of Legrand and colleagues (2018), who also found that these four phyla were highly abundant in the skin mucosa of Yellowtail Kingfish [83]. Interestingly, the relative abundances of bacterial phyla noted in the present work show more similarity to what these authors report for wild Yellowtail Kingfish, whereas farmed fish raised in sea-cages had higher proportions of *Bacteroidetes* [83]. High-throughput skin microbial community analyses in other farmed fish species have also found *Proteobacteria* to be the dominant phylum associated with the skin mucosa, followed by differing proportions of *Firmicutes, Actinobacteria*, and *Bacteroidetes* [76, 78, 79, 130].

The skin mucosa of Yellowtail Kingfish was associated with 982 unique OTUs and shared only 92 OTUs with the estuarine water samples, indicating that the skin microbiome is independent of the surrounding water (Figure 3). This finding is consistent with that of Chiarello and colleagues (2015) who noted that the skin microbial communities of both gilthead seabream and European seabass (*Dicentrarchus labrax*) shared few OTUs with the water [78]. Given that specific phyla are consistently dominant across a range of fish species and the communities are distinct from the surrounding water, it is hypothesised that the skin mucosa plays a role promoting these core microbes, although the mechanisms for this remain unclear [26]. Future studies combining mucosal glycomics and microbial community analysis may be helpful in gaining an understanding of how the mucosa interacts with and influences microbial composition [32].

The relative abundance of OTUs assigned to *Photobacterium* and *Lawsonella* were increased in fish fed SPC at both 22°C and 26°C when compared to the control FM treatment. *Lawsonella* belongs to the family *Corynebacteriaceae* and increases in this family have also been reported in response to altered diet [131]. Rimoldi and colleagues (2018) noted that the relative abundance of *Corynebacteriaceae* was increased in the gut of rainbow trout fed a high proportion of plant protein when compared to the other diets, indicating that alternative feeds may specifically impact this family [131]. Low levels of *Photobacterium* have been noted in the skin of marine fish; however, this genus showed higher relative abundance in the skin microbiome in this study than previously reported [76, 78, 83, 132]. The relative abundance of *Photobacterium* was particularly high in the three experimental treatments compared to the FM control, indicating that diet and temperature

are influencing the levels of these bacteria on the skin, possibly by inducing alterations to the skin mucosa.

OTUs assigned to *Salegentibacter* and *Chitinophagales* were the only OTUs that showed lower relative abundance in fish fed SPC across both temperatures when compared to the control treatment. There is little available literature describing either *Salegentibacter* or *Chitinophagales* in the fish microbiome, and therefore little inference can be drawn from the altered relative abundance of these OTUs. Neither *Salegentibacter* or *Chitinophagales* were assigned to the top 100 most abundant OTUs in previous investigations of the Yellowtail Kingfish skin microbiome [83].

Elevated temperature, regardless of diet, was associated with a decrease in the relative abundance of OTUs assigned to *Alteromonadaceae* when compared to the control. Lower relative abundance of *Alteromonadaceae* has previously been associated with poor health, with lower levels observed in Yellowtail Kingfish exhibiting enteritis and in Atlantic salmon infected with Salmonid alphavirus [83, 133]. Given that fish housed at 26°C in the present study were associated with decreased growth and altered immune parameters, the relative abundance of *Alteromonadaceae* in the skin is potentially an indicator of poor fish performance and reduced health. Increased prevalence of the genus *Photobacterium* in the skin may also be a marker of reduced fish performance and may potentially be used in conjunction with *Alteromonadaceae* to non-invasively define fish health status.

Comparing changes in the microbiome in each treatment provides information on how diet and water temperature influence microbial composition independently and in concert. In comparison to the control, the 22 SPC treatment was associated with higher relative abundance of 21 OTUs, whereas the 26 SPC treatment was only associated with 2. This variation indicates that diet influences the relative abundance of microbiota in the skin; however, the surrounding water temperature further influences which OTUs are impacted by diet. This further supports the hypothesis that the diet related shifts in the microbiome and health of fish are exacerbated by additional environmental stressors, such as non-optimal water temperature.

4.5 Diet and Temperature Influence the Microbiome of the Digesta

The digesta was dominated by OTUs assigned to the family *Vibrionaceae* regardless of treatment. The dominance of this family is in line with a previous meta-analysis of marine fish microbiomes and experimental work in Atlantic cod and gilthead sea bream [20, 127, 134]. Although the high relative abundance of *Vibrionaceae* and the genus *Photobacterium* is not without precedent, it is important to address the question of whether the abundance of these bacterial groups may have been inflated

due to issues with the methodology applied. Processing of all samples, including digesta, was designed to minimise handling time and collected samples were all placed on ice within five minutes of collection to reduce the chance of subsequent overgrowth of species, including *Vibrio* representatives, known for their fast doubling times [135, 136]. Samples were placed on ice immediately on collection and transferred from ice to -80°C within 20 minutes of collection. The sampling, DNA extraction, and PCR processes were standardised across samples and body sites, and all sequencing was performed as a single run. As the high relative abundance of OTUs assigned to *Vibrionaceae* was observed only for digesta and gut mucosa samples, it is posited that the high relative abundance of certain OTUs indeed reflects the community profile in these samples and is not due to biases and/or contamination introduced during sample processing.

The inclusion of 30% SPC for fish housed at 22°C resulted in a substantial increase in the relative abundance of OTU 1622 (assigned to *Photobacterium)* relative to the FM control, along with increases in three other bacterial OTUs assigned to *Clostridiales* and *Streptococcus*. Only one OTU declined significantly in the 22 SPC treatment relative to the control, OTU 1626 which is assigned to genus *Vibrio*. In contrast, the combination of SPC at 26°C was associated with an increase in OTU1622 alone, while nine OTUs were found to be significantly reduced in their relative abundance compared to the control. This suggests that the SPC diet, when combined with the additional stress of elevated temperature created an opportunity for OTU 1622 to dominate in this environment and out-compete other OTUs. Whilst OTU 1622 appears to be out-competing other OTUs in the 26 SPC treatment, there was only a minor reduction in alpha diversity. The slight reduction in alpha diversity suggests that many OTUs were reduced in relative abundance under the 26 SPC treatment; however, the majority remained present in the population at low levels.

Analysis of the sequence variants within OTU 1622 indicates that this likely represents the species *Photobacterium damselae* but does not distinguish between different variants of this species which are reported to differ in their pathogenicity [111]. Well-characterised subspecies of *Photobacterium damselae,* subsp. *damselae* and subsp*. piscicida* are common pathogens of fish thought to infect internal organs through invasion across the gastrointestinal epithelia [111, 122, 137]. In order to determine if OTU1622 is likely to represent a pathogenic *Photobacterium* strain, assays were performed to determine whether a chromosomally encoded phospholipase (plpV) was present in the samples [111]. As plpV has haemolytic activity and is thought to contribute to pathogenicity, the presence of the gene has been previously used as a marker for identifying pathogenic strains of *Photobacterium* [111]. Of the 43 final digesta samples, all 37 samples with a relative abundance of

Photobacterium above 20% produced a PCR product of the expected size, while the other five samples that had a relative abundance of *Photobacterium* of less than 2% gave no PCR product. Three of the PCR products of the expected size were sequenced and found to share 100% amino acid identity with sequenced thermolabile haemolysin proteins found in *Photobacterium Damselae*. The results of the PCR indicate that a pathogenic subspecies of *Photobacterium* was present in the digesta and that this is associated with the relative abundance of OTU 1622. As such, it appears that dietary inclusion of SPC and elevated water temperature has created an opportunity for a potentially pathogenic strain of *P. damselae* to dominate the digesta, out-competing other microbial species.

4.6 Diet Influences the Immune Status of the Gut

Myeloperoxidase (MPO) is a component of the fish innate immune system produced by neutrophils and acts as a primary defence against pathogenic bacterial infection [73]. MPO catalyses the production of the antimicrobial hypochlorous acid [138]. Levels of MPO were significantly reduced in fish fed SPC when compared to the control. There are a number of explanations as to why MPO is reduced in fish fed the SPC diet: firstly, network analysis highlighted a statistically significant correlation between reduced MPO in the digesta and high relative abundance of OTU 1622, indicating that this OTU may be directly influencing MPO production in the gut. Virulent strains of *P. damselae* ssp. *piscicida* have been shown to induce apoptosis of sea bass neutrophils in what appears to be a key pathogenic strategy for successful infection [122]. Given that neutrophils are the primary producers of MPO, it is possible that the reduction in measurable MPO is due to *Photobacterium* induced apoptosis of neutrophils. Alternatively, soy-based products have previously been shown to induce alterations in the histology of the gut, and therefore may be directly influencing neutrophil recruitment and the level of MPO [7, 139]. It is also possible that a combination of increased abundance of OTU 1622 and the direct impact of SPC on the gut both acted to influence the levels of MPO in the digesta. The correlation between reduced levels of MPO and increased OTU 1622 abundance indicates that suppression of the fish immune system through these intimated or other mechanisms may have contributed to the dominance of the genus *Photobacterium* observed in the digesta.

4.7 The Gut Mucosal Microbiome Appears Relatively Resistant to Changes in Diet and Temperature

Unlike the digesta microbiome, the overall composition of the gut mucosa microbiome was not significantly impacted by diet or water temperature. The digesta and gut mucosa microbial profiles

were similar, both being dominated by *Vibrionaceae*; however, the relative abundance of OTU 1622 (assigned to *Photobacterium*) did not reach the same levels in the gut mucosa as in the digesta. The lower relative abundance of OTU 1622 in the gut mucosa samples indicates that *Photobacterium* was not able to outcompete other bacteria or colonise the mucosa as effectively as in the digesta. As with the skin microbiome, this shows that the mucosal microbiome is not simply a reflection of its surrounding environment (i.e. the digesta) and indicates that the mucosa influences microbial composition.

The only OTU that was significantly impacted by diet or temperature was OTU 1619, which is only classified to the family level of *Vibrionaceae*. Given that members of the family *Vibrionaceae* are diverse and include both commensal and pathogenic bacterial strains, it is not currently possible to comment on the potential functionality of this OTU [23, 68]. As the gut mucosal microbiome was less impacted by treatment than the skin mucosal microbiome, it would be helpful to understand whether these changes were reflected in the mucosal composition. Proteomic and glycomic studies would provide insight into the gut and skin mucosal composition, allowing comparison of the relative impact of treatment on each body site.

4.8 Conclusions

The skin, digesta, and gut mucosa microbiomes of Yellowtail Kingfish were significantly impacted by inclusion of soy protein concentrate (SPC) in the diet. These effects were amplified at elevated water temperature. The SPC diet and elevated temperature also significantly reduced the growth of fish and altered measured innate immune parameters. In the digesta of fish fed the SPC diet and housed at 26°C a single OTU assigned to *Photobacterium* dominated the microbial communities when compared to the FM control reared at 22°C. The high relative abundance of the OTU assigned as *Photobacterium* was correlated with the presence of the gene plpV which encodes a phospholipase that contributes to virulence, indicating that this OTU may have pathogenic capacity. This OTU was also linked to a reduction in levels of the innate immune defence molecule MPO. Given the significant reduction in growth and the major alterations to the microbiome observed in this study, it would be prudent to limit the amount of SPC included in commercial diets for Yellowtail Kingfish until further research is undertaken.

An elevated water temperature of 26°C independently influenced the response of the microbiome to diet and contributed to reduced growth. Water temperature also influenced the effect of diet, with the impact of SPC on the skin and digesta microbial composition being most pronounced at 26°C. Similarly, the relative abundance of *Photobacterium* was highest in the 26 SPC treatment.

Taken together, these results indicate that dietary changes that may be tolerated in optimal conditions may impact health and growth if another stressor commonly associated with commercial fish production (such as non-optimal water temperature) is encountered. As such, the impact of stressors should be carefully considered by aquaculture practitioners before they commit to using alternative raw feed materials or making significant changes to the diet of the fish, such as changing brand of feed or moving from weaning feeds to production feeds.

Diet had a significant impact on the skin despite having no direct interaction with this mucosal surface. It is hypothesised that changes in the Yellowtail Kingfish mucosal environment, potentially through diet induced alterations of mucins and secretions of immune-related molecules, caused these shifts in the microbiome. Further studies combining glycomics and proteomics with microbial composition analysis would allow investigation of this hypothesis. As the skin microbiome appears to be impacted by diet and health, the composition of the skin mucosa provides a potential non-invasive biomarker for fish monitoring.

Fishmeal replacement is an economic imperative for the global aquaculture industry. Further research should continue to be directed at understanding the impact that alternative raw feed materials have on Yellowtail Kingfish microbiome and associated health. Increasingly, research is providing evidence that suggests replacement of fishmeal in diets for carnivorous fish will remain challenging. Nonetheless, a greater understanding of what a healthy microbiome for species such as Yellowtail Kingfish looks like, coupled with a greater understanding of how the microbiome reacts to perturbations in feed source and the environment will lead to advances in this area of research.

5. References

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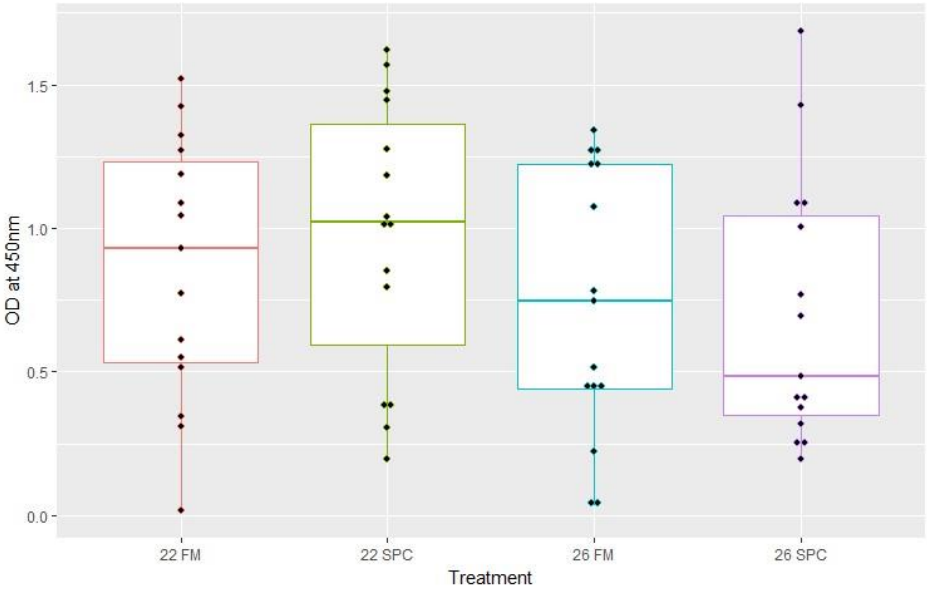
6. Supplementary Information

Supplementary Table 1 | Ingredients and compositions of the experimental diets

	FM	SPC
Ingredients		
Fishmeal	682.4	475.1
Soy Protein Concentrate	-	300.0
Wheat flour	270.0	188.0
Fish oil	35.0	24.4
Choline chloride (70%)	6.0	6.0
Vitamin C (Stay-C 35®)	0.6	0.6
Vitamin / mineral premix	6.0	6.0
Total dry matter	1,000.0	1,000.0
Nutrient composition		
Nitrogen	91.3	93.6
Crude protein	570.8	585.2
Ash	125.0	109.8
Lipid	83.7	61.2
NFE	220.5	243.8
Gross energy (MJ kg^{-1})	20.8	20.8
Amino acid composition		
Alanine	34.7	31.4
Arginine	26.4	31.2
Aspartic acid (+ asparagine)	52.8	61.1
Cysteine	5.8	5.3
Glutamic acid (+ glutamine)	83.3	94.6
Glycine	38.9	33.9
Histidine	9.8	9.6
Isoleucine	19.9	21.1
Leucine	39.7	40.9
Lysine	35.2	36.5
Methionine	13.1	12.8
Phenylalanine	23.9	25.6
Proline	26.6	28.2
Serine	28.1	30.5
Taurine	13.6	12.5
Threonine	24.0	24.7
Tyrosine	18.8	21.1
Valine	26.4	26.8
Total reported amino acids + taurine	520.9	547.9

Values are given in g kg^{-1} dry matter unless otherwise stated.

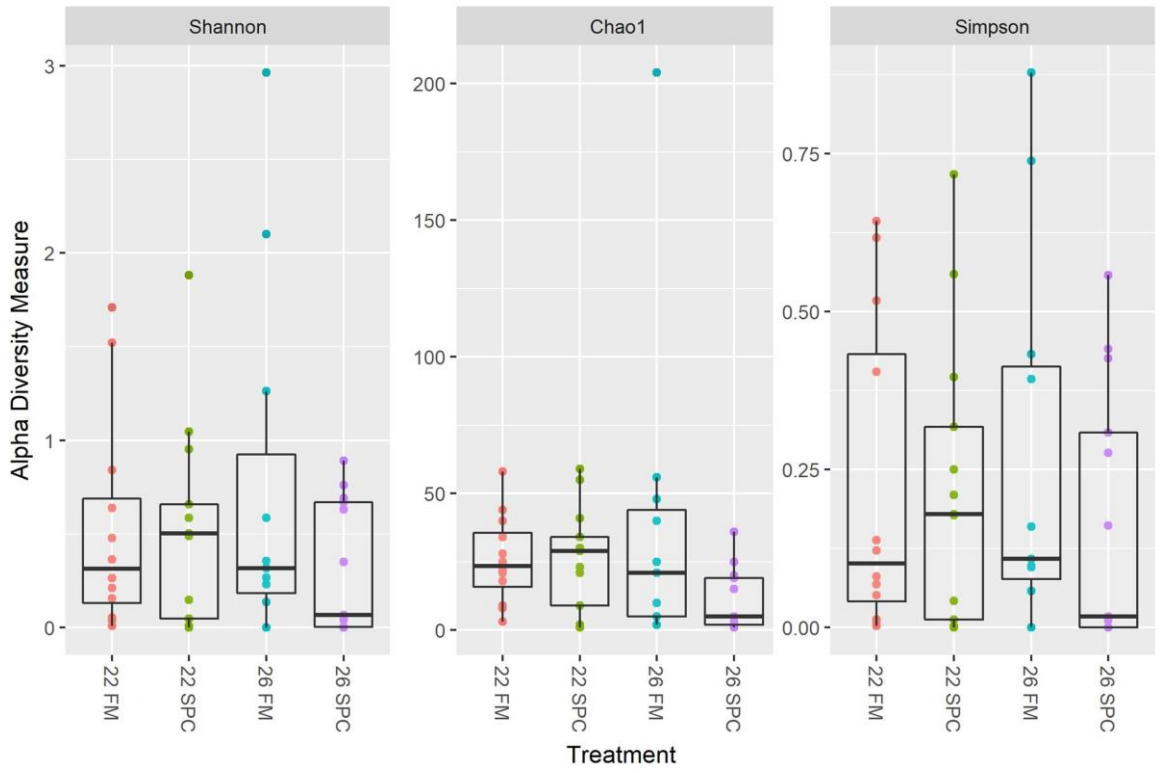


Supplementary Figure 1 | Levels of plasma peroxidase and in Yellowtail Kingfish for each treatment.

Supplementary Table 2 | PERMANOVA comparing the baseline microbial communities of the skin, digesta, and gut mucosa by treatment.

Body site	r2	p
Skin	0.079	0.181
Digesta	0.065	0.286
Gut Mucosa	0.894	0.387

Pairwise PERMANOVA with 999 permutations was performed on a Bray-Curtis dissimilarity matrix.



Supplementary Figure 2 | Alpha diversity (Shannon), richness (Chao1), and evenness (Simpson) of digesta microbial communities for each treatment.

Shannon, Chao1, and Simpson alpha diversity metrics for each digesta sample, grouped by treatment.

Supplementary Table 3 | Nucleotide sequence identity of isolated plpV PCR products

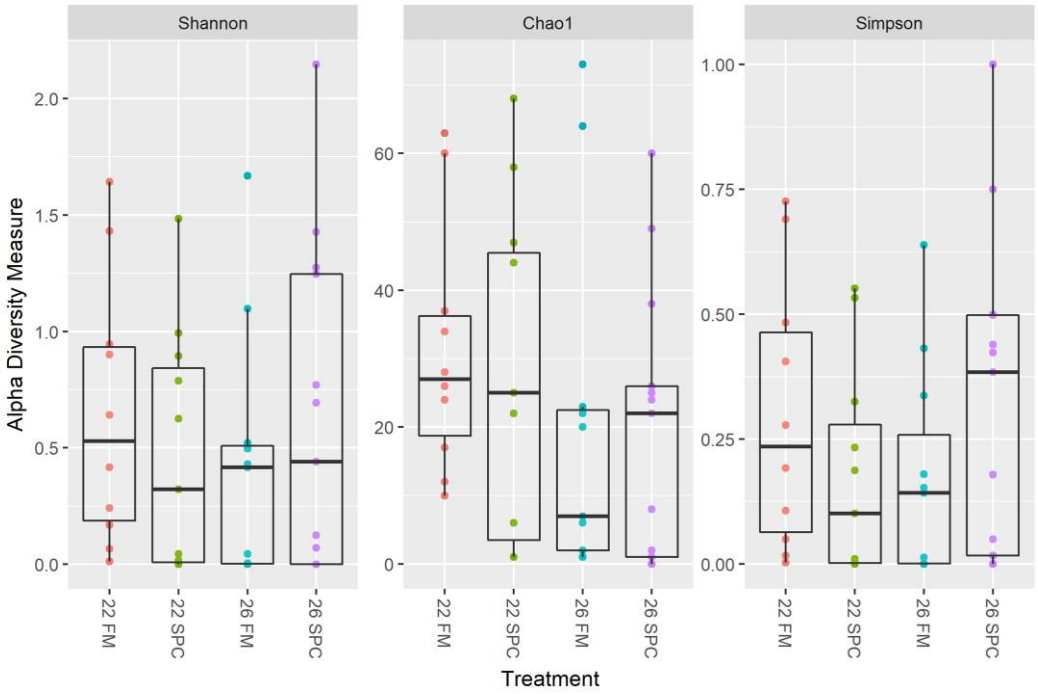
Sample Name	Alignment	Accession	Identity
124	*Photobacterium damselae* subsp. *damselae* strain KC-Na-1	CP021151	99.8%
	Photobacterium damselae strain Phdp Wu-1	CP018297	99.7%
	Photobacterium damselae subsp. *piscicida* DNA	AP018045	99.8%
180	*Photobacterium damselae* subsp. *damselae* strain KC-Na-1	CP021151	95.80%
	Photobacterium damselae strain Phdp Wu-1	CP018297	95.80%
	Photobacterium damselae subsp. *piscicida* DNA	AP018045	96%
188	*Photobacterium damselae* subsp. *damselae* strain KC-Na-1	CP021151	99.7%
	Photobacterium damselae strain Phdp Wu-1	CP018297	99.7%
	Photobacterium damselae subsp. *piscicida* DNA	AP018045	99.8%

Supplementary Table 4 | Translated amino acid sequence identity of isolated plpV PCR products

Sample Name	Protein Alignment	Accession	Identity
124	Thermolabile hemolysin [Photobacterium damselae]	WP_106261769	100%
180	Thermolabile hemolysin [Photobacterium damselae]	WP_065172159	100%
188	Thermolabile hemolysin [Photobacterium damselae]	WP_106261769	100%

Supplementary Table 5 | Taxonomy of OTUs significantly correlated with the level of digesta peroxidase.

OTU	Phylum	Class	Order	Family	Genus
1622	*Proteobacteria*	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Photobacterium*
209	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Porphyromonas*
1553	*Proteobacteria*	*Gammaproteobacteria*	*Pasteurellales*	*Pasteurellaceae*	*Pasteurella*
196	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
631	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*
725	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptostreptococcaceae*	*Peptostreptococcus*
772	*Fusobacteria*	*Fusobacteriia*	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacterium*
1562	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*
1619	*Proteobacteria*	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Unassigned*
207	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Falsiporphyromonas*
774	*Fusobacteria*	*Fusobacteriia*	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacterium*
634	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*
662	*Firmicutes*	*Clostridia*	*Clostridiales*	*Family XI*	*Gallicola*
632	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*
195	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*



Supplementary Figure 3 | Alpha diversity (Shannon), richness (Chao1), and evenness (Simpson) of gut mucosa microbial communities for each treatment.

Shannon, Chao1, and Simpson alpha diversity metrics for each gut mucosa sample, grouped by treatment.

Supplementary Table 6 | Pairwise PERMANOVA comparing the microbial communities of experimental treatments to the control in the gut mucosa.

Control	Treatment	Differ by	R^2	p
22 FM	22 SPC	Diet	0.105	0.412
22 FM	26 FM	Temp	0.115	0.689
22 FM	26 SPC	Diet + Temp	0.135	0.516

Pairwise PERMANOVA with 999 permutations was performed on a Bray-Curtis dissimilarity matrix.